Effects of long-term callus culture on stability and agronomic performance of maize regenerants among three Kenyan hybrids and an Open Pollinated Variety

By

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A thesis in partial fulfillment for the requirement of the award of the Degree of Master of Science (Biotechnology) of the School of Pure and Applied Sciences Kenyatta University.

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DECLARATION

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

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I dedicate this work to my late father Mr. Omar Makenzi A. and my late mother Mrs. Zainab Kassim for their sacrifice, encouragement and educating me. This has enabled me to pursue education up to this level.
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ABSTRACT

Maize (Zea mays) is a member of the maydeae tribe of the grass family Poaceae. It is planted by over 80% of the rural smallholder farmers in East and Central Africa. Commercial maize production is concentrated in the Kenya highland and moist transitional zones, which produce 90% of the maize that is marketed in the country. However over 70% of Kenya’s land falls under the arid and semiarid zones which is unsuitable for rain fed agriculture. The major abiotic factor affecting Kenya’s maize production is inadequate rainfall in the ASAL areas or erratic and unfavorable distribution of rainfall in other areas. The resulting drought conditions in these areas leads to yield loses of up to 71% high, due to lack of suitable drought resistant varieties. It is therefore important to produces high yielding maize that is tolerant to drought. Biotechnology application in maize improvement is a promising option that is suitable for the production of new varieties specifically to overcome the constraints facing maize production in the different Kenyan agroecological zones. In this study three Kenyan hybrids Pwani Hybrid 1(PH1) and its hybrid, Dryland Hybrid 2 (DH02), and an Open Pollinated Variety; Katumani (KAT), calli were maintained culture media for a long time (6 to 12 months) and then used for plant regeneration. The regenerated plants R₀ (primary regenerants) and R₁ (progeny of primary regenerants) were then evaluated for the degree of somaclonal variation in their phenotypes and how they performed agronomically. Dehydration test on both germination of (R₁, R₀ and control) seeds and maize plants was also done to assess the effect of the culture process on the ability of the seeds and plants to withstand drought stress. In this study regeneration was observed to be genotypic dependent with rates of 177.4%, 94%, and 27% for KAT, PH1, and DH02 respectively. Regeneration was also observed to decrease with callus age with a negative correlation (R² = 0.85). The R₀s revealed many phenotypes but only the grained tassel was transmitted to the R₁s, most of which bear similarities in the maize database and other published data. The mutants are important in the study of the effect of different genes on the phenotype and their effects in biochemical pathways in plant function. Data showed that all parameters tested in the R₀ genotype performed significantly from the control. However no significant difference was observed in the agronomic performance of the R₁ genotype compared to the control from commercial seeds. The R₁s matured earlier than those of the controls, had a short ASI and when stressed they performed better than the controls.
ABBREVIATIONS

2, 4, D 2, 4, dichlorophenoxy acetic acid.
ASAL Arid and semi-arid lands.
ASI Anthesis Silking Interval
CAN Calcium Ammonium Nitrate.
CIMMYT Center for the improvement of maize and wheat.
DAP Double ammonium phosphate.
DH02 Dryland Hybrid 02.
DNA Deoxyribonucleic acid.
F1 First filial generation.
GDB Genetic Database.
KAT Katumani Hybrid.
MAS Marker assisted selection.
MS Murashige and Skoog medium
MSV Maize Streak Virus.
N Nitrogen.
NAA Naphthalenic acetic acid.
NPK Nitrogen Phosphorous Potassium.
OPV Open Pollinated Variety
P Phosphorous
PCR Polymerase chain reaction.
PEG Polyethylene Glycol.
pH Hydrogen potential.
PH1 Pwani Hybrid 1.
PTL Plant transformation laboratory.
QTL Quantitative trait loci.
R0 Primary regenerant
R1 First generation of the regenerants.
SNP Single nucleotide polymorphism.
SPSS Statistic Package for Social Sciences.
SSR Simple sequence repeats.
TC Tissue culture.
UV Ultra Violet light.
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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the study

Maize (Zea mays) is a member of the maydeae tribe of the grass family Poaceae. Maize is monoecious. It bears separate male (tassels) and female (ear) inflorescence on the same plant and with the ear producing seeds. Maize is a cross pollinating (allogamous) species such that a natural population is usually heterogeneous. In four to six months in the low tropics, a maize plant can grow up to 2.5-3.5 m high and produce 400-700 seeds on one ear. The maize kernel is a hard one seeded fruit called a caryopsis and consists of a pericarp, endosperm and germ or embryo (Kling and Edmeades, 1997).

Maize is the number one staple cereal crop in East and Central Africa. It is consumed as boiled or roasted maize, cooked maize meal or as thick porridge. Maize is grown from 0 to over 2300m above sea level mainly under rain fed conditions, with traditional low productivity technologies and is planted by over 80% of the rural population. Small-scale farmers account for 85% of the total maize production with medium scale farmers producing the remaining (Hilbeck and Andow, 2004).

The maize production areas in Kenya can be divided into six agro ecological zones. These are based on elevation and climate (Appendix 1). These regions include the lowland tropics, comprising the coastal strip and adjoining inland area, the dry mid-
altitude and dry transitional zones in the southeast, the highland tropics, the moist transitional zone to the east and west of the highland tropics, and the moist mid-altitude zone around Lake Victoria (Corbett, 2005). The moist transitional zone is the most important maize production zone, followed by the highland tropics. These zones mostly lie above 1200 m and have rainfall above 550 millimeters per year with current maize yields of 2.7-5 t/ha (Hassan et al., 1998).

Individual farm holdings of 2 ha or less account for more than 75% of the total area of Kenya's agricultural land, while farms within the 2.1-10 ha size category accounting for another 13% (Government of Kenya, 1995, 2000, 2001). Commercial maize production is concentrated in the higher yielding highland tropics and moist transitional zones, which produce 90% of the maize that is marketed in Kenya. Despite the fact that virtually all rural households in Kenya grow maize, over 60% of them are net maize buyers because they do not produce enough for their domestic consumption (World Bank, 1995; Argwings-Kodeck et al., 1998).

Over half of the maize area in Kenya is planted to hybrid seed (Argwings- Kodeck et al., 1998; Pingali, 2001). In the high-yielding zones almost all farmers purchase certified hybrid seed every year but with large regional and individual differences, (DeGroote et al., 2001). In all the other zones, half to three quarters of farmers use recycled seed of improved or local cultivars. (Argwings-Kodeck et al., 1998; IRMA, 2002). In the dry transitional zones, smallholder farmers consider early maturity and drought tolerance to
be more important criteria for seed selection than high yield with a preference to local varieties.

Maize production in rural Africa is grown in small plots using negligible amounts of inputs or no improved germplasm (Morris 1998). Such non-availability of suitable varieties and erratic rainfall conditions have significantly affected the production and productivity of maize in Africa, with yield not exceeding 1.3 t/ha compared to a potential of over 10 t/ha (CIMMYT, 1999).

In addition to poor varieties maize productivity is hampered by both abiotic (Boyer 1982) and biotic constraints. Biotic factors like weeds e.g. *Striga* (Kanampiu et al., 2002); diseases, such as maize cob rot (Ajanga and Hillocks, 2000) and northern corn leaf blight (Schechert et al., 1999); pests such as maize stem borers (Ajanga, 2000) and grain borers are the major constraints that cause crop losses in Kenya. Abiotic factors that can result in water deficit stress in plants include; heat, drought, salinity and freezing (Boyer, 1982). Average yield losses due to drought are estimated at 17% (equivalent to 2-4 million t/year) but values as high as 70% (10 million t/year) have been documented in Africa (Edmeades et al., 1994).

Somaclonal variation is defined as genetic, cytogenetic and phenotypic variation among clonally propagated plants of a single donor clone (Olhoft and Phillips, 1999). This can offer an attractive alternative to conventional breeding (Hajela and Sticklen, 1994). Epigenetic changes are transient and appear due to the effect of the culture process. They
are physiological in origin perhaps the effect of growth regulators in the medium (Fluminhan et al., 1996). Such changes are of no value for crop improvement, as they are not heritable. Media components have been reported to induce chromosome instability as well as changes in chromosome number and structure in particular explants tissue (Peschke and Phillips 1992). Variation in regenerants can be attributed to the direct effect of culture constituents on DNA replication and activation of transposable elements. Rapid cell cycling which does not give time for replication of DNA (like heterochromatin) leading to chromosome breaks.

Tissue culture induced variation has been described for many phenotypes including plant height, plant biomass, grain yield, and agronomic performance (Dahleen et al., 1991; Bregitzer et al., 1998). Variations with desirable morphological traits in cultivars have been harnessed for disease resistance such as Southern leaf blight (Wise et al., 1987), herbicide tolerance such as imiordazole (Anderson and Georgeson, 1986), antibiotic tolerance such as methotrexate (Tuberose and Philips 1986) and salt tolerance in rice cultivars (Zhu et al., 2004).

Plant tissue culture could thus offer an alternative source of variation for agronomic trait selection supplementing the process of varietal development. Variants with desirable traits such as drought, herbicide tolerance or disease resistance selected fro.
1.2 Problem statement

Production of maize by smallholder farmers is very low in Kenya with yields rarely exceeding 1.3t/ha. This is due to both biotic and abiotic constraints. The major abiotic factor affecting Kenya’s maize production is inadequate and erratic or unfavorable distribution of rainfall (Bonhof et al., 2001). Average yield losses in Africa due to drought is estimated to be 17% (equivalent to 2-4 million t/year) but values as high as 70% (10 million t/year) have been documented (Edmeades et al., 1994). Of the total Kenya’s land area, 85% is semi-arid or arid and it is unsuitable for rain fed agricultural purposes requiring irrigation. It is therefore important to produces high yielding maize that is tolerant to drought.

1.3 Justification

The occurrence of genetic and cytogenetic somaclonal variations in plants regenerated \textit{in vitro} may be useful for maize crop improvement. The high frequency of recombination resulting from tissue culture might be utilized to introgress traits or break undesirable linkages in plant genotypes. Such mutants when analyzed could thus provide important information that can be used in maize improvement. This could supplement the process of varietal development and precise characterization and selection of desirable genotypes occurring in low frequency in our local maize genotypes. This study was justified since it aimed at selecting progenies of primary regenerants (R0) that are more tolerant to drought than non-regenerants form calli maintained over a long period of time. In addition R1 were compared to non-regenerants in terms of agronomic performance. This work has not been reported for any maize genotype in Kenya.
1.4 Null hypothesis

1. Long-term callus culture has no effect on phenotypic progeny of maize regenerants
2. Long-term culture process of callus has no effect on genetic stability of sexual progeny of maize regenerants.

1.5 General objective

To evaluate the genetic and/or phenotypic stability of sexual progeny of maize regenerants recovered from long-term callus cultures.

1.5.1 Specific objectives

1. To regenerate maize from calli maintained over long periods of time (at least six months).
2. To analyze the morphological phenotypes of maize primary regenerants (R0) and their selfed progeny (R1).
3. To compare the performance of tissue culture (TC) regenerants and non-TC plants with respect to selected agronomic traits, with emphasis on dehydration/drought stress tolerance.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Sporogenesis in maize plant

The mature maize plant is the spore-producing or saprophytic phase, which forms microspores from male meiosis in the anthers and megaspores in the ovules. Microspores give rise to the male gametophytes (pollen grains). The megaspores give rise to the female gametophytes (embryo sacs), which produce the egg cells. The gametophytes are haploid. Maize plants are naturally out crossing but can be selfpollinated by dusting pollen from a pollen donor onto the female recipient (Kling and Edmeades, 1997).

2.1.1 Classification of Maize

Cultivated maize, Zea mays subsp mays is well established; however, that of its wild relatives, called "teosintes", is well known (Doebley and Iltis, 1980). Based on isoenzymatic studies and geographic distribution, four subspecies are now recognized (Doebley, 1990). These are Zea mays subsp. mays (maize), Zea mays subsp. parviglumis, Zea mays subsp. Huehuetenanguensis and Zea mays subsp. mexicana, all of them with 2n = 20 chromosomes.

For a long time maize was considered to be a diploid species; however, it is also thought of as an ancient polyploidy (Ting, 1985). This is supported by the existence of
chromosome pairing in haploids (McClintock, 1933; Ting, 1985), the secondary association of bivalents (Vijendra Das, 1970) and the three-dimensional chromosome distribution in four groups of five chromosomes each in somatic metaphases (Bennett, 1995). Cytogenetic evidence and molecular studies further confirms the cryptic polyploid nature of the Zea genus except for the octoploid Zea perennis. All Zea species have a basic chromosome number of five; $x = 5$ (Poggio and Naranjo, 1995). Genetic mapping provide also compelling evidence that maize is an allopolyploid that has undergone extensive chromosomal rearrangement (Moore et al., 1995; White and Doebley, 1998; Soltis and Soltis, 1999).

2.1.2 The Origin of Maize

Maize is thought to have come from domesticated annual teosintes Zea mays subsp. Mexicana or Zea mays subps. parviglumis (Doebley, 1990, 1992). The molecular similarity observed between Zea mays subsp. parviglumis and modern maize was interpreted as proof that maize descended directly from this teosinte (Matsuoka et al., 2002). The discovery of the diploid perennial teosinte, Z. diploperennis favored the "Wilkes hypothesis", that annual teosinte was derived from a cross between Z. diploperennis and wild extinct maize, and that domesticated maize arose from subsequent introgressive hybridization (Mangelsdorf, 1986).

The other hypothesis states that maize is a composite of the genome teosinte and Tripsacum (Eubanks, 1997). This intergeneric hypothesis is based on the basis of the
close correspondence of the intergenomic map with unique *Tripsacum* restriction fragments that are stably inherited in *Tripsacum*-teosinte hybrids and on the finding that the recombinant progeny obtained through experimental crosses between gamagrass *Tripsacum* and *Z. diploperennis* shows ears with all the features of ancient maize (Benz, 2001; Eubanks, 2001). Labeled DNA from *Tripsacum dactyloides* hybridized to maize chromosomes indicates some level of similarity between both species (Poggio et al., 1999). This level of hybridization could be due to shared retroelements (Meyers et al., 2001), but it could be the consequence of some introgression of *Tripsacum* on the maize genome during the domestication process as well (Gonzalez et al., 2004).

### 2.2 Maize improvement

#### 2.2.1 Conventional maize breeding

Maize is one of the most highly bred crops in the world. Maize improvement up to the early 1900s was limited to recurrent selection methods. Population improvement through a series of recurrent selection procedures is aimed at maximizing percentages of favorable alleles at each locus of importance to crop performance in a given environment. Population improvement remains the primary means of improving levels of performance in base populations from which inbred lines are developed (Sprague *et al.*, 1988).

Backcrossing is a breeding method designed to move genes from one maize line, the donor line, to another the recipient, without genetically changing the recipient. The number of backcrosses determine how closely related the progeny resemble the recipient.
The number of backcrosses determine how closely related the progeny resemble the recipient. The complexity of the backcross method depends on type of trait being transferred (single gene vs. several genes), level of expressivity, type of inheritance, and the parents included in the crosses. For single gene traits that are relatively easy to classify, the backcross method is effective and relatively easy to manage. For traits that have a more complex inheritance, the backcross method requires greater selection pressure for the desired trait (Sprague et al., 1988).

Gamete selection is a scheme devised by Stadler (1944) for sampling elite gametes from a population. The procedure involves crossing an elite line with a random sample of pollen from a source population. Each of the F₁ plants arising and the elite line are test crossed to a common tester. Testcrosses are evaluated in replicated trials; and the F₁ plants that exceed the elite line by tester are presumed to have obtained a superior gamete from the source population. Gamete selection has some intrinsic features that interest breeders, and, consequently, gamete selection is included in some breeding programs.

2.2.2 Mutation breeding

The term mutation refers to a sudden phenotypic change in a character of an individual, not due to crossing or segregation and or an alteration in the macromolecule of DNA. Included under the term mutation is the augmentation of genetic material through nucleotide or gene copies, through addition of individual chromosomes, as well as through the multiplication of whole genomes resulting in polyploidy. However recombination of genes can also provide additional genetic variation. To date the
processes of mutation induction by UV light, ionizing radiation and also by certain chemical mutagens is well understood but the causes of spontaneous mutations are still to some extent uncertain (Hall, 1990).

The results from induced mutation are useful in self-pollinating plant species as the trait is maintained within the genotype. However heterosis in mutants has also been repeatedly reported (Maluszynski et al., 1989). Specific mutations e.g. male sterility (Daskalov and Michailov, 1988) or grain quality traits (Robbelien, 1990) proved useful in cross-pollinating species. In vegetatively propagated ornamental crops, which are usually heterozygous mutation breeding has been successful (Broertjes and Van Harten, 1988).

2.2.3 Breeding for drought

Drought tolerance is generally defined as the property of a given cultivar to show a relatively small yield reduction upon exposure to drought. This implies that breeding for stress adaptation is at the expense of yield potential. Drought stress is highly heterogeneous in time and space and is extremely unpredictable often with irreproducible results. Since the phenotype is the product of genotype and environment, assessment of the desired genotype is highly dependent on the proper environmental conditions. The unpredictable nature of drought stress makes the selection of drought tolerance plants extremely difficult (Banzinger et al., 2000).

Drought tolerance has been shown to be a highly complex trait, influenced by many different genes (Flowers and Yeo, 1995; Winicov, 1996). In fact, drought tolerance
should not be regarded as a unique heritable trait, but as a complex of often fully unrelated plant traits. Drought can hardly be separated from other abiotic stresses such as temperature and salinity since they have similar effects.

Plant water deficit occurs when the rate of transpiration exceeds water uptake. At the Cell level, water deficit can result in concentration of solutes, changes in cell volume and membrane shape, disruption of membrane integrity and denaturing of proteins (Holmberg and Bullow, 1998). Complete loss of free water results in desiccation or dehydration and impairs photosynthesis (Bray, 1997).

Resistance to water deficit occurs when a plant withstands the imposed stress and may arise from a mechanism that permits avoidance of the situation at the cellular or whole plant level. This response depends on species and genotype, the length and severity of water loss, the age and stage of development of the plant (Bray, 1997).

2.2.4 Adaptive plant mechanisms

The general mechanism that plant employ to cope with drought stress fall in three general categories: the ability of a plant to escape periods of drought, in particular during the most sensitive periods of its development is the first mechanism. One breeding strategy is therefore to shorten the life cycle of a crop to enable it to mature safely during a rainfall period (Banzinger et al., 2000). The ability of a plant to endure or withstand a dry period by maintaining a favorable internal water balance under drought condition is another mechanism. Osmotic adjustment, in which the plant increases the concentration of
organic molecules in the cell water solution to 'bind' water, is one good example (Holmberg and Bullow, 1998). The ability of a plant to recover from a dry period by producing new leaves from buds that were able to survive the dry spell is the last category.

2.2.5 Drought and maize crop development

Drought affects maize development at almost all growth stages, but the crop is most susceptible during flowering (Grant et al., 1989). Extreme sensitivity seems confined to the period 2 days before to 22 days after silking, with a peak at 7 days. Complete bareness can occur if maize plants are stressed in the interval from just before tassel emergence to the beginning of grain filling period (Grant et al., 1989). Maize is more susceptible during flowering than other rain fed crops because both male and female florets develop virtually at the same time. The male and female flowers are separated by as much as 1 m and pollen and stigmatic tissue are exposed to a dry and hostile atmosphere for pollination to occur (Banzinger et al., 2000).

Silk growth and kernel number depend directly on the flow of photosynthetic products during the three weeks of flowering and extreme sensitivity (Schussler and Westgate, 1995). When photosynthesis at flowering is reduced by drought or other abiotic stresses, silk growth is delayed, leading to an easily measured increase in the anthesis- silking interval (ASI), as well as kernel and ear abortion (Bolanos and Edmeades, 1996, Nesmith and Ritchie, 1992).
2.3 Tissue culture, organogenesis and somatic embryogenesis

The regeneration ability of any plant is influenced by many factors. The type of explant is the main factor (El-Itriby et al., 2003). Immature embryos are the most widely used in many cereals including maize (Shillito et al., 1989). Regeneration is possible from calli initiated from anthers (Ting et al., 1981), glumes (Suprasanna et al., 1986), immature inflorescence (Pareddy and Petolino, 1990), immature tassels (Rhodes and Gray, 1992; Songstad et al., 1992), leaf segments (Ray and Gosh, 1990) seedling segments (Santos et al., 1984), shoot tips (Zhong et al., 1992 and O’Connor-Sanchez et al., 2002), shoot apical meristems (Zhang et al., 2002) and mature embryos (Huang and Wei, 2004). Plant regenerability is also influenced by the media composition (Carvalho et al., 1997), and genotype of the explant (Bohorova et al., 1995).

2.3.1 Organogenesis and somatic embryogenesis

Explant can regenerate into an entire plant through organogenesis and somatic embryogenesis. Generally, in the first case, shoots and roots form sequentially and in response to appropriate culture conditions. This type of development is also characterized by the presence of vascular connections between the mother tissue and the regenerating section (Faure et al., 1998).

Somatic embryogenesis can be described as the process by which haploid or diploid somatic cells develop into structures that resemble zygotic embryos through an orderly
series of characteristic embryological stages without fusion of gametes (Emons, 1994, Raemakers et al., 1995). Somatic embryos are capable of continuous growth resulting from the absence of developmental arrest (Faure et al., 1998). Both processes, organogenesis and somatic embryogenesis, can occur in the same explant (He et al., 1990), but originate from particular tissue layers or cells within explants (Osternack et al., 1999).

2.3.2 Applications of somatic embryogenesis

Somatic embryogenesis is used in mass propagation of plants through multiplication of embryogenic propagules (Merkle et al., 1990). Somatic embryogenesis has many advantages over organogenesis. It permits the culture of large numbers of reproductive units with the presence of both root and shoot meristems in the same element. Additionally the plants from somatic embryos are less variable than those derived by way of organogenesis (Osuga et al., 1999).

Somatic embryogenesis is used in the production of plants with different levels of ploidy (Lo Schiavo et al., 1989). Embryogenic cultures are a source of regenerable protoplasts in grass species (Funatsuki et al., 1996) and Citrus species (Jiménez, 1996). Somatic embryogenesis is an efficient pathway for studies involving production of genetically transformed plants (Vicient and Martínez 1998).
2.3.3 Transformation biotechnology

*Agrobacterium* mediated transfer of genes into an explant is the preferred method of transforming dicotyledonous species. Co-cultivation of callus, suspension cultures or leaf discs with *Agrobacterium* has been used to successfully transform many species (Komari, 1989), such as potato (Visser, 1991), tomato (McCormick, 1991) and sugar beet (Lindsay et al., 1991). The most recalcitrant of cereal species is wheat and maize, where difficulty in developing reliable regeneration systems (Vasil, 1990) is exacerbated by low frequency of transformation (Vasil et al., 1992). Other problems associated with tissue culture of cereals are loss of regenerative capacity with increasing number of subcultures (Rhodes and Gray, 1992) and occurrence of abnormalities such as sterility in regenerated plants (Vasil, 1990).

Since the first reports of gene transfer with species that are relatively easy to tissue culture, petunia and tobacco (Horsch et al., 1983), transformation procedures have been published for a wide range of species including tree crops, such as apples (James and Dandekar, 1991) and papaws (Fitch et al., 1992). However there are public concerns about transformation biotechnology (Machuka, 2004)

Biotechnolog has been used to fortify cereals and sweet potato with enhanced levels of vitamins or proteins in their seeds and tubers (Wu et al., 2003). Other areas of application include lowering the levels of antinutrients, toxins and allergens in seeds, food grains, leaves and tubers (Graham et al., 2001). Transgenic plant production of edible vaccines
and antibodies (plantibodies), as well as products of therapeutic, pharmaceutical and industrial value, is also possible (Hood et al., 1997).

2.3.4 Somaclonal variation and application in plants

Somaclonal variation (SV) caused by the process of tissue culture is also called tissue culture-induced variation. It is manifested as either somatic variation that cannot be transmitted to subsequent generation or meiotically heritable variation (Larkin and Scowcroft, 1981).

Epigenetic control of gene expression can be defined as a somatically or meiotically heritable alteration in gene expression that is potentially reversible and is not due to sequence modification. Epigenetic aspects of somaclonal variation would therefore involve mechanisms of gene silencing or gene activation that were not due to chromosomal aberrations or sequence change (Kaeppler et al., 2000).

Tissue culture variation has also been harnessed in some cases to confer desirable traits to cultivars including desirable morphological traits, disease resistance e.g. Southern leaf blight (Wise et al., 1987), herbicide tolerance e.g. imidazole (Anderson and Georgeson, 1986), antibiotic tolerance e.g. methotrexate (Tuberose and Philips 1986) and salt tolerance in rice cultivars (Duncan, 1997; Veilleux and Johnson, 1998; Zhu et al., 2004).
CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Plant materials

Embryogenic type II calli of DH02, PHI, and Katumani from immature zygotic embryos that were maintained in culture by subculturing in fresh media after every three weeks, at The Plant Transformation Laboratory (PTL) Kenyatta University, (Oduor et al., 2006) for six (6) to twelve months (12) was used for plant regeneration. Commercial seeds from Kenya Seed Company Limited; KAT, DH02 and PHI were used as controls. Calli of PHI X HII were also used in plant regeneration but was not part of the experiment.

3.2 Methodology

3.2.1 Callus maintenance

Calli maintained in culture (see also subsection 3.1 above) were sub cultured to fresh N6 media (Chu et al., 1975), supplemented with 2.0mg/l glycine, 2.9gm/l proline, 0.01gm/l casein hydrolysate, and 2mg/l 2,4D 3% gm sucrose, 3gm/l gelrite. The pH was adjusted to 5.8 before autoclaving at 15psi for 20 minutes. The calli were subculture after every 3 weeks and kept in the dark.
3.2.2 Callus maturation

Embryogenic calli in maintenance media were transferred to maturation media. This was the same as maintenance media but supplemented with 6% sucrose and 0.1mg/l NAA without proline. Calli were cultured for 2 weeks in the dark before they were transferred to shoot induction media.

3.2.3 Plant regeneration

After four weeks, embryogenic calli were transferred to a modified MS (Murashige and Skoog, 1962) media containing MS basal salts and vitamins for shoot induction and kept in light. This media was supplemented with 3g/l gelrite, 3% sucrose and 2.0mg/l glycine. The culture was maintained at 27°C under 16-h photoperiod and transferred to fresh media after every two weeks.

3.2.4 Rooting of regenerants

When the shoots attained a height of 1-2cm in the shooting medium, they were transferred into half strength MS (Murashige and Skoog, 1962) rooting medium. This is the same as the shooting medium but with half the amount of MS basal salts and vitamins supplemented with 2% sucrose, 3g/l gelrite and 0.1mg/l NAA. Shoots were maintained in rooting media until roots were properly developed before being taken for acclimatization.
3.2.5 **Plant hardening**

Shoots with roots were hardened in small pots 5cm diameter, containing sterile peat moss (Kekilla Oyj Finland) or vermiculite and phymix\textsuperscript{R} (Phytomedia International, Kenya) supplemented with DAP fertilizer. The shoots were covered with a polythene paper bag with adequate ventilation to prevent excessive evapotranspiration during acclimatization.

3.2.6 **Transfer of maize plants into larger pots**

When the maize regenerants attained 2-3 leaves in the small pots they were transplanted into the soil in larger flowerpots in the green house. They were watered after every two days until they reached maturity. Top dressing using 10 grams of CAN (26% N) or NPK (17:17:17) was applied weekly to avoid confounding effects of N or P deficiency due to leaching. FURADAN (5G FMC Corporation) was applied during transplanting to control nematodes, stem or root borers and other soil parasites. The maize regenerants were also sprayed periodically using DANADIN (Dimethoate 40EC, Chemova Agro A/s Denmark) to control the spread of Maize Streak Virus (MSV) by red mites and other leaf parasites.

3.2.7 **Morphological analysis**

The phenotypic characteristics of the regenerants were visually evaluated and were compared with that of the non-regenerants controls. Morphological characteristics such as plant height, tussle morphology such as length, presence of grains on tassels, tassel branching; general color of plant, phylotaxi of the regenerants and grain morphology
were evaluated. Photographs of plants showing the observed characteristics were recorded using Mercury 3.1 mega pixels camera. The phenotypes were measured and compared with similar characteristics of the controls. Mutant phenotypes were also compare with those in the maize database; www:// maizeGDB.org. and in Mutants of Maize (Neuffer et al., 1997)

3.2.8 Controlled pollination of maize plants

Pollination of regenerants was done according to established procedures for maize. Where no pollen was produced, sibbing was done. The fertilized ears were covered immediately afterwards with the pollen paper bags and allowed to grow to maturity. Data collected include days to 50% anthesis and days to 50% silking. Anthesis - Silking Interval was calculated by subtracting days to silking from days to anthesis (Banzinger et al., 2000).
3.2.9 Agronomic performance of R₀ and R₁

At maturity both the R₀ and R₁ secondary traits for selection of drought tolerance were evaluated. The following selected agronomic parameters measured or scored; tassel length, number of ear per plant, presence of grains on tassel, plant height, ear height, tassel branches and sub branches, tassel color and silk color (Banzinger et al., 2000).

3.2.10 Germination tests

Germination test for R₀ seeds from the regenerated maize genotype and commercial seeds were carried out in large buckets for the purposes of comparing their viability. Two seeds from each line of the four R₀ genotypes and their commercial controls were planted in buckets in duplicates and arranged in a complete randomized block design. The experiment was replicated twice. The percentage germination of the R₀ and control were calculated.

3.2.11 Dehydration of R₀ Seed tests

Seeds of primary regenerants (R₀) maize plants were surface sterilized with 70% ethanol for 3min then 3% sodium hypochlorite for 30min, then washed with sterile distilled water three times. The sterile seeds were soaked overnight in sterile distilled water and then placed in culture bottles containing cotton wool saturated with MS salt solution or MS
salt solution with 30% (w/v) PEG-6000 or 29% (w/v) NaCl at 25°C in the growth room (Rudang et al., 2004). The germination percentage was recorded on a daily basis. The germinating seedlings were washed with sterile distilled water three times before planting into small flowerpots containing peat moss and later transplanted into soil in larger flowerpots.

### 3.2.12 Screening for drought tolerance

Maize was grown under rain exclusion shelter in the soil of flowerpots with one plant per pot. Duplicate maize plants arranged in a complete randomized design experiment was set up to test for drought tolerance in the R₁ and non-regenerated maize plants. A total of four genotypes and 20 lines were tested. They were sufficiently watered after every two days to maturity. Water was withdrawn one week before tassel emergence in the experimental block for two weeks to induce severe stress to the plants during flowering. They then watered as necessary to prevent zero-yield. The control block was watered normally throughout the experiment. The ASI of both the experimental block and control was calculated. The length of maize cobs was measured and photographs was taken (Banziger et al., 2000).
3.2.13 Data analysis

Analysis of variance (ANOVA) of the data collected was done by use of GENSTAT DISCOVERY edition computer statistical package. Calculation of means and standard error of the means, percentages, regression analysis and graphical presentation of the data collected was done using MS EXCEL computer programme. Frequency of occurrence of the different observed phenotypes were determined and expressed as percentages.
CHAPTER FOUR

4.0 RESULTS

4.1 Plant regeneration

When calli were transferred to shoot induction media, they started showing patches of green coloration after one week and in the second week shoots start forming (Figure 4.1a). Some patches of brown calli were also observed when the calli were sub cultured in shoot induction media (Figure 4.1a). When the shoots were fully formed they were transferred to root induction media for root formation. It was observed that after one week in the shoot induction media, the root length was long enough for the plants to be taken out for hardening (Figure 4.1c). It was observed that shoot formation varied from callus to callus and from genotype to genotype but in general shoot formation rate was higher with Katumani maize than any other genotype (Figure 4.1b).

Figure 4.1 Calli in shoot induction and root media.
(a) KAT calli in shoot induction media green and brown calli shown by red and blue arrows respectively. (b) Multiple shoots fully formed in shoot induction media after 3 weeks. (c) Maize shoots in root induction media; red arrow shows roots formed after 1 week. Bar = 2 cm.
The results of plant regeneration shows that regeneration rates of KAT, PH1, PH1 X HII, and DH02 were 177.4%, 94%, 37%, and 27% respectively (Figure 4.2). The regeneration rate of KAT was significantly different from that of the other genotypes but there was no significance difference between PH1 X HII and DH02 at \( p<0.05 \). Multiple shoots formation were also observed from the four genotypes of up to 4 per callus on average but KATUMANI produced the highest number of shots from one callus of up to 8 plants (Figure 4.1b). The rate of regeneration was observed to decrease with an increase in the number of subculture (calli age) in all the four genotypes. Regression analysis of plant regeneration and number of subculture was done and a negative correlation was observed in all the four genotypes. Overall Pearson’s regression correlation coefficient of \(-27.367\) was observed with an \( R^2 = 0.933 \) (Figure 4.3).
Figure 4.2 Regeneration rate of four genotypes used in plant regeneration
KAT had the highest rate followed by PH1 and its hybrids then DH02 in that order
Figure 4.3 Plant regeneration against number of subculture.

Negative correlations exist between number of plants that are regenerated and number of subculture with a negative Pearson correlation and $R^2 = 0.933$. 

$$y = -27.367 \ln(x) + 98.948$$

$R^2 = 0.933$
4.2 Hardening of $R_0$ maize plants

When the $R_0$ were taken out for hardening majority of them survived. However some of the regenerants from all the four genotypes were too delicate that they could not survive the hardening process. It was also observed that when the polythene paper bag was removed before 5 days the regenerants died. (Figure 4.4)

Figure 4.4 KAT $R_0$ maize during hardening (acclimatization)
Polyhene paper bag covering the plant is to prevent excessive evapo-transpiration. Bar = 3cm.
A total of 564 maize plants were regenerated, 446 survived on acclimatization to the external environment. This is equivalent to 79.1% survival of the total plant regenerated.

When the multiple shoots were separated from each other many could not survive the external environmental conditions and died since the maize plants did not have roots of their own. A total of 57 plants died on separation out of the 446 plants that survived acclimatized. From this data, it is only 67% of the regenerated plants that survived to maturity (Table 4.1).

Table 4.1 Plant regeneration survival on acclimatization and at maturity

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Plant regeneration</th>
<th>Acclimatization survival (AS)</th>
<th>% AS</th>
<th>No of plants at maturity (PM)</th>
<th>% PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Katumani</td>
<td>264</td>
<td>211</td>
<td>80.0</td>
<td>182</td>
<td>69.0</td>
</tr>
<tr>
<td>D/Hybrid 02</td>
<td>099</td>
<td>074</td>
<td>75.0</td>
<td>054</td>
<td>55.0</td>
</tr>
<tr>
<td>P/Hybrid 01</td>
<td>201</td>
<td>161</td>
<td>81.0</td>
<td>153</td>
<td>76.1</td>
</tr>
<tr>
<td>Grand total</td>
<td>564</td>
<td>446</td>
<td>79.1</td>
<td>379</td>
<td>67.2</td>
</tr>
</tbody>
</table>
4.2 phenotype analysis of the $R_0$

In this study somaclonal variation was assessed on the morphological appearance of the whole maize plant. The mutant phenotype observed includes height, tassel morphology, chlorophyll deficiency mutant, and coloration mutant.

Dwarf phenotype was observed 23% and 37% more frequently in the PH1 and its hybrids and 13% in KAT and none in DH02 (Figure 4.5). This phenotype was also observed to have tillers that proliferated at the sides of the parent plant.

Figure 4.5 Dwarf mutant compared with normal height regenerant.
(a) Normal height PH1 maize regenerant. Bar = 10cm. (b) Dwarf plant with a height of less than 30cm. Bar = 5cm. Note the ears height of 1-2 cm above ground.
In the grained tassel mutant the terminal inflorescence was observed to be completely pistillate but in some cases pollens were also produced. Some mutants had the maize cob at the top of the maize plant and no ear was produced while others had few grains on tassels (Figure 4.6 a and b). The kernel placements on some of the grained tassels were observed to be irregular. Grained Tassel phenotype was found in all the four hybrids that were regenerated. PH1 hybrid had the highest frequency of 75% followed by 60%, 50%, 40% for KAT, DH02 and PH1 hybrid respectively. (Table 4.2)

Figure 4.6 Grained tassel mutant compared with normal tassel regenerant. (a) PH1 regenerant with branched cob grained tassel. Bar = 2cm. (b) PH1 maize regenerant with normal tassel. Bar = 4cm.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number (n)</th>
<th>Dwarf(^1) R(_0) (%) Cont(^2) (%)</th>
<th>Grained tassel R(_0) (%) Cont (%)</th>
<th>Unbranched tassel R(_0) (%) Cont (%)</th>
<th>Pink coloration R(_0) Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>KAT</td>
<td>85</td>
<td>13 0</td>
<td>50 &lt;0.1</td>
<td>71 0</td>
<td>L(^3), e(^4), s(^5) 0</td>
</tr>
<tr>
<td>DH02</td>
<td>20</td>
<td>00 0</td>
<td>60 0</td>
<td>43 0</td>
<td>Wp(^6) 0</td>
</tr>
<tr>
<td>PH1</td>
<td>25</td>
<td>23 0</td>
<td>40 0</td>
<td>67 0</td>
<td>S, t(^7) 0</td>
</tr>
<tr>
<td>PHI X HII</td>
<td>35</td>
<td>37 0</td>
<td>75 0</td>
<td>05 0</td>
<td>S, t 0</td>
</tr>
</tbody>
</table>

1. Plants < 30 cm height.
2. Non tissue culture maize
3. Leaves only
4. Ears only
5. Silk only
6. Whole plant
7. Tassel
Regenerated plants of the four genotypes showed varying frequency of tassel morphology at maturity. Occurrence of similar mutation in the controls was very rare. It was observed that KAT maize genotype had the highest frequency followed by PHI, DH02, and PHI XHII hybrid of 71%, 67%, 43% and 5% respectively (Table 4.2).

Figure 4.7 Unbranched tassel mutant compared with normal tassel maize. (a) PHI maize regenerant with unbranched tassel. Bar = 4cm. (b) PHI maize regenerant with normal branched tassel. Bar = 4cm.
Chlorophyll deficiency mutant was also observed from the primary regenerants from the four genotypes (Figure 4.8). These occurred on rare occasions but did not survive to maturity because they could not photosynthesize.

![Chlorophyll deficiency mutant. Only PH1 maize regenerants had this mutant. Bar = 2cm.](image)

**Figure 4.8 Chlorophyll deficiency (albino) mutant.**
*Only PH1 maize regenerants had this mutant. Bar = 2cm.*

Pink coloration of regenerated maize plants was observed in all the four genotypes. This includes pink coloration of the tassels, leaf, silk, stems, ears and leaf sheath (Figure 4.9). The color varied from dark to light tint variation of the same color. DH02 and KAT regenerated maize plants had the most of the vegetative parts colored pink. In PHI, pink coloration was only observed in the silk and in some cases the tassels. In most cases the coloration was independent of the number of subculture of the embryogenic calli the maize plants were regenerated from.
At maturity some of the regenerated maize plants produced ears with no silk while others had tassels that did not produce pollen grains (Figure 4.10). This was observed with all the four genotypes regenerated.

Figure 4.10 Sterile tassel mutant compared with normal plant.
(a) Tassels with glumes that fail to open to expose the anthers. Bar = 3cm (b) Normal tassel. Bar = 5cm (c) Sterile ear without silk. Bar = 5cm
Regenerants from KAT and PHI Hybrid maize genotype were observed to have the ears located at opposite positions of the stem as opposed to alternate positioning (Figure 4.11). This phenotype occurred rarely and was only observed twice in KAT maize genotype.

Figure 4.11 Maize regenerants showing opposite and normal ear arrangement. (a) Opposite ear location in KAT maize regenerants. Bar = 10cm (b) Normal ear location in PHI maize regenerants. Bar = 10cm.

Additionally some of the plants showed terminal ear phenotype whereby the ear was produced just below the tassel. This phenotype was observed in the KAT maize genotype as well as the PHI maize. Sometimes the ear was wrapped in the tassels such that it could be confused with grained tassel phenotype. The occurrence of this phenotype was rare when compared to other morphological aberrations that were observed (Figure 4.12).
Tillers were also observed in the R₀ plants mostly in the PH₁ and its Hybrid. When the tillers were left to grow, they formed long and weak stalks that sometimes produced ears with silk. This trait was heritable and was observed in the R₁ plants as well (Figure 4.13).
Figure 4.13 Tillers in $R_0$ and $R_1$ maize.

(a) Tillers $R_1$ PH1 maize genotype (b) Tillers in the $R_0$ PH1 maize some had terminal ear and silk as seen in the photograph.
4.7 Controlled pollination

Some of the $R_0$ did not produce tassels but ears with silk were produced as such cross-pollination was done. Anthesis silking interval (ASI) of the $R_0$ could not be calculated because of lack of silk emergence in some while in others no tassels were produced. Controlled pollination of the $R_1$s was done so as to prevent contamination of the maize lines with pollens from other maize plants from the surrounding areas. They were then self-pollinated and anthesis silking interval calculated.

4.8 Agronomic performance of $R_0$

The effect of tissue culture on the primary regenerants ($R_0$) maize was determined using the agronomic performance of tissue culture maize compared with non tissue culture controls. Maize plant parameters were selected on the basis of their ease of observation, value to the crop, measurement and heritability.

Significant difference between the $R_0$ and the control was observed in plant height, ear height, tassel length and number of tassel branches ($p<0.05$). There was no significant difference in the number of ears per plant and number of maize cobs harvested between the $R_0$ and control ($p>0.05$). Significant difference was observed between the genotypes of the $R_0$ and control used in all the parameters tested ($p<0.05$) Table 4.3.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>DH02 R₀</th>
<th>Cont*</th>
<th>PH1 R₀</th>
<th>Cont</th>
<th>KAT R₀</th>
<th>Cont</th>
<th>PH1XH11 R₀</th>
<th>Cont</th>
<th>G.M.</th>
<th>LSD (5%)</th>
<th>F.Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height</td>
<td>167.6(3.74)</td>
<td>218.9(9.0)</td>
<td>126(2.83)</td>
<td>217(14.4)</td>
<td>121.2(16.6)</td>
<td>238.9(21.1)</td>
<td>128.6(1.94)</td>
<td>229.1(11.8)</td>
<td>136.1(8.57)</td>
<td>24.22</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Tassel length</td>
<td>41.1(2.46)</td>
<td>44.8(2.4)</td>
<td>29.8(2.48)</td>
<td>44.6(3.3)</td>
<td>32.6(5.0)</td>
<td>53.8(3.0)</td>
<td>29.7(0.91)</td>
<td>46.8(3.4)</td>
<td>33.3(2.23)</td>
<td>0.631</td>
<td>0.001*</td>
</tr>
<tr>
<td>Ear height</td>
<td>64.4(2.4)</td>
<td>102.6(5.0)</td>
<td>60.6(0.53)</td>
<td>91.7(10.8)</td>
<td>49.6(5.5)</td>
<td>115.7(15.7)</td>
<td>59.9(1.0)</td>
<td>111.0(7.4)</td>
<td>58.6(5.53)</td>
<td>15.59</td>
<td>0.279</td>
</tr>
<tr>
<td>Tassel branch</td>
<td>9.56(1.73)</td>
<td>18.7(1.7)</td>
<td>12.4(0.17)</td>
<td>15.2(1.7)</td>
<td>7.83(1.35)</td>
<td>15.7(1.9)</td>
<td>9.28(0.47)</td>
<td>16.9(2.4)</td>
<td>9.78(1.13)</td>
<td>3.192</td>
<td>0.041*</td>
</tr>
<tr>
<td>Number of ears/plant</td>
<td>1.67(0.24)</td>
<td>1.7(0.22)</td>
<td>1.67(0.53)</td>
<td>2.4(0.22)</td>
<td>2.22(0.37)</td>
<td>1.7(0.24)</td>
<td>2.0(0.22)</td>
<td>2.2(0.22)</td>
<td>1.89(0.22)</td>
<td>0.629</td>
<td>0.225</td>
</tr>
<tr>
<td>Number of cobs harvested/plant</td>
<td>1.056(0.29)</td>
<td>1.6(0.24)</td>
<td>1.44(0.20)</td>
<td>1.4(0.24)</td>
<td>1.33(0.11)</td>
<td>1.2(0.15)</td>
<td>2.0(0.29)</td>
<td>1.7(0.24)</td>
<td>1.45(0.16)</td>
<td>0.459</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

N = 64

*Significant difference from grand mean LSD =5% (ANOVA II p < 0.05)

# plants derived from commercial seeds.

Figures Mean (±SE)
On average the R₀ had lower values for all the parameters compared to the controls except the number of cobs harvested per plant which had approximately equal values (Figure 4.14). Comparison of the individual parameters tested: plant height, the R₀ had an average of 46.1cm while controls had 226.9cm; ear height, the R₀ had an average of 12.0cm while controls had 105.2cm; tassel length, the R₀ average was 17.8cm while controls had 48.0cm; tassel branches, R₀ had an average of 2.94 while that of the control was 16.6cm; number of ears per plant, the R₀ had an average of 1.75 while the control had 2.03 and finally the number of cobs harvested per plant, the R₀ had an average of 1.44 while the control had 1.47. (Table 4.3)
Figure 4.14 R₀ height compared to the control.

All the R₀ parameters tested had lower values for all the genotypes. Bars (±SE)
4.10 Agronomic performance of R₁

To find out whether the R₁ performed differently from their parents, the agronomic parameters evaluated were the same as those used in the evaluation of R₀ maize and their control. Seeds of the individual regenerated maize plants were planted in buckets to give rise to the R₁. The number of seeds restricted the number of R₁ maize plants that were used in this experiment.

Significant difference was observed in ear height, tassel length and tassel branches ($p < 0.05$) while there was no significant difference in the number of ears per plant and the number of cobs harvested per plant between the R₁ and the control ($p > 0.05$). On the genotype used, no significant difference was observed in all the parameters tested between the R₁s and their control ($p > 0.05$). Again no significant difference in the interaction between the genotypes and control in the R₁s and control was observed in all the parameters tested ($p > 0.05$) (Table 4.4).
Table 4.4 Summary of agronomic performance of R1 and control.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DH02 R1</th>
<th>PHI R1</th>
<th>KAT R1</th>
<th>PH1XH11 R1</th>
<th>G.M*</th>
<th>LSD (5%)</th>
<th>F.Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height</td>
<td>211.0(14.0)</td>
<td>215.0(31.3)</td>
<td>183.5(9.0)</td>
<td>206.0(10.5)</td>
<td>213.7(4.0)</td>
<td>211.7(23.3)</td>
<td>188.7(12.6)</td>
</tr>
<tr>
<td>Ear height</td>
<td>92.8(9.0)</td>
<td>108.7(21.6)</td>
<td>75.5(7.8)</td>
<td>93.0(6.1)</td>
<td>103.3(3.1)</td>
<td>112.7(14.5)</td>
<td>94.8(9.9)</td>
</tr>
<tr>
<td>Tassel length</td>
<td>46.2(1.3)</td>
<td>48(4.1)</td>
<td>46.8(2.3)</td>
<td>50.0(2.3)</td>
<td>43.2(1.8)</td>
<td>46.0(4.0)</td>
<td>41.3(5.7)</td>
</tr>
<tr>
<td>Tassel branches</td>
<td>18.2(1.9)</td>
<td>20(3.2)</td>
<td>15.0(0.9)</td>
<td>17.3(3.9)</td>
<td>18.5(1.2)</td>
<td>23.7(5.2)</td>
<td>16.3(2.8)</td>
</tr>
<tr>
<td>Number of ears/plant</td>
<td>3.17(0.3)</td>
<td>3(1)</td>
<td>2.33(0.3)</td>
<td>3.0(0.57)</td>
<td>3.17(0.6)</td>
<td>3.67(0.8)</td>
<td>2.67(0.6)</td>
</tr>
<tr>
<td>Number of cobs harvested/plant</td>
<td>1.67(0.7)</td>
<td>1.7(0.3)</td>
<td>1.50(0.3)</td>
<td>1.7(0.3)</td>
<td>1.67(0.3)</td>
<td>2.0(0.5)</td>
<td>1.67(0.3)</td>
</tr>
</tbody>
</table>

N = 64

* Group mean for R1 and control

1. Seeds were not available hence not be done.
On average the R₁s recorded lower values compared to the controls in plant height, ear height, tassel length, tassel branches and the number of cobs harvested per plant. However, it was only in the number of ears per plant that a higher average of the R₁s than that of the controls was observed (Figure 4.1).

Figure 4.15 R₁ genotype ear height compared with control.
All R₁ had lower values for all parameters tested except number of ears. PH X HII Control was not done because there were no commercial seeds sold.
**Anthesis Silking Interval (ASI)**

Anthesis silking interval is important since it is used as an indicator of the ability of a particular maize genotype to tolerate drought; the shorter the interval the more drought tolerant the genotype. This ensures that pollination occurs before silk are desiccated or after the ovaries have exhausted their starch reserves.

In this experiment no significant difference in the anthesis date between the genotype used and control was observed ($p > 0.05$) while the silking date for the R1s and control was not significantly different between the genotype used either ($p > 0.05$). Anthesis Silking Interval (ASI) of the R1 compared to the controls showed no significant difference among the genotype used ($p > 0.05$) (Table 4.5). On average, the tasseling (anthesis) of the R1s occurred earlier than the controls by 6 days. The average tasseling date of the R1s was $68.46 \pm 0.92$ days while that of the controls was $74.08 \pm 1.21$ days. KAT maize genotype tasseled and silked earlier than the other genotypes used (Table 4.5). The average number of days to silking of the R1s was lower than the controls by 7 days. On average, the tasseling (anthesis) of the R1s occurred earlier than the controls by 6 days.

The ASI of the R1s ($2.79 \pm 0.34$) was lower by 2 days from that of the controls ($4.33 \pm 0.34$). The average ASI of the R1s genotypes was 3.5, 2.18 and 2.78 days for PHI, DH02 and KAT respectively. While that of the control were 4.38, 5.0 and 3.63 days for PHI, DH02 and KAT respectively (Table 4.5).
Table 4.5 Tasseling, Silking dates and Anthesis Silking Interval (ASI) of R₁ and control.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Average number of days to tasseling (Anthesis A)</th>
<th>Average number of days to silking (S)</th>
<th>Anthesis silking interval (ASI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R₁</td>
<td>Cont</td>
<td>R₁</td>
</tr>
<tr>
<td>PH₁</td>
<td>71.50(1.15)</td>
<td>77.0(1.53)</td>
<td>75.00(1.13)</td>
</tr>
<tr>
<td>DH02</td>
<td>67.88(2.05)</td>
<td>76.5(1.46)</td>
<td>69.75(2.16)</td>
</tr>
<tr>
<td>KAT</td>
<td>66.00(0.73)</td>
<td>68.8(1.94)</td>
<td>68.75(0.77)</td>
</tr>
<tr>
<td>PH1XHII</td>
<td>74.03(1.21)</td>
<td>nd²</td>
<td>78.00(1.77)</td>
</tr>
<tr>
<td>AVERAGE</td>
<td>68.46(0.92)</td>
<td>74.0(0.93)</td>
<td>71.17(0.99)</td>
</tr>
</tbody>
</table>

N=24
1 Means ± (SE)
2 commercial seeds were not available
Figure 4.16 R_{1} KAT maize genotype in the field showing relative earliness of R_{1} compared to control. Bar = 20cm
4.10 Germination tests of the $R_0$ seeds

There was no significant difference between the $R_0$ seeds and their commercial counterparts ($p > 0.05$). It was also observed that there was no significant difference between the germination rates of the $R_0$ genotypes used as well ($p > 0.05$). PH1 x HII was the highest rate of germination followed by DH02, then KAT and PH1 of 100%, 85%, 80% and 80% respectively (Table 4.6).

Germination under dehydration of $R_0$ seeds was significantly different from that of the controls ($p < 0.05$). The rate of germination was observed to be highest in KAT $R_0$ seeds followed by DH02 and PH1 Hybrid while PH1 and Control had 00% growths in PEG (Table 4.6).
Table 4.6 Percent germination of R₁ and commercial seeds in PEG and control.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Normal R₀</th>
<th>Control</th>
<th>Dehydration (0.05% PEG)* R₀</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>KAT</td>
<td>80</td>
<td>90</td>
<td>45</td>
<td>00</td>
</tr>
<tr>
<td>DH02</td>
<td>85</td>
<td>100</td>
<td>20</td>
<td>00</td>
</tr>
<tr>
<td>PH1</td>
<td>80</td>
<td>85</td>
<td>05</td>
<td>00</td>
</tr>
<tr>
<td>PH1 X H11</td>
<td>100</td>
<td>nd#</td>
<td>20</td>
<td>00</td>
</tr>
</tbody>
</table>

*Seeds grown in cotton wool saturated with PEG (8000)
# No commercial seed available thus not done.
4.12 Screening of the R₁ genotypes for drought tolerance

The R₁ genotypes were significantly different from their respective controls. Delayed ASI of tested maize genotype and controls was observed under dehydration. Anthesis Silking Interval of the control was 10 days on average compared to 8 days in the R₁s. When watering was resumed few controls maize plants recovered (figure 4.17b) while most of the R₁ recovered and set seeds. The survival rate of KAT R₁s after dehydration was 100% and 80% of them had silked ear and were successfully pollinated; 93% of the DH02 lines survived but only 39% produced silked ears and were successfully pollinated; of the PH1 lines 80% survived of which only 28% were successfully pollinated and set seed. The controls had lower survival and pollination rates compared to the R₁s (Table 4.7). The kernel and ear abortion rates were lower in the R₁s and higher in the control which translates to lower yield lose in R₁s but higher loses in the controls (Figure 4.17).
Table 4.7 Summary of drought tolerance of $R_1$ compared to Controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of plants</th>
<th>Plant survival (%$R_1$)</th>
<th>Plant survival (%$CONT$)</th>
<th>Pollination success (%$R_1$)</th>
<th>Pollination success (%$CONT$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KAT</td>
<td>30</td>
<td>30(100)</td>
<td>18(60)</td>
<td>15(80)</td>
<td>2(10)</td>
</tr>
<tr>
<td>DH02</td>
<td>30</td>
<td>28(93)</td>
<td>15(50)</td>
<td>11(39)</td>
<td>3(20)</td>
</tr>
<tr>
<td>PH1</td>
<td>30</td>
<td>24(80)</td>
<td>10(33)</td>
<td>5(28)</td>
<td>2(20)</td>
</tr>
</tbody>
</table>

1. Numbers (percent observation)
Figure 4.17 Stress experiments of R₁ and control.

(a) Control after 14 days of dehydration. Bar = 20cm (b) Rehydration of controls after 5 days. Bar = 20cm (c) Experimental setup of the R₁ and control. Bar = 20cm (d) Relative loss in yield due to water stress. 1 Stressed R₁, 2 Stressed control, 3 Unstressed control. Bar = 3cm.
CHAPTER FIVE

5.0 Discussion

In this study it was observed that regeneration of the Kenyan maize hybrid, DH02 and PH1 and PH1 X HII, as well as KATUMANI from calli is genotype dependent (Figure 4.2). The findings in this study are in agreement with earlier studies on callus induction and regenerability of Kenyan hybrid by Oduor et al., (2006). The fact that different maize genotype have different regeneration potential have been reported by Tomes and Smith, (1988), Bohorova et al., (1995) and Matthys-Rochon et al., (1998).

Although culture environment was maintained uniform for all genotypes at the time of regeneration the rate was low. It is possible that regeneration of plant from callus was affected by other cytoplasmic factors as suggested by Tomes and Smith, (1985).

in all the four genotypes regenerated some type II embryos from the calli that were put in shoot induction media did not form shoots even after subculturing into fresh media several times. Some of the embryos turned brown and died while others proliferated with subsequent subcultures. This was also reported by Bohorova et al., (1995) in tropical maize genotype.

Data presented in this study indicate that calli that had stayed in maintenance media for over six month were difficult to form shoots in all the four maize genotypes used. It was also observed that there was a negative correlation between calli age and rate of regeneration. This suggests that there is a decline in regenerability of cultures over time (figure 4.3). This is in agreement with studies by Carvalho et al., (1997) who observed similar results using calli of tropical inbred
lines maintained over long period of time. Kaepler et al., (2000) also observed that plant regeneration decreases with callus age. In addition to callus age other factors that have been reported to affect plant regeneration include the number of somatic embryos during callus subculture (Carvalho et al., 1997).

The mutant phenotype observed in this study such as dwarf plants, unbranched tassel, grained tassel, chlorophyll deficiency mutants and purple colored vegetative plant parts in the R₀ maize plants could be attributed to somaclonal variation and or to environmental influence. Such observation could be attributed to the fact that tissue culture induces stress to the genome leading to mutations (Filipecki et al., 2006). Several of the unstable or non-transmissible mutations that have been observed, indicate that transposable elements may be the basis of tissue culture induced mutations (Phillips et al., 1993) as well as allelic variation of gene expression (Guo et al., 2004).

Similar phenotypes to the ones observed in this study are found in the maizeGDB database http://www://maizeGDB.org. (Accessed April 20^{th} 2006) and Neuffer et al. (1997). The reports contain phenotype that have been characterized and obtained from studies done with induced mutation on either the seeds or pollen grains by use of ethylmethanesulfonate (EMS).

The dwarf mutant phenotype observed is similar to that in the maize database controlled by the dwarf (dwf) gene. The dwarf gene has been reported to control early flowering in temperate maize (Leitizia et al., 2006) thus they may be useful in conferring early flowering in our local maize hybrids which is necessary for drought tolerance.
The observation that many primary maize regenerants have seeds on the tassel is consistent with reports by Kaeppler et al., (2000). This may be an indication that developmental patterns are less tightly followed in plant development during tissue culture.

Only the tassel seed mutant and the pink coloration of vegetative plant parts was observed in the \( R_1 \) an in indication that these traits are genetic and inherited from one generation to the other. Tillers were also observed in the \( R_1 \) that was found to occur with the dwarf phenotype in the \( R_0 \). \( R_1 \)s showing tillering were relatively intermediate in height an indication that there was segregation in the height phenotype. Tillering is an inheritable variation that was passed from the \( R_0 \) to the \( R_1 \) maize plants (Figure 4.13).

Germination test of the \( R_0 \) seeds showed that the viability of the seeds from the regenerated maize performed equally compared to their commercial controls. The fact that the \( R_0 \) regenerants yielded viable seeds in this study is consistent with other studies reported by Lee et al., (1988).

Evaluation of the agronomic performance of the \( R_0 \) in this study showed that the plant height of the maize plants tended to decrease with the culli age. This is in agreement with the results reported by Lee et al., (1988) who observed that culture age of callus affect grain yield and plant height. It was also observed that both the \( R_0 \) and \( R_1 \) plants matured earlier than the controls (Figure 4.17) particularly KAT maize genotype an indication that tissue culture may generate variation for agronomic traits that may be useful. This observation is also in agreement with
findings by Lee et al., (1988). It was observed that the R₀ had an inferior agronomic performance in terms of plant height, ear height, tassel length, tassel branches and number of ears per plant. This is attributable to the stress induced by tissue culture process (Filipecki et al., 2006) and the degree of somaclonal variation in the maize genotype.

Evaluation of the R₁ revealed that the agronomic performance was relatively inferior to that of the controls (Table 4.3). The R₁ maize plants were observed to have a higher number of ears per plant than that of the control that might lead to higher yield. This observation is consistent with report by Diallo et al., (2001). Data presented in this study shows that ear height which is highly heritable remained relatively similar in the R₀ and R₁. The R₁ maize plants were also observed to have small tassels (short tassels with few branches) than the control an indication that they could be more droughts tolerant. (Banziger et al., 2000).

A short ASI is one of the characteristics of drought tolerant maize plants (Banziger et al., 2000). When the R₁s were tested for drought tolerance it was observed that the ASI of the maize plants increased due to delayed silking leading to kernel and ear abortion as a result of drought stress. Similar results were reported by Bolanos and Edmeades (1996) as well as NeSmith and Ritchie (1992). The observation that the different genotypes have different ASI under drought stress is in agreement with reports on the existence of genetic variability to drought in maize. (Vasal et al., 1997; Banzinger et al., 2000)

Germination test under dehydration using PEG showed that the different R₁ genotypes behaved differently under osmotic stress. This observation is in agreement with reports that species differ
in their ability to germinate under osmotic stress (Pesqueira et al., 2006). Salt tolerance in R₁ could also be by resistance to toxic effect of ions, but not to osmotic stress alone (Dolgykh et al., 1992). Genetic studies have shown that the relative ability to germinate in salt or osmotic solutions is controlled by many genes and is heritable (Flowers and Yeo 1995; Winicov, 1996).
5.2 Conclusion and Recommendation

5.2.1 Conclusion

Plants regenerated from calli that had been maintained for a long period of time had a negative correlation between number of plants regenerated and the time callus had been sub cultured. The degree of somaclonal variation of the R0 maize plants was assessed by using the morphological phenotypes observed. Many phenotypes generated by the tissue culture process had similarity to other reported phenotypes that had been characterized. The mutant phenotype could be harnessed if their genetic constitution could be ascertained to improve drought tolerance in Kenyan dry land maize hybrid. Sterility affected the performance of the R0, as most could not be self-pollinated in cases where male or female was observed sibing was done. The agronomic performance of the R0 was found to be inferior to that of the control. This could be attributed to the variation induced by the tissue culture process. Some of the R0s did not bear any cobs hence leading to lower yields in the regenerants. The R1 plants showed an improvement in terms of the parameters tested. They had a short ASI, higher numbers of ears per plant and small tassel size (short tassel and few branches) than the controls that is consistent with enhanced drought tolerant phenotypes.
5.2.2 Recommendation

The fact that tissue culture generated numerous and varying morphological phenotypes is of great importance to research and their value to plant improvement should be explored. More work should be done in the characterization of the mutants observed to ascertain whether their genotypes correspond to similar morphological phenotypes that have been characterized elsewhere. This could identify novel genotypes that could assist in the improvement in Kenyan dry land maize. More research needs to be done particularly the field-testing of the $R_0$ and $R_1$ to exhaustively evaluate the agronomical performance of the selfed progenies and even test the $R_2$ of the tested genotype. Plants tolerant managed stress need to be characterized to establish the genes responsible for the enhanced trait. Such maize adapted to drought will be desirable to most parts of the country that experience inadequate and erratic rainfall and will go a long way in the increased production of food in the ASAL of Kenya so as to make our country self sufficient in food production.
REFERENCE


Hall, B G (1990). Spontaneous point mutations that occurs more often when advantageous than when neutral. *Genetics* 126, 5-16.


Appendix

Appendix 1 maize growing zones of Kenya