Competence of Tropical Maize Lines to *Agrobacterium*-Mediated Transformation and the Expression of Maize Poly (ADP-Ribose) Polymerase (*PARP2*) Gene under Drought Stress.

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DECLARATION

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

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To Beatrice Ngami Maluki and Agnes Wayua Ndeleva.

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ACRONYMS AND ABBREVIATIONS

µg	Microgram
µl	Microliter
2,4-D	2,4-Dichlorophenoxyacetyl monohydrate
ABA	Abscisic acid
Agro	*Agrobacterium tumefaciens*
ANOVA	Analysis of variance
ASI	Anthesis silking interval
CaMV	Cauliflower mosaic virus
CCM	Co-cultivation Media
cDNA	Complementary DNA
CIM	Callus Induction Media
CIMMYT	Centro Internacional de Mejoramiento de Maíz y Trigo
CML	CIMMYT maize line
CYS	Cysteine
DAP	Days after Pollination
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DPE	Days Post-Emergence
GUS	β glucuronidase
H_2O	Water
H_2O_2	Hydrogen peroxide
IM	Infection Media

LS	Linsmaier and Skoog (1965) media
MARS	Marker-Assisted Recurrent Selection
MAS	Marker assisted selection
MS	Murashige and Skoog (1962) media
N6	Chu (1975) media
NaCl	Sodium chloride
ºC	Degrees celsius
OD	Optical Density
PARP	Poly (ADP-ribose) polymerase
PCR	Polymerase Chain Reaction
PRO	Proline
QTL	Quantitative trait locus
RM	Regeneration Media
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse-transcriptase Polymerase Chain Reaction
T_0	Zero generation transformant
Vir	Virulent
x-Gluc	5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid
YEP	Yeast Extract peptone

ABSTRACT

Drought stress affects many maize growing regions in sub-Saharan Africa. As population increases, the gap between maize supply and demand increases. There is, therefore, need to develop maize lines that are tolerant to drought as well as amiable to *Agrobacterium*-mediated transformation. This study aimed to assess the competence of tropical maize lines after co-cultivation on Yeats Extract Peptone (YEP) media to *Agrobacterium*-mediated transformation and determine the relative expression of *PARP2* gene under drought during development. Tropical inbred maize lines CML 144, CML 216, A04, and E04 as well as *Agrobacterium* strain EHA101 harbouring vector pTF102 containing the *GUS* reporter gene were used in this study. The ability of immature maize embryos to form embryogenic calli was determined after pre-culturing these embryos on YEP and Murashige and Skoog (MS) media. Transient *GUS* assay was used to evaluate the competence of the inbred maize lines to *Agrobacterium*-mediated transformation using YEP as co-cultivation media or YEP that was supplemented with growth regulator 2,4-D (YEP+2,4-D), Cysteine (YEP+CYS), Proline (YEP+PRO) or in combination (YEP+ALL). In all cases, MS media was used as the control. The optimum *Agrobacterium* concentration for infection of immature maize embryos before co-cultivation was determined. The physiological response of tropical maize lines to drought stress was evaluated and the expression of the *PARP2* gene at different drought levels determined by Polymerase chain reaction (PCR). The data collected was analysed using ANOVA at 95% confidence interval with SAS statistical computer software (version 9.1.3). Separation of means was carried out using Tukey's pairwise comparison at 5% probability level. Callus formation frequency and regeneration were genotype dependent (P= 0.0001). Immature embryos from the four inbred maize lines exhibited high transient *GUS* expression when co-cultivated with *Agrobacterium* on YEP (12.31%), YEP+PRO (13.75%) and YEP+ALL (8.68%) media than when co-cultivated on MS media (6.76%). Co-cultivation in YEP+2,4-D and YEP+CYS, however, resulted in lower transient *GUS* expression than on MS media. *Agrobacterium tumefaciens* at a concentration of 0.07 (OD_{660}) gave the highest transient *GUS* expression (20.90%) while higher concentrations of 0.2 and 0.8

resulted in low transient *GUS* expression (9.17% and 12.22%), suggesting that YEP media is superior to MS media in enhancing the competence of immature embryos to *Agrobacterium*-mediated transformation. Thus YEP media is proposed as an alternative media in in *Agrobacterium*-mediated transformation protocols. Growth rate, fresh weights of seedlings and dry weights of seedlings was low in seedlings subjected to severe drought stress compared to seedlings subjected to moderate drought and unstressed conditions. Severe and moderate drought stresses induced the expression of maize *PARP2* gene, suggesting that deregulation of maize *PARP2* gene is likely to improve the ability of tropical maize to resist severe drought stress condition.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the study

Maize plays a major role in the diet of more than 400 million people in the world. It is the most preferred crop in southern and eastern Africa and is cultivated on about 35 million Ha in Africa alone. Worldwide production of maize is estimated to be slightly over 1 billion tonnes with Africa contributing only 70 million tonnes (FAO, 2015). Third to wheat and rice, the importance of maize as a food crop in the world and especially Africa cannot be overlooked. It contributes significantly to food either as grain or flour although it has other uses including; ethanol production and as feed for animals (Ranum et al., 2014). In economic terms, maize generated about 35.5 billion and 38.8 billion US dollars in 2013 as export and import revenue worldwide hence its importance (FAO, 2015).

1.1.1 Origin and domestication of maize

Maize (*Zea mays* L. ssp. *mays*) is a member of the grass family Poaceae (Gramineae) together with other grasses like sorghum, oats, barley, rice and sugar cane which are thought to have originated from a common ancestor over 55-70 million years ago (Bennetzen et al., 2001). Maize originated 8700 years ago from a group of grasses in the genus *Zea* native to Mexico and South America which includes wild taxa commonly called Teosinte (*Zea mays ssp. parviglumis*). A more recent theory on the origin of maize is the *Tripsacum–Z. diploperennis* hypothesis developed in 1995 by Eubanks which challenges the idea that maize originated from Teosinte. In this theory, maize is a progeny of the cross between *Z. diploperennis* and *T. dactyloides* (Eubanks, 1995). Strong evidence has refuted this claim based on the chromosome numbers of the parents and that of the present day maize (Buckler & Stevens, 2005). The domestication of maize began in the Balsas River Basin of Mexico. Later, maize was introduced to America, Europe, Africa and Asia (Wilson et al.,1970). After the domestication of maize, many improvements have been done which have

subsequently ended up with today's maize hybrids which have the desired traits suited for modern agriculture. These improvements include kernel colour and size, texture, ear size and tolerance to insects and diseases (Johannessen et al., 1970). Today, maize is grown throughout the world as an alternative crop to rice and wheat. About 35% of the maize produced in the world is consumed by humans, 25% consumed by cattle and poultry while 15% is used as processed food (Eubanks, 1995).

1.1.2 The biology and genetics of maize

Maize is a tall, determinate, monoecious and annual C4 plant that produces large alternate leaves along the length of the stem and varies in height from 1 to 4 metres. It has a determinate growth with the main shoot terminating into a staminate tassel (Figure 1.1). Each male flower spikelet consists of two functional florets with each floret containing the lamella and palea, two lodicules, three anthers and a non-functional pistil. The female inflorescence commonly referred to as the ear/pistil develops from one of the lateral branches that originate from the auxiliary shoot buds. Normally, maize has three types of roots: the seminal roots which grow from the radicle, adventitious roots which develop from the lower stem nodes below the ground and the prop roots which develop from the lower nodes above ground for support. The root system grows rapidly outwards and downwards and in a good soil they can grow up to 60 cm long. Generally, a fully grown maize stem attains a maximum thickness of 3 to 4 cm with fairly short and thick internodes at the base and relatively longer and thicker internodes up the plant. The internode that bears the ear usually has a groove which allows for proper positioning of the cob. The maize grain is a caryopsis and contains two important structures: the germ which contains the radicle and plumule and the endosperm which stores food for the growing seedling (Buckler & Stevens, 2005). Maize has 10 chromosomes which consist of over 32000 genes. The qualitative genes control morphological traits like grain maturity, yield as well as pest and disease resistance but are very difficult to manipulate although easy to breed (Wei et al., 2007).

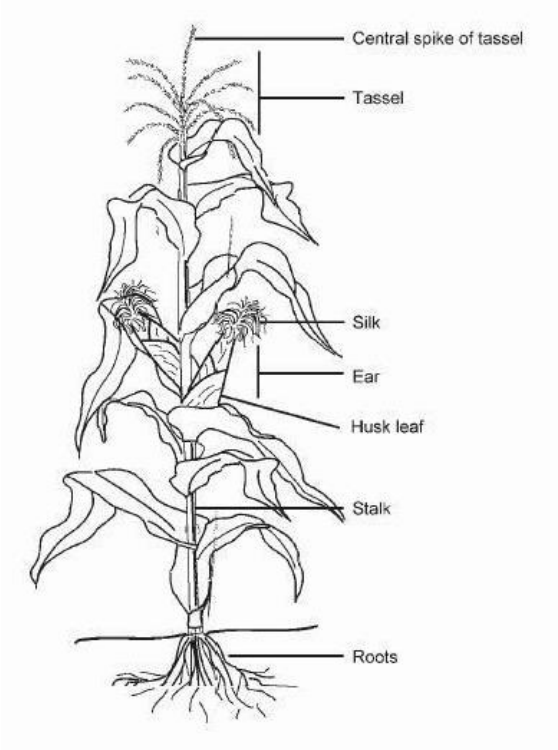

Figure 1.1: The maize plant.

(Source) http:/www.farmwest.com /book/export/html/30

1.1.3 Production, economics, and utilization of maize

Although maize is grown almost in the whole world, there exist significant differences in yield as shown in table 1.1 (FAO, 2016). World production estimates by 2013 stood at about 1,018,111,958 Tons with the United States, China, Brazil, Argentina and Ukraine harvesting over 71% of the world production. The entire Eastern Africa contributed only about 2.7% of the 1bn tons produced globally with the entire African continent contributing only 6.9%. The differences observed in production between developed and developing countries could be attributed to factors/practices by developed countries but lacking in the developing countries. These include the use of proper seed stocks, herbicides and pesticides, fertilizers and mechanisation compared to traditional technology in developing countries such as traditional breeding techniques and multi-cropping (Izuchu et al., 2009).

In Kenya, production estimates by 2014 stood at about 3,513,171 Tons, translating to just over 39.04 million bags. The crop is grown in all regions but the Rift Valley produces approximately half of the country's total maize production. Other key production regions include Western, Nyanza and Eastern which produce an average of 14% each of the total produce while the Central region contributes about 6% of the national tally. Annual consumption of maize is on the increase and currently stands at 49 million bags per year. The resulting shortfall is met through imports from other countries. (FAO, 2015)

Table 1.1: Maize production by country in 2013 (FAOSTAT, 2015).

Country	Maize production in 2013 (M. tons/year)
United states of America	353,699,441
China, mainland	218,489,000
Brazil	80,273,172
Argentina	32,119,211
Ukraine	30,949,550
India	23,290,000
Mexico	22,663,953
Indonesia	18,511,853
France	15,053,000
Canada	14,193,800
South Africa	12,486,000

1.1.4 Threats to maize production in sub-Saharan Africa

Maize production in sub-Saharan Africa is affected by biotic and abiotic conditions. Abiotic stresses adversely affect plant growth and trigger morphological, physiological, biochemical as well as molecular changes in the plant. Drought, temperature extremes and saline soils are the most common abiotic stresses in plants (Bhatnagar-Mathur et al., 2008). When plants are dehydrated, the production of

reactive oxygen intermediates/species (ROS) such as oxygen, superoxide anions, hydrogen peroxide (H_2O_2) and hydroxyl radicles increase. This eventually leads to the breakdown of vital cellular components such as proteins, lipids and nucleic acids, and subsequent cell death (Carvalho, 2008). Increasing temperatures, changing rainfall patterns and extreme weather conditions worsen the inability to meet maize demand. Soil salinity is another threat to maize production. Over 800 million hectares of land in the world are affected by either salinity or sodicity. Saline levels of more than 0.25M NaCl have been observed to severely affect maize growth, causing stunted growth and permanent wilting (Farooq et al., 2015). The problem is further aggravated by certain agronomic practices such as irrigation without appropriate drainage systems. Increasing salinization in agricultural fields is expected to reduce potentially arable land by 30% in the next 15 years and up to 50% by 2050 (Wang et al., 2003).

1.1.4.1 Abiotic stresses

Abiotic stress refers to an environmental condition that reduces the growth and eventually plants yields to levels below the optimum. It is difficult to quantify how these stresses affect plants but their impact cannot be ignored (Cramer et al., 2011). The most common abiotic stress affecting maize production in Africa includes water stress, high temperatures and unfavourable soil conditions.

Water stress is mostly manifested as drought and affects vast regions in sub-Saharan Africa (SSA), with 22% of sub-tropical regions and 25% of lowland tropical regions experiencing drought annually. Drought is responsible for yield losses of up to 15%, 53% and 30% in SSA when it occurs at pre-flowering, flowering and post flowering in maize (Buckler & Stevens, 2005). Although it is difficult to measure the economic impacts of drought stress, the obvious effects include reduced maize yields, which may cause 100% crop loss under severe drought.

Heat stress refers to the increase in temperatures beyond a certain point sufficient to cause irreversible damage to plant growth and development. Tolerance to heat, on the other hand, refers to the plant's ability to produce significant yields irrespective of the high ambient heat (Wahid et al., 2007). Heat causes crop damage as a result of the destruction of cellular organization. These damages may both be direct or indirect and include; protein denaturation, increase in fluidity of lipid membranes, inactivation of enzymes and inhibition of protein synthesis. Maize has an upper heat threshold of 38ºC. Towards and beyond this limit, many physiological aspects are affected and eventually yield decreases. Known physiological changes include; increased pollen damage, reduction in the rate of grain filling as a result of reduction in the rate of cell division, reduced starch biosynthesis, and sugar metabolism as well as reduced growth rate (Cairns et al., 2012).

Soil stresses in crop production are diverse. However, there are some common soil stresses encountered in SSA such as excess salts, water logging and metal toxicity. Salts pose a great challenge to plants since their excessive accumulation causes cytotoxicity, prevents the synthesis of enzymes and proteins, affects membranes structure and permeability, causes the production of reactive oxygen species (ROS) in plants and can lead to plant death as a result of inhibition of photosynthesis (ISAAA, 2007).

1.1.4.2 Biotic stresses

Any stress that occurs due to substantial damage to a plant by a living organism is referred to as biotic stress. Biotic stresses account for a great portion of maize yield losses in Africa where many farmers cannot afford the cost of controlling the stress causing agent. Pests and diseases are the most common biotic stresses in any crop production system in the world (Cairns et al., 2012). Most microbial pathogens causing maize crop diseases of economic importance in Africa are of fungal origin although viruses and bacteria also cause significant losses. Maize diseases such as

the Turcicum leaf blight, Gray leaf spot (Northern leaf blight), Crazy top disease (Downy mildew), Head smuts and the common rusts are all caused by fungal pathogens. The maize streak virus, maize dwarf mosaic virus and the maize lethal necrosis are caused by viruses. The Stewart's disease (Bacterial wilt) and bacterial leaf spots are caused by bacteria (Guantai et al., 2010). Insects such as moths, the African maize stalk borer and earworms, grain borers and weevils, leaf bugs, maize fleas and aphids have been reported to cause devastating losses in maize (Ortega, 1987). In 2013 alone, 25% of maize crop yield in Africa was lost to diseases. Coupled with pests, some countries have reported up to 45% crop loss (Magomba, 2013). Striga (witchweed) is a parasitic weed which constraints maize production and yield significantly in SSA. It infests over 40 million hectares in Africa and causes yield losses of 20-80% and sometimes total crop failure (Kim et al., 2002).

1.1.5 Approaches to enhance maize production

Maize improvement has for a long time depended on conventional breeding and selection, which in most cases aims at only improving yield. This strategy is limited by the existing low genetic variation, high costs and the long generation time it takes to produce a single improved selection (Gao et al., 2013). Grain yield as a trait is influenced by many factors such as growth rate and nutrient supply. Since many conventional breeding programs include grain yield as a parameter for selection, there is need to understand the components that contribute to higher yields so as to improve the entire selection process (Bruce, 2002). Recent improvement strategies include resistance to biotic and abiotic stresses in addition to grain yield as selection strategies. Today, use of molecular techniques in maize improvement is on the increase. Most of the commonly used techniques include; marker assisted selection (MAS), functional genomic tools and transformation. *Agrobacterium tumefaciens* mediated and biolistic gun methods have been used as transformation techniques with great success in maize. The first method being preferred to the latter because it results in a greater portion of stable, low copy number transgenic events and is highly efficient (Buckler & Stevens, 2005).

1.2 Statement of the problem

Agrobacterium-mediated transformation is rapidly gaining use in maize improvement to deliver important genes to maize systems. To achieve this, *Agrobacterium tumefaciens* carrying the gene of interest are co-cultivated together with immature maize embryos to deliver these genes to the explant. However, data indicates that there is very low efficiency of T-DNA transfer to immature maize embryos when using MS based co-cultivation media (Ombori et al., 2013b; Yu et al., 2013). Attachment of *Agrobacterium* to the immature embryo is key to the transfer of T-DNA and therefore, lack of attachment contributes to the recalcitrance of immature maize embryos to *Agrobacterium*-mediated transformation. When subjected to drought stress, maize accumulates PARP through an energy depleting process (De Block et al., 2005). However, the precise level of drought stress which triggers the expression of this gene remains unknown and the fundamental mechanisms of drought tolerance in maize are not fully understood.

1.3 Justification

MS, LS, and N6 media have been used at the co-cultivation stage in tissue culture during *Agrobacterium*-mediated transformation. However, although this method promises stable integration among other advantages, resulting transformation frequencies are low. Immature tropical maize embryos continue to show high recalcitrance which could be attributed to the continued use of these media which do not support the optimal growth of *Agrobacterium*. YEP does support the optimal growth of *Agrobacterium* and therefore it would be important to determine whether YEP media alone and in combination with other media components could be used as an alternative co-cultivation media in tissue culture during *Agrobacterium*-mediated transformation. This could enhance *Agrobacterium* attachment and consequently transfer of T-DNA.

Maintenance of energy balance in plants through down regulating the expression of *PARP* genes is one of the approaches which has been used to achieve drought tolerance in maize (Vanderauwera et al., 2007). Although this approach promises success there is need to first determine the precise levels of drought stress which induce the expression of *PARP* genes. This knowledge would determine the necessity to down regulating the gene in maize, which would lead to saving on resources. This study sought to determine the precise levels of drought stress that trigger the expression of *PARP2* gene in maize as well as optimise YEP as a co-cultivation and callus induction media for use in maize transformation. It also sought to assess the competence of tropical maize to *Agrobacterium*-mediated transformation.

1.4 Null hypotheses

1. YEP media does not affect callus induction of tropical maize.
2. YEP media does not enhance transformation efficiency in immature maize embryos.
3. *PARP2* gene expression does not differ under different stress conditions.

1.5 Objectives

1.5.1 Broad objective

To determine the competence of tropical maize lines to *Agrobacterium*-mediated transformation and relative expression of maize *PARP2* gene under drought stress.

1.5.2 Specific objectives

1. To optimise YEP as an alternative co-cultivation and callus induction media using immature tropical maize embryos.
2. To determine the competence of immature tropical maize embryos to *Agrobacterium*-mediated transformation using YEP media.

3. To determine relative expression of *PARP2* gene under different drought conditions in maize during development.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Drought tolerance mechanisms in maize and other cereals

Drought refers to the prolonged lack of precipitation over a period of time. On the other hand, drought tolerance in plants is the ability of a plant to grow, flower and eventually bear yields at water levels below the optimum (Farooq et al., 2009). For a plant to register water deficit, its rate of transpiration must exceed its rate of absorption and once this happens, damage or adaptation responses are triggered.

2.1.1 Physiological mechanisms of drought tolerance

Plants respond to water deficit through wilting, reduction in leaf area, leaf abscission, stimulating the growth of roots and induction of stomatal closure by the production of abscisic acid (ABA). These observable changes are enabled by a number of mechanisms such as water conservation by cells and tissues, antioxidant defences, maintenance of cell membrane stability, use of plant growth regulators and through osmotic adjustments (Waseem et al., 2011).

When there is water deficit, osmotic adjustments by cells and tissues allow the plant to decrease osmotic potential and maintain turgor pressure which consequently increases the gradient of water influx into these cells. These osmotic adjustments enable cells to maintain water balance by the accumulation of solutes in their cytoplasm and thereby reduce the harmful effects of drought such as dehydrative damage. The resulting positive effects of osmotic adjustments by cells include better translocation of carbohydrates during grain filling, higher photosynthesis and a higher growth rate (Farooq et al., 2009).

An antioxidant refers to the activity possessed by substances like vitamins, secondary metabolites and other phytochemicals present in the plant to protect it from the

damaging effect of reactive molecules such as ROS. These antioxidant defences include enzymatic components such as superoxide dismutase, catalase, peroxidase, ascorbate peroxidase and glutathione reductase. They also include non-enzymatic components like cysteine, reduced glutathione, ascorbic acid, α-tocopherol, carotenoids and phenolic compounds (Jaleel et al., 2009). Generally, antioxidant enzymes as well as lipid/water soluble scavenging molecules remove ROS from plants. The non-enzymatic antioxidants are the most efficient in plant defence against oxidative stresses and most of them do this by electron or hydrogen donation. They are mostly involved in the ascorbate-glutathione cycle, a superoxide radicals and H_2O_2 scavenging pathway (Sharma et al., 2012).

Since most abiotic stresses initially target cell membranes, maintaining the stability and integrity of these membranes under stress is a vital component of drought tolerance in plants. As shown by Premachandra et al. (1990) potassium nutrition in maize improves drought tolerance due to increasing stability of cell membranes and osmotic adjustments.

Plant hormones influence the physiological processes of plants at very low concentrations. When plants are subjected to drought, auxins, gibberellins and cytokinin decrease while there is an increase in abscisic acid (ABA) and ethylene (Buckler & Stevens, 2005). Auxins induce the formation of new roots by reducing the root apical dominance caused by cytokinins. The increase in root-to-shoot ratio helps the plant cope with drought. In maize, drought and application of ABA increase the synthesis of indole-3-butyric acid (IBA), a naturally occurring auxin which increases net photosynthesis and stomatal conductance (Kumar et al., 2001). ABA and cytokinins have opposing roles in drought stress. Increase in ABA and decrease in cytokinins cause stomatal closure and reduce the amount of water lost through transpiration under drought stress. Under drought stress, cereals produce ethylene which brings about the onset of drought-mediated leaf senescence.

2.1.2 Morphological mechanisms

Drought response in plants involves changes at the whole plant, tissue or molecular levels. Therefore, the ability of the plant to survive drought depends on the manifestation of one or a combination of these changes. The morphological mechanisms to drought tolerance include escape, avoidance and phenotypic flexibility. Plants escape drought by shortening their life cycles and therefore reproduce before the onset of drought. Escape from drought will only occur when the plant development is synchronised with periods of water/moisture availability in regions where the plant's growth period is shorter and the terminal drought stress period is longer (Araus, 2002). In other plants, leaf shedding in response to drought stress occurs. In coffee, for example, leaf shedding occurs beginning with the older leaves and subsequently younger leaves with drought tolerant clones experiencing lower extents of leaf shedding than susceptible clones (DaMatta, 2004).

Drought avoidance mechanisms involve all those mechanisms that enable the plant reduce the loss of water due to enhanced stomatal control of transpiration as well as maintaining a constant water uptake through an extensive root system (Turner et al., 2001; Kavar et al., 2008). Root characteristics such as biomass, length, density and depth are known avoidance traits that enable a plant to survive in drought environments. Wax on leaves or glaucousness is another trait known to contribute significantly to plants ability to withstand drought. It has been shown that in wheat different degrees of glaucousness increase the plant's water use efficiency at the same time not affecting the plants harvest index (Richards et al., 1986).

Under drought conditions, morphologically, the plant's shoot and root systems play an important role towards its survival. Plants tend to reduce the number and the size of leaves to reduce the net transpiration rate as well as increase the density, size and growth rate of roots to increase its water scavenging ability (Richards et al., 1986; Kavar et al., 2008). Plants with smaller leaves are well adapted for environments

with less water although their growth rate and biomass are relatively lower. Leaf pubescence is an important phenotypic trait which enables plants to cope with drought environments. The hairs on plant leaves enable the plants to withstand higher temperatures as they protect the leaves from excessive water loss (Buckler & Stevens, 2005). These hairs also increase light reflectance thereby reducing water loss by increasing the resistance of the boundary layer to water vapour movement from leaf surfaces (Farooq et al., 2009).

2.1.3 Molecular mechanisms

When a plant experiences drought stress, certain changes in gene expression occur. There is up and down regulation of different genes which leads to the desired response by the plant. Different genes are induced in response to drought stress in cereals and other plants, whose proteins function to effect tolerance to the growth limiting water deficit (Kavar et al., 2008; Briggs and Bent, 2011). Whether gene expression is triggered directly by drought stress or other secondary stresses such as injury, it is generally accepted that response to drought is a complex phenomenon which involves the action of many genes (Cattivelli et al., 2008). Aquaporins, stress proteins and certain chemical signals are examples of gene products synthesised by plants in response to drought.

Aquaporins are integral membrane proteins conserved in bacteria, plants and animals with the ability to facilitate the passive exchange of water and other solutes across membranes (Tyerman et al., 2002). The discovery of aquaporins in plants led to very important contributions to the understanding of plant water relations although to this date their relation to plant drought tolerance is not fully understood. However, It has been shown that aquaporins play a role in the water uptake by plants as well as osmoregulation which could possibly help plants cope with drought and salinity stresses (Aharon et al., 2003; Maurel & Chrispeels, 2015).

Plants under stress have been shown to synthesize several stress proteins and transcription factors to enable them cope with these stresses. Some of these include the dehydration-responsive element binding gene2 which has been shown to be induced by dehydration stress (Choi et al., 2002). Studies have shown that cloning dehydration-responsive element binding gene transcriptional factor improves drought tolerance significantly (Tran et al., 2004). Another group of stress proteins produced in response to stress are the heat shock proteins which play a role in the stabilization of other proteins under heat. These proteins are generally produced in response to high environmental temperatures though other studies have shown that they are also produced under drought, low temperatures and in environments with insufficient oxygen. These proteins protect others from heat damage by participating in ATP-dependent protein assembly/disassembly reactions, subsequently preventing protein denaturation due to heat stress. Other proteins produced under stress include the membrane-stabilizing proteins and late embryogenic abundant (LEA) proteins. These two groups of proteins increase the ability of other proteins to bind water by synthesizing dehydrins/group 2 LEA proteins which accumulate in maturing seeds or vegetative tissues under drought or salinity stress. Membrane-stabilizing proteins and LEA proteins also function in sequestrating concentrated ions which accumulate when cells dehydrate (Gorantla et al., 2007).

The signalling process which senses stress in plants involves a complex of chemical substances. These include chemical signals such as reactive oxygen species (ROS), calcium and plant hormones which activate genomic reprogramming, mitogen-activated protein kinases which connect external stimuli to cellular machinery and calcium which acts as a secondary messenger. Other chemical substances involved include; calcium-dependent protein kinases which decode calcium ion signals in plants and several phospholipid systems which are activated by osmotic stress to generate a series of messenger molecules (Chen et al., 2002).

2.2 Progress in improving drought tolerance in maize

The identification of genetic variants which are heritable and respond to drought stress in any breeding population is the first and most important requirement to achieving progress toward drought stress tolerance. Drought stress alleles occur at very low frequencies in a breeding population, hence the importance to evaluate these populations before any work can commence (Blum, 2011). Although natural populations may possess these unique alleles, they are often limited by their undesirable traits such as low yields and poor adaptability (Edmeades, 2013). The most important primary trait in any breeding population is grain yields under stress and unstressed conditions. Other important secondary traits to consider include; leaf erectness, canopy temperature, the number of kernels per cob and kernel weight (Jansen, 2012). The most commonly used strategies to transfer drought tolerance to maize include conventional breeding, marker-assisted selection, and the genetic engineering approaches. The collective effort of molecular biology, plant physiology and classical breeding are required to develop stress tolerant plants.

2.2.1 Conventional breeding

After the advent of plant biotechnology and molecular biology, plant breeding began being referred to as "conventional breeding" or "classical breeding". In classical breeding, new gene combinations are created by crossing parents that possess desirable traits or otherwise by introducing new germplasm from a new environment or a breeding program followed by selection of new variants that perform better in the target environment (Blum, 2011). Conventional breeding in maize for drought stress tolerance has been achieved through intra-population and inter-population improvement schemes as well as backcross breeding. Through these approaches, drought tolerance and increased yields in tropical maize populations has been achieved at the International Maize and Wheat Improvement Centre (CYMMYT) (Edmeades, 2013). From these improved populations, inbred lines which are tolerant to drought and with other desirable traits such as reduced Anthesis Silking Interval (ASI), barrenness and senescence are picked and used as parents in breeding

programs to develop drought-tolerant hybrids (Banziger et al., 2004). After this, drought-tolerant hybrids are constantly selected after being subjected to controlled drought conditions.

2.2.2 Marker Assisted Selection (MAS)

Molecular mapping for tolerance to drought has been in practice since the 1990s, addressing plant traits which are related to drought such as high yields under drought, favourable root morphology as well as osmotic adjustments (Blum, 2011). In maize, this technique started with the discovery of the association between markers and traits in the early 1970s which later enabled the development of genetic maps. In addition to marker technologies, phenotyping and statistical tools are essential in the development of quality marker-trait associations, commonly referred to as Qualitative Trait Loci (QTL) (Ribaut & Ragot, 2007). In MAS, genetic markers are used instead of phenotypic traits to select desirable plants which eventually end up accelerating the breeding cycle and the eventual release of new cultivars. The commonly used MAS approaches to improve drought tolerance in maize are Marker Assisted Back Crossing (MABC) and Marker Assisted Recurrent Selection (MARS). MAS has enabled breeders to significantly reduce the amount of field testing required to develop a new cultivar. The genotype-phenotype associations upon which this technique is based heavily depends on accurate phenotyping (Edmeades, 2013).

MABC is a breeding technique which involves the transfer of only a few genes such as a drought tolerance transgene or a loci into an adapted variety. In most cases, the backcrossing parent has a large number of desirable characteristics and lacks only a few characteristics of interest to the breeder. This technique was applied in the improvement of an inbred line CML 247 which is susceptible to drought by backcrossing it with a drought tolerant line Ac7643 by Ribaut and Ragot, (2007). CML 247 is a tropical inbred line which is high yielding under good watering

conditions but has a very large ASI under drought conditions which might lead to the loss of the desired traits. After backcrossing, the resulting hybrid was evaluated and found to yield at least 50% higher yield than the control hybrids under high water stress although less than the controls at low stress (Ribaut & Ragot, 2007).

MARS, involves combining several genes into one genotype by identifying and selecting genomic regions with relatively complex traits like drought tolerance and yield, as well as, disease resistance within a population. This technique is mostly used to breed for disease resistance in maize with an aim of developing stable disease tolerant genotypes since it has been observed that most pathogens overcome disease resistance driven by single genes as a result of the emergence of new pathogen races (Collard & Mackill, 2008). A pathogen might not easily overcome more than one effective gene by just mutating but may easily overcome the resistance of a single gene after it mutates. Previously, it was difficult to combine more than one resistance gene in a single genotype because these genes have a similar phenotypic resemblance. However, with the advent of DNA markers, the number of resistance genes in a single plant can now be identified with ease. This technique was used to improve maize kernel colour in maize line L-14-4B by crossing it with maize inbred lines L-08-05. The donor parent (L-08-05) was early-maturing and had orange flint kernels while the recurrent parent was also early maturing but had yellow dent kernels. The progeny obtained after backcrossing using the recurrent parent phenotypically resembled the recurrent parent but had orange flint kernels (Benchimol et al., 2005).

MAS techniques in plant breeding experiences a number of drawbacks. The most critical challenge being the inability to predict a phenotype of a given genotype in a segregating population based on the constitution of alleles at a given loci. In addition, QTL mapping is not very accurate for complex traits like yield which are controlled by many QTLs (Ribaut and Ragot, 2007).

2.2.3 Genetic engineering

Several attempts have been made to enhance maize tolerance to drought through the genetic engineering approach. Transgenic maize highly expressing C4 phosphoenolpyruvate carboxylase (C4-PEPC) gene showed increased water use efficiency and dry weight when subjected to moderate drought conditions (Jeanneau et al., 2002). Shou et al. (2004) produced transgenic maize plants expressing the Nicotiana Protein Kinase (*NPK1*) gene which exhibited improved tolerance to drought. The production of the *NPK* protein induces the expression of genes responsible for the production of Heat Shock Proteins (HSPs) and Glutathione-*S*-transferases (GSTs) in maize which protects the photosynthetic machinery from drought-induced damage. Engineered maize with *betA* gene by Quan et al. (2004) showed drought tolerance at seedling, germination and reproductive stages. Maize transformed with the maize transcription factor ZmNF-YB2 exhibited an improved tolerance to drought stress and an increase in yield under severe water stress (Nelson et al., 2007). Transgenic maize referred to as DroughtGard was engineered with a gene *cspB* which codes for proteins that stabilize the mRNA under stresses like low temperature in the common soil bacteria *Bacillus subtilis*. DroughtGard maize has exhibited increase productivity during the vegetative growth and grain yield under water-limited conditions (Castiglioni et al., 2008).

2.3 Poly (ADP-Ribose) Polymerase genes in maize

Poly ADP-Ribose (PAR) is a protein mediated by the Poly (ADP-Ribose) Polymerase (PARP) enzyme which plays a role in cellular response to stresses such as drought, salinity and heat. Its synthesis is almost always directly proportional to the level of stress and determines one of the many possible responses depending on the severity of the stress. Possible responses include; cellular defence under mild stress, DNA repair under moderate stress and cell death under severe stress. PARP is formed from two pathways which are both energy consuming hence one of the main causes of cell death under severe stress. Both pathways involve depletion of nicotinamide adenine dinucleotide (NAD^+) as a substrate hence subsequent

utilization of ATP (Hashida et al., 2009). *PARP* genes in plants are homologous to their mammalian counterpart. In transgenic *PARP*-deficient plants, disruption of the gene results in a broad spectrum abiotic stress tolerance. The reduction of ATP consumption avoids extensive respiration in the mitochondria and therefore inhibits the formation of Reactive Oxygen Species (ROS) which form under almost all abiotic stress conditions (Vanderauwera et al., 2007). Maize and other higher plants have two genes which encode PARP homologous proteins. The maize *PARP1* gene product is 55% identical and 64% similar to the human PARP1 homologue, with two zinc-finger DNA binding domains and is about 110 kDa. The other gene, maize *PARP2*, encodes a gene product which is similar to the catalytic domain of PARP2 protein in mammals, except for its N terminal which does not show any homology to other known proteins. The maize PARP2 has a molecular mass of about 73 kDa and is 61% identical and 69% similar to the *A. thaliana* PARP2 protein (Babiychuk et al., 1998).

2.3.1 Role of PARPs in stress and energy consumption

Energy depletion in drought stressed maize plants occurs as a result of the accumulation of PARP1 and to some extend PARP2. The resulting energy depletion is due to the poly (ADP-ribosyl) ation of PARP through a pathway which entails the transfer of ADP-ribose moieties from NAD^+ to residues of glutamic acid in proteins followed by additional transfer of ADP-ribose monomers into the newly formed products. This reaction is reversible and is catalysed by glycohydrolase (PARG) enzymes which catalyse the hydrolysis of the glycosidic bonds of the ADP-ribose polymers to produce free ADP-ribose (Lamb et al., 2012). Free ADP-ribose metabolites are quite toxic and cause lethal mutations to *PARG* genes hence in most cases they work to promote the activity of *PARPs*.

PARGs have not been extensively analysed in plants although, in *Arabidopsis thaliana*, two genes have been shown to encode putative PARGs. It has been shown

in that *A. thaliana PARG1* is important in plants in coping with oxidative and osmotic stress. This was done by analysing the activity of *PARG1* mutants and comparing their response to drought, stomatal activity under stress as well as oxidative stress tolerance in comparison to wild-type plants (Li et al., 2011). Accumulation of PARPs, therefore, leads to depletion of cellular ATP in plants, necrosis in cells and consequently death of the whole plant.

2.3.2 Tolerance to multiple stresses in plants by inhibition of *PARP*

Studies have shown that inhibition or down-regulation of *PARP*s confers tolerance to abiotic stresses in plants (Block et al., 2004; Vanderauwera et al., 2007; Schulz et al., 2012). Different strategies have been used to disrupt *PARP* such as the RNA interference technique which was used to downregulate the expression of *PARP2* in *Arabidopsis thaliana* and *Brassica napus* and thereby increased tolerance of these plants to multiple oxidative stresses (Vanderauwera et al., 2007). This was confirmed by an increase in NAD^+ content in stressed plants as well as the up regulation of ABA-related gene expression which consequently increased the amount of secreted ABA by the transgenic plants. This resulted in the expression of a wide set of defence related genes and improvement in energy homeostasis thereby conferring tolerance to the transgenic plants (Vanderauwera et al., 2007). Chemical inhibition of *PARP* using 3-Methoxybenzamide (3 MB) has also been done in *Arabidopsis thaliana*. The resulting effects were an improvement of growth and abiotic stress tolerance in the transgenic plants. This was a result of alteration of gene expression and levels of metabolites enabling the plants to cope with these stresses (Schulz et al., 2012).

2.4 *Agrobacterium tumefaciens* mediated transformation in maize

Conventional breeding of maize is limited by many factors such as the intensiveness of the labour involved, the time it takes and the complicated genetics that controls the traits to be improved (Yang et al., 2010). Today, genetic modification of maize is

possible due to advances in technology. Two major gene delivery techniques have been developed to enable genetic modification. These are the direct DNA delivery technique which includes electroporation and polyethylene glycerol methods, particle bombardment and silicon carbide whiskers. The other technique is the *Agrobacterium*-mediated transformation (Wang et al., 2009). The first fertile transgenic maize was produced in the late 1980s through particle bombardment (Gordon-Kamm et al., 1990) and at the time, direct DNA transfer techniques were preferred to the *Agrobacterium*-mediated technique due to *Agrobacterium* recalcitrance to monocots. In 1996, a new protocol was developed for *Agrobacterium*-mediated maize transformation which was superior in terms of high frequency of transformation and its ability to insert single copies of inserts (Ishida et al., 1996). Today, *Agrobacterium*-mediated transformation remains the preferred method by many scientists.

2.4.1 The genetic basis of *Agrobacterium* pathogenesis

Agrobacterium tumefaciens is a Gram-negative, rod-shaped widespread soil bacteria which naturally causes crown galls in over 41 families of plants (Kado, 2014). Today, disarmed strains of these bacteria have found use in agricultural biotechnology by providing a way of producing genetically modified crops. The natural *A. tumefaciens* mediated genetic transformation from the plant's viewpoint is a six step process which begins by *Agrobacterium* attachment and ends when a segment called the T-DNA from the Tumour inducing (Ti) plasmid is transferred into the nucleus of the infected cell (Gelvin, 2010) as shown in Figure 2.1.

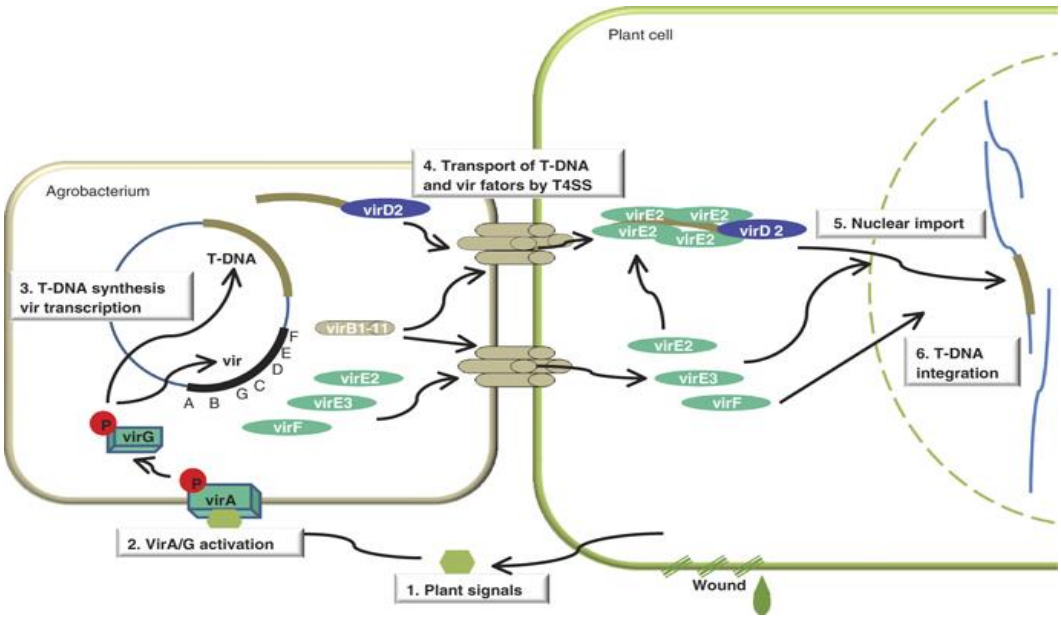

Figure 2.1: Major events in *Agrobacterium*–plant interaction.

1. Signal induction by the plant 2. Activation of VirA/G 3. T-DNA synthesis and Vir gene expression. 4. T-DNA and Vir transfer into the plant cell 5. T-DNA complex importation into the host cell nucleus 6. T-DNA integration into the host chromosomes. Source (Pitzschke & Hirt, 2010).

During attachment, it is thought that capsular polysaccharides produced by the *att* region play an important role (Reuhs et al., 1997). After attachment, induction of the bacterial virulence system follows. This *vir* region contains six essential and two non-essential operons. *Vir A* proteins detect phenols produced by the wounded plant such as acetosyringone and other monosaccharides which act synergistically with it (Winans, 1992). *Vir G* controls the expression of the other *vir* genes after phosphorylation by *Vir A*. The third step involves generation of the T-DNA transfer complex. At this stage, a single-stranded nucleic acid molecule which is a copy of the bottom T-DNA strand is generated, controlled by *vir D1* and *Vir D2* gene. Once the ssT-DNA-VirD2 complex is ready, the next step involves its transfer into the nucleus of the host plant. During the transportation process, *Vir B* proteins form a channel through which the T-DNA-protein complex passes from the bacteria to the

host while *Vir D4* generates ATP for the transfer process (De La Riva et al., 1998). Once the T-DNA is inside the plant host cell, it has to find its way into the nucleus. This is facilitated by a number of Vir and host plant proteins. The Vir proteins involved are VirD2 and VirE2 which code for plant-active nuclear localization signal (NLS) sequences. Importin α and cyclophilin family of proteins are the plant proteins involved in this process (Pitzschke & Hirt, 2010). Once the T-DNA is inside the nucleus, stable integration into the host genome is directed by *VirD2* which ligates the T-strand 5' end of the host chromosome.

2.4.2 Plants defence against *Agrobacterium tumefaciens* attack

There are many factors that affect *Agrobacterium*- mediated transformation such as the strain of *Agrobacterium* in use, the density/concentration of the bacteria, plant growth hormones and antibiotics, plant species/genotype, explant and explant wounding, light and temperature (Karami 2009). The multiplicity of the factors in monocots especially maize could be a contributing factor as to why this mode of transformation in maize has been limited. Other factors thought to reduce *Agrobacterium* transformation efficiency are based on the plant's inherent defence mechanisms such as the production of some chemical components which inhibit the growth of *Agrobacterium*. As observed by Carvalho et al. (2004), immature embryos of most sorghum genotypes produce chemicals, hypothesised to be phenolic compounds which inhibit the growth of *A. tumefaciens*.

2.5 Regeneration of tropical maize

Several attempts have been made towards the regeneration of tropical maize genotypes. In Kenya, all the tested maize genotypes resulted in successful callus formation and subsequent regeneration through different basal medium compositions and growth regulators (Abebe et al., 2008; Akoyi et al., 2013; Anami et al., 2010; Gorji et al., 2011; Odour et al., 2006). However, in cases where growth regulators were omitted in basal media when using both mature and immature embryos, there

was no callus formation, hence no regeneration. Regenerating maize plantlets through somatic embryogenesis of obtained calli remains the most difficult task, in view of genotype dependent nature of the regeneration of tropical maize.

2.6 Yeast Extract Peptone media in growth of *Agrobacterium*

In order to transform any plant tissue with a transgene harboured in *Agrobacterium*, an explant must be co-cultivated with *Agrobacterium* in a suitable medium and the appropriate conditions provided for a period of time. The composition of media is, therefore, an important factor towards achieving acceptable transformation frequency and efficiency. All successful plant tissue culture systems depend greatly on the choice of nutrient medium used (Frame et al., 2006). Murashige and Skoog (MS), Linsmaeir and Skoog (LS) and Chu (N6) media have all been used in *Agrobacterium*-mediated transformation of maize for infection, co-cultivation and regeneration (Du et al., 2010). Optimal *Agrobacterium* growth is however not supported by all these media at the co-cultivation stage hence this could be a contributing factor to the high recalcitrance of inbred maize embryos to *Agrobacterium*-mediated transformation. Yeast Extract Peptone (YEP) media has been shown to support the growth of *Agrobacterium* and promising results have been obtained based on transient *Gus* expression when *Agrobacterium* is grown on YEP before infection (Ombori et al., 2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Plant Materials

Four white seeded tropical maize inbred lines; CML 216, CML 144, E04 and A04 used in the study were planted in plots in the research fields of the Jomo Kenyatta University of Agriculture And Technology. The seeds of all the maize inbred lines were provided by the Kenyatta University Plant Transformation Laboratory. On reaching physiological maturity, these plants were self-pollinated and some ears were harvested 14 days after pollination (DAP) and used either immediately or stored at 4^{o}C for a maximum of three days as described by Frame et al. (2002). The remaining ears were bulked to provide seeds for drought stress experiments. The maize lines were selected due to their desired traits. CML 144 and CML 216 are generally late maturing, resistant to maize streak virus and insect pests, are tolerant to acidic soils as well as adapted to the tropical climate. These lines do not grow very tall hence are easy to pollinate. Previous studies show that they are regenerable through tissue culture as well as relatively better to transform than other inbred lines. In this study, CML 216 was used as the control for all transformation and regeneration experiments. A04 and E04 are Kenyan inbred lines commonly used as parental lines of hybrids and are successful in highland environments (Beyene et al., 2016).

3.2 Drought stress experiments

Well dried seeds harvested after self-pollinating inbred lines were soaked in distilled water overnight and planted in vermiculite in a large plastic pots in a clear glass screen house with average day temperatures of 19 ºC. After the 5th day when all seeds had germinated, and after the emergence of the first leaf, individual seedlings were transferred into small plastic pots measuring 10cm by 10cm by 20cm containing about 250g of soil. The soil had first been air dried and filled into the small pots to fill two-thirds of the perforated pots. These pots were then put in

containers with water overnight to allow them to absorb the maximum amount of water. The following day, the pots were removed from the water containers and seedlings transplanted into them. They were then allowed to lose the excess water for another 24 hours to field capacity and the weights of the pots determined. The amount of water in the soil was determined by the following equation:

$$water\ content\ (g) = (pot\ weight + wet\ soil) - (pot\ weight + dry\ soil)$$

This determined water content was maintained throughout as the water content for the control experiments. Water content for moderate stress experiments was maintained at 50% that of the controls and water content for severe stress maintained at 25% that of the controls. Therefore, after seedling transfer, pots containing seedlings to be subjected to drought were dried down to the desired weights for both moderate and severe stresses by withholding watering. Pot weights were monitored on a daily basis and water was carefully added only after the target weight had been reached. The amount of water added was ensured to be just enough to readjust pot weights to the target level and compensate for evaporation. Water applied to re-adjust pot weights was always applied at the edges of the pots and never directly at the plant vicinity and was always done at 10 am every day (Verelst et al., 2013). A completely randomised design was used with three replications, hence each genotype had 21 pots.

3.3 Plant growth analysis

Plant growth was monitored daily by measuring the length of the fourth leaf beginning when the leaf immediately appeared. The distance between the base and tip of the leaf were measured for all plants at 10 am and these measurements continued until no further growth was recorded for all plants in the four maize inbred lines. After the fourth leaf reached maturity, all the collected data points were entered in excel sheets. Leaf elongation rate per day was determined by obtaining the difference in leaf length from one day to the next. The resulting data was used to determine the performance of each maize inbred line at the different stress levels.

3.4 *Agrobacterium* strain and maintenance

Agrobacterium tumefaciens strain EHA 101 with the standard binary vector pTF 102 (Figure 3.1) was used to infect the embryos of the different maize lines. The T-DNA region of this construct consists of a spectinomycin resistance marker gene for bacteria selection and a CaMV 35S promoter to drive the *GUS* reporter gene (Frame *et al*., 2014). The bacteria strain containing the vector was maintained on Yeast Extract Peptone (YEP) media containing 100 mg/L spectinomycin and 100 mg/L kanamycin. Bacteria cultures for weekly experiments were drawn from the mother plate refreshed from long term stored glycerol stocks. Before every experiment, bacteria cells were grown overnight in liquid YEP medium and adjusted to an optical density (OD_{660}) 0.8. This was designated the 100% *Agrobacterium* concentration which was diluted serially to 75%, 50% and 25% respectively, corresponding to 0.1, 0.03 and 0.007 OD's respectively.

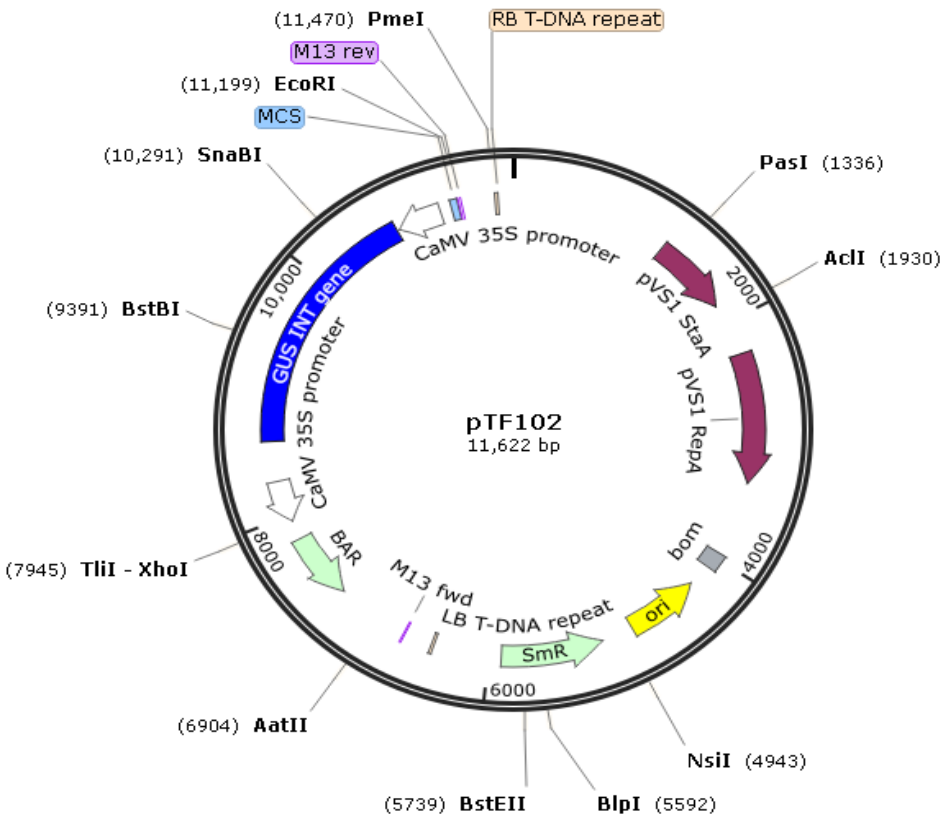

Figure 3.1: Map of EHA 101habouring standard binary vector pTF 102.

3.5 Isolation of immature embryos

Harvested ears were de-husked and the tip of a long forceps inserted at the top end to enable ease of handling during the embryo isolation process. For sterilization, the ears were first placed in a container with 70% ethanol for 3 minutes then moved to another container with commercial bleach (3.85% sodium hypochlorite) for 20 minutes inside a laminar flow bench after which they were rinsed three times with sterile distilled water. After the ears were surface sterilised, the kernel crowns were cut off using a new sterile blade and the immature embryos excised by inserting the narrow end of a spatula between the endosperm and pericarp at the basipetal side of the kernel to pop out the endosperm from the seed coat exposing the embryo. Using the spatula tip, the embryos were picked and put in 2 ml Eppendorf tubes with sterile distilled water to avoid desiccation (Frame et al., 2011).

3.6 Culture media and modifications

The infection media used was as described by Frame et al. (2002) with slight modifications on the amount of Acetosyringone (AS) to 300 mM. Co-cultivation of infected embryos was done on two types of media (Table 3.1). The first co-cultivation media consisted of 2g/L Yeast extract, 2g/L peptone, 1.5g/L NaCl, 220 mg/L casein hydrolysate, 20g/L sucrose, 10g/L glucose, 8g/L phytoagar and pH adjusted to 5.2. After autoclaving, 16mg/L $AgNO_3$, 300mM AS, 100mg/L kanamycin, and 100mg/L spectinomycin were added. The effects of adding 2-4,D, proline and cysteine on transient *GUS* expression in this media were also analysed. The second co-cultivation medium consisted of MS Macro and Micro salts and vitamins, 1.5mg/L 2-4-D, 20g/L sucrose, 10 g/L glucose, 2.31g/L L-proline, 220mg/L Casein hydrolysate 8g/L phytoagar and pH adjusted to 5.2 (Murashige & Skoog, 1962). After autoclaving, 16mg/L $AgNO_3$, 300 mg/L L-cysteine, 300 mM AS, 100mg/L kanamycin, and 100mg/L spectinomycin were added. After co-cultivation, callus induction was done on media consisting of MS Macro and Micro

salts and vitamins, three varying levels of (0, 1.5 and 3 mg/L) 2-4-D, 20g/L sucrose, 20 g/L glucose, 2.31g/L L-proline, 220mg/L Casein hydrolysate 3g/L gelrite and pH adjusted to 5.6. After autoclaving, 16mg/L $AgNO_3$, 300 mg/L L-cysteine and 500mg/L Cefotaxime were added. Shooting and rooting of embryogenic calli were done in media consisting of MS Macro and Micro salts and vitamins, 100mg/L myoinositol, 60g/L sucrose, 3g/L gelrite and pH adjusted 5.8. After autoclaving, 250 mg/L Carbenicillin was added. All media regimes used in this research were slightly modified from Frame et al. (2002) and Gorji et al. (2011).

Table 3.1: Media composition at different stages.

Component	IM	CCM		CIM	RM
		YEP	MS		
MS salts and vitamins premix (g/L)	4	-	4	4	4
Yeast extract (g/L)	-	2.5	-	-	-
Peptone (g/L)	-	5	-	-	-
Sodium chloride (g/L)	-	2.5	-	-	-
L-proline (g/L)	0.7	-	0.7	2.31	-
Cysteine (mg/L)	-	-	300	300	-
2,4-D (mg/L)	1.5	-	1.5	(0, 1.5, 3)	-
Casein hydrolysate (mg/L)	-	220	220	220	-
Sucrose (g/L)	68.4	20	20	20	60
Glucose (g/L)	36	10	10	20	-
pH	5.2	5.6	5.6	5.8	5.8
Acetosyringone (µM)	300	300	300	-	-
$AgNO_3$ (mg/L)	-	16	16	16	-
Myo-inositol (mg/L)	-	-	-	-	100
Kanamycin (mg/L)	-	100	100	-	-
Spectinomycin (mg/L)	-	100	100	-	-

Cefotaxime (mg/L)	-	-	-	500	-
Carbenicillin (mg/L)	-	-	-	-	250
Phytoagar (g/L)	-	8	8		-
Gelrite (g/L)	-	-	-	3	3

3.7 Infection of immature embryos and co-cultivation.

After the excision of the immature maize embryos was complete, the excess water was sucked out of the Eppendorf tubes using a pipette and fresh infection medium containing the bacteria at different concentrations added into the tubes. This was followed by gently inverting the tubes several times and allowing them to rest for five minutes. The contents were transferred to the different co-cultivation media and the embryos turned to ensure that the scutellum side was facing upwards and the embryo axis in complete contact with the media. At least 10 immature embryos from every inbred line infected with a specified *Agrobacterium* concentration were co-cultivated alone in three replicates such that every inbred line had three plates for every *Agrobacterium* concentration. These plates were properly labelled and incubated in the dark at 18^{o}C for three days after which transient *GUS* assays were done. For regeneration experiments, embryos were allowed to rest in the two different co-cultivation media with varying levels of 2,4-D for three days after which they proceeded to callus induction media, regeneration medium and eventually hardening followed by acclimatization. A completely randomized design was used for these experiments.

3.8 Induction of embryogenic calli, shooting and rooting

To induce embryogenic calli, immature embryos were first pre-cultured on co-cultivation media based on YEP and MS in order to study the possible effects of YEP media on the regeneration ability of tropical maize compared to MS. To study the effects of 2,4-Dichlorophenoxyacetic acid (2,4-D) on development of embryogenic calli, embryos from co-cultivation media were plated into callus induction media

with different concentrations of 2,4-D (0.0. 1.5 and 3 mg/L). All embryos were then incubated in the dark at 25^{o}C for 14 days before being sub-cultured into fresh media.

Only embryogenic calli from 1.5 and 3 Mg/l 2,4-D levels were transferred to the RM media and incubated at a 16/8 light and darkness regime to induce shoots and roots concurrently. After 2 weeks, the number of shoots that formed was counted and recorded. Three-week-old plantlets were transferred from culture bottles to peat moss in 10 cm diameter plastic pots and covered with a plastic paper for 48 hours for gradual acclimatization and hardening. After hardening, the plantlets were transferred into 20L buckets and allowed to grow to maturity.

3.9 Histochemical GUS assays

Histochemical analysis of the *GUS* gene was done as described by Ishida et al. (2007) and Jefferson et al. (1987) though with some adjustments on the tissue fixative and the staining process. After three days of co-cultivation, 5 embryos per genotype were transferred into 2 ml Eppendorf tubes containing 300 µl tissue fixative (mannitol (300mM), Mopholine ethane sulphonate (10mM) and 750 µl Formaldehyde dissolved in 250 ml of sterile distilled water and pH adjusted to5.6), 500 µl 200mM $NaPO_4$, and 300 µl X-gluc solutions and incubated for 24 hours at 37^{o}C. After hydrolysis of the X-gluc substrate by the β- glucuronidase enzyme, and reacting with oxygen, only positive embryos for the *GUS* gene turned blue and were counted under a microscope. A completely randomized design with three replications was employed. Leaves from a previously transformed tobacco plant with the *GUS* gene served as the positive control with non-infected embryos serving as the negative controls.

3.10 RNA isolation and cDNA synthesis

Maize tissues from the third leaf were cut, approximately 21 days after appearance of the fourth leaf. Only 50 mg of the tissues was placed into 2ml Eppendorf tubes containing two metal beads. The tubes were immediately placed in a container with

liquid nitrogen. The samples were ground into fine powder; 1 ml of trizol reagent was added and mixed vigorously by brief vortexing. The mixture was incubated for 5 minutes at room temperature. Thereafter, 0.2 ml of chloroform was added to each tube then shaken vigorously by hand and incubated for 5 minutes at room temperature. After incubation, the samples were centrifuged for 15 minutes at 12000xg at room temperature and the upper aqueous phase transferred to a fresh 1.5 ml Eppendorf tube. To these tubes, 0.5 ml of isopropyl alcohol was added, mixed and incubated at room temperature for 5 minutes then centrifuged at 12000xg for 10 minutes at room temperature. The supernatant was discarded and the RNA pellet washed twice with 1 ml of 75% chilled ethanol by mixing and vortexing for 15 seconds then centrifuging at 12000xg for 5 minutes at room temperature. The pellet was air-dried for 5 minutes and dissolved in 60 µl DEPC water then stored at -20ºC (Sah et al., 2014).

Synthesis of RNA to cDNA was done according to the user manual (Thermo Fisher Scientific, Waltham, USA). The RNA was treated with DNase to eliminate any traces of genomic DNA. To 1 µg of RNA, 1 µL of 10X Reaction Buffer with $MgCl_2$ and 1 µL of RNase-free DNase I were added and the volume adjusted to 10 µL. This was incubated for 30 minutes at 37ºC and 1 µL 50 mM EDTA added and incubated at 65ºC for 10 minutes to inactivate DNase I. The prepared RNA was used as template for reverse transcriptase, where 5 µg of the RNA was added to a reaction mix of 1 µL Oligo (dT) 18 primers and nuclease-free water to a volume of 12 µL, 4 µL 5X Reaction Buffer, 1 µL RiboLock RNase Inhibitor, 2 µL 10 mM dNTP Mix and 1 µL RevertAid M-MuLV RT. The mixture was incubated for 60 minutes at 42ºC and the reaction terminated by heating at 70ºC. The resulting cDNA used as a template for PCR.

3.11 Reverse Transcriptase Polymerase Chain Reaction (rtPCR)

The standard PCR conditions were used to detect genes in cDNA. PCR reagents were mixed in a 200 µl tube to a final reaction volume of 25 µl (Table 3.2). PCRs were done using the Eppendorf Mastercycler Pro (Eppendorf AG, Hamburg) programmed as shown in Table 3.3. Optimizing reaction conditions for the PCR conditions, in particular the annealing temperature was done using the cycler's gradient PCR feature. Three samples from each drought level were obtained from all the maize lines to constitute nine samples per maize genotype.

Table 3.2: Composition of master mix for PCR amplification.

Reagent	Final concentration	Final volume
Buffer (× 10)	× 1	5
dNTPs(10 mM)	0.5mM	2.5
$MgCl_2$ (50 mM)	2.5 mM	2.5
Primer1(2 µM)	0.25 µM	6.25
Primer2(2 µM)	0.25 µM	6.25
Taq(5 U/µl)	1 U/rxn	0.5
Template (10 ng/µl)		2
dH2O		To 25 µl

Table 3.3: Conditions for PCR reactions.

Step	No. of cycles	Temperature	Time
Initial denaturation	1	95	5 min.
Denaturation		95	30 sec.
Annealing }	25	48.9	30 sec.
Extension		72	30 sec.
Final extension	1	72	8 min.

3.12 Agarose gel electrophoresis

To prepare 1.5% agarose gel, 1.5 grams of agarose was weighed and placed into a conical flask containing 100 ml of 1× TAE buffer. The flask was placed in a microwave and heated for 5 minutes to melt the agarose completely. The Gel was then allowed to cool to 50ºC before casting into a gel tray with a comb and left to solidify. The gel was then placed in the electrophoresis tank containing 1× TAE buffer and the comb removed. 5 µl of a sample was loaded in each well after staining with 1 µl of 10000× SYBR green and 5 µl of 6× DNA loading dye. Electrophoresis was done at 80V until the dye front had migrated 2/3 of the gel. The gel was placed in a UV transilluminator box and documented using a digital camera.

3.13 Data analysis

Data on growth parameters such as leaf length of stressed and unstressed plants and other parameters such as transformation frequencies and callus formation frequencies were stored in excel sheet and analysed using ANOVA at 95% confidence interval with SAS statistical computer software. Mean separation was done using Tukey's pairwise comparison test at 5% probability level. Transient transformation frequencies were calculated as the number of embryos that showed *GUS* expression over the total number of embryos infected and expressed as a percentage. Additionally, all percentage data counts were arcsine transformed to fit a normal distribution before ANOVA was done. Callus formation frequency was calculated as the number of embryos forming embryogenic calli over the total number of embryos plated on callus induction media and expressed as a percentage.

CHAPTER FOUR

4.0 RESULTS

4.1 Effect of YEP media on callus formation from immature embryos of tropical maize

Immature embryos from all maize inbred lines on YEP callus induction media even when supplemented with either 2,4-D, Proline and cysteine failed to form callus. In order to mimic a transformation process which uses YEP media for co-cultivation, immature embryos were first pre-cultured on YEP and MS media separately for three days before proceeding to callus induction media based on MS media. The effects of pre-culturing immature maize embryos on MS and YEP media before transferring them to callus induction media were then studied.

Calli formed after pre-culturing embryos on both media types though there were differences in appearance based on the media they were pre-cultured on. In all maize genotypes under study, immature embryos left for 72 hours on YEP media formed both friable embryogenic and non-embryogenic calli which were cream in colour unlike calli formed from MS media which were compact and hard, white in colour and most of them being embryogenic (Plate 4.1). Data analysis revealed that there was a significant difference ($F_{2, 71} = 76.93$, $P = 0.0288$) on callus formation frequency based on the media type on which embryos were pre-cultured, irrespective of the growth hormone and the genotypes. Embryos pre-cultured on MS media registering a higher callus formation frequency (64.34%) than those pre-cultured on YEP media (61.64%). Immature embryos of inbred line CML 144 had a callus formation frequency of 66.18% when pre-cultured on MS media compared to 60.89% when pre-cultured on YEP media while E04 had a callus formation frequency of 65.25% on MS media compared to 60.63% on YEP media. The callus formation frequency of inbred line A04 was 62.11% after pre-culture on MS media and 61.04% after pre-culture on YEP media. Except for CML 216 which registered a callus formation frequency of 63.99% on YEP media compared to 63.83% on MS media, callus

formation frequency in all other maize inbred lines was higher in the embryos pre-cultured on MS media than in embryos pre-cultured on YEP media (Table 4.1).

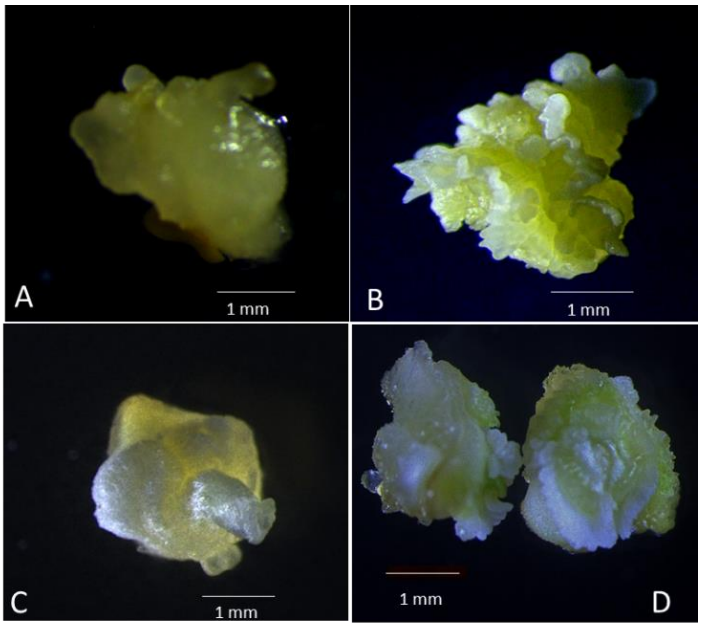

Plate 4.1: Embryogenic calli forming from E04 immature embryos pre-cultured in YEP and MS media.

(A) 14-day old embryo pre-cultured in YEP media supplemented with 3Mg/l 2,4-D. (B) 21-day old embryo pre-cultured in YEP supplemented with 1.5 Mg/l 2,4-D. (C) 7-day old embryo pre-cultured in MS supplemented with 1.5 Mg/l 2,4-D. (D) 21-day old embryo pre-cultured in MS supplemented with 3Mg/l 2,4-D.

4.2 Effect of 2,4-D on callus induction

The effect of growth regulator 2,4-D on callus induction when supplemented in MS based callus induction media was assessed. At 1.5 and 3.0 mg/l 2,4-D concentrations, immature embryos were responsive and formed calli. The optimal concentration of the growth regulator was 1.5 mg/l which resulted in 95.81% callus formation frequency. Analysis of variance revealed that there was a significant difference in the concentration of the hormone on callus formation ($F_{2, 71}$ = 2708.81,

P = 0.0001) with most calli forming at 1.5mg/l (95.81%) hormone concentration and the least forming at 0 mg/l (0.00%) hormone concentration (Table 4.2). Of the immature embryos pre-cultured on MS media at 1.5 mg/l 2,4-D concentration, inbred line E04 had the highest frequency of callus formation (99.38%) followed by CML 144 (99.35%) and CML 216 (93.70%) while inbred A04 had the least frequency of callus induction (90.43%). Inbred line CML 144 had the highest callus formation frequency of (98.72%) on YEP media followed by E04 (96.90%), A04 (94.38%) and CML 216 (93.65%). When the growth regulator concentration was increased to 3 mg/l 2,4-D immature embryos from CML 144 that were pre-cultured on MS media had the highest callus formation frequency (99.21%) followed by CML 216 (97.78%), E04 (96.37%) and A04 (95.92%). However, when immature embryos pre-cultured on YEP media were subjected to callusing at 3 mg/l 2,4-D concentration, the highest formation frequency was observed in inbred line CML 216 (98.33%) and the least in CML 144 (83.94%) (Table 4.3). Analysis of variance also revealed that there was no significant difference in callus formation frequency based on the four maize inbred lines ($F_{3, 71} = 0.05$, P = 0.985).

Table 4.1: Callus induction frequencies of tropical maize inbred lines pre-cultured on MS and YEP media.

Maize line	Media	Callusing frequency (%)
CML 144	MS	66.18±16.55
	YEP	60.89±15.38
CML 216	MS	63.83±16.02
	YEP	63.99±16.12
E04	MS	65.25±16.33
	YEP	60.63±15.35

A04	MS	62.11±15.63
	YEP	61.04±15.29
Totals	MS	64.34±16.13
	YEP	61.64±15.54

Means with ±SE

Table 4.2: Effects of 2,4-D concentration on callus formation frequency of tropical maize embryos on CIM media.

Hormone concentration	Callus formation frequency
2,4-D 0mg/l	0.00±0.00[b]
2,4-D 1.5mg/l	95.81±1.18[a]
2,4-D 3.0mg/l	93.16±1.43[a]

Values with the same letter are not significantly different by Tukey's pair-wise comparison ($P \leq 0.05$). Means with ±SE

Table 4.3: Callus formation frequencies of tropical maize lines pre-cultured on MS and YEP media at different 2,4-D concentrations.

Media before CIM	**MS****			**YEP**		
Line/2,4-D conc.	0 (Mg/l)	1.5 (Mg/l)*	3(Mg/l)*	0(Mg/l)	1.5(Mg/l)*	3(Mg/l)*
CML 144	0.00±0.00[a]	99.35±0.65[a]	99.21±0.79[a]	0.00±0.00[a]	98.72±1.28[a]	83.94±1.43[a]
E04	0.00±0.00[a]	99.38±0.62[a]	96.37±1.53[a]	0.00±0.00[a]	96.90±2.05[a]	84.99±5.70[a]
CML 216	0.00±0.00[a]	93.70±3.97[a]	97.78±2.22[a]	0.00±0.00[a]	93.65±6.35[a]	98.33±1.67[a]
A04	0.00±0.00[a]	90.43±5.27[a]	95.92±2.10[a]	0.00±0.00[a]	94.38±0.73[a]	88.73±1.51[a]

Values with the same letter in the same column are not significantly different by Tukey's pair-wise comparison ($P \leq 0.05$). Means with ±SE. * - shows significant effect of media on callus formation

4.3 Regeneration Analysis

After embryogenic calli were placed on regeneration media, the regeneration process started with embryogenic calli turning green after placing them in the light for about 10-14 days (Plate 4.2a). Shoots appeared approximately 7 days post embryogenic calli turning green. Data analysis revealed that there was a significant difference in the regeneration frequency based on the different maize lines ($F_{3, 63} = 8.28$, P= 0.0001) with CML 216 forming most shoots and T04 forming the least shoots (Figure 4.1). E04 embryos pre-cultured on YEP and MS media formed 3.9 and 3.8 shoots per callus respectively while CML 144 calli from embryos pre-cultured on both YEP and MS media formed 4.2 shoots per callus. A04 calli from embryos pre-cultured on YEP media formed 3.1 shoots per callus while those from embryos pre-cultured on MS media formed 3.4 shoots per calli. CML 216 had the highest number of shoots forming using the two media regimes, with calli from embryos pre-cultured on YEP media forming 4.3 shoots per calli while those from MS forming 4.7 shoots per calli. Analysis of variance revealed that based on the media previously used for pre-culture embryos, there was no significant difference on the regeneration ability of the calli ($F_{2, 63} = 0.51$, P= 0.4783). After hardening and acclimatizing 10 regenerated plants per line, only two plants of maize inbred line E04 showed somaclonal variation. These variations included folding during flowering, dwarfing of some plants and in other cases production of multiple cobs at one nodes.

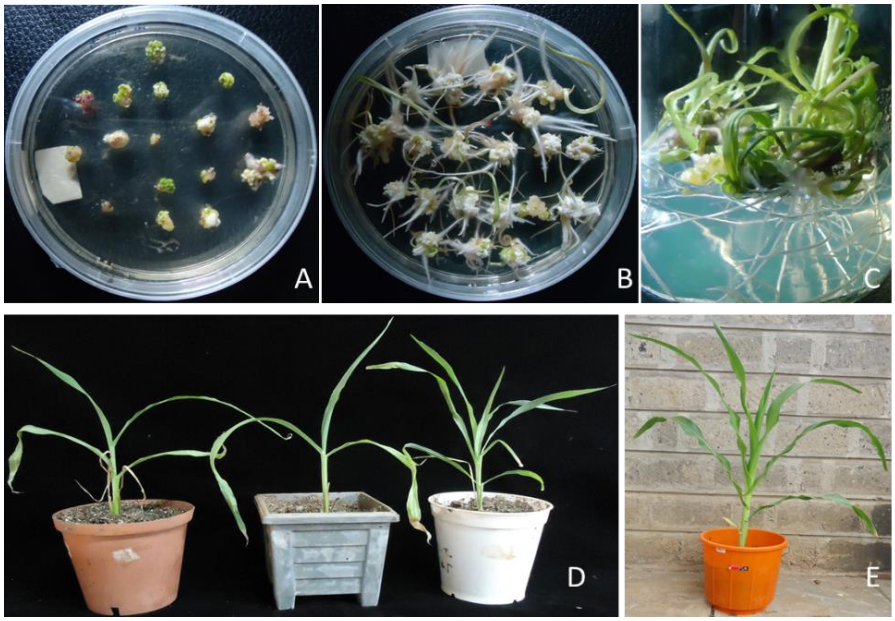

Plate 4.2: Somatic embryogenesis and regeneration of tropical maize inbred line E04 from immature embryos pre-cultured on YEP media.

(A) Green somatic embryos growing in light. (B) Shoot and root structures forming. (C) Plantlets forming in regeneration media. (D) Plantlets at acclimatization pots. (E) Fully grown regenerated plant.

4.4 Transient *GUS* expression on different media

After co-cultivating embryos in the different media, transient expression of the *GUS* transgene was indicated by blue colour (Plate 4.3). The concentrations of *Agrobacterium* strain EHA101 were optimized ranging between 0.8, 0.2, 0.07 and 0.03 at OD_{660} using first immature embryos of inbred line CML 216 and later with other lines in order to determine the optimal concentrations for transient *GUS* expression. Using CML 216, *Agrobacterium* densities of OD_{660} = 0.07 resulted in the highest transient *GUS* expression of 36.67% after co-cultivating on YEP media for three days compared to 23.33% when co-cultivated on MS media (Fig 4.2). A similar pattern was recorded at *Agrobacterium* concentrations of 0.02 with immature embryos co-cultivated on YEP media resulting in high transient *GUS* expression of 20% while those co-cultivated on MS media was 10%, Concentrations of 0.8 and

0.03 on YEP and MS media recorded transient *GUS* expression of 16.67% and 6.67% respectively.

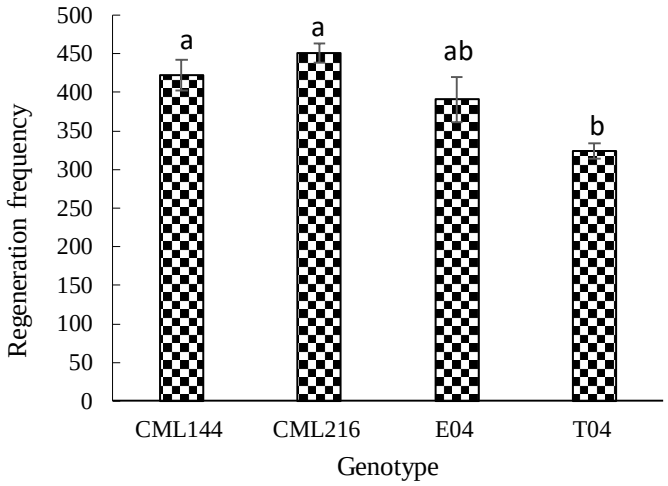

Figure 4.1: Regeneration frequencies of different maize inbred lines.
Vertical bars represent the standard errors. Columns with the same letter are not significantly different (P≤ 0.05).

Transient *GUS* expression was also high in CML 144, E04 and A04 at *Agrobacterium* concentrations of 0.07 on YEP media than on MS media (Table 4.4). Thus, the optimal concentration of *Agrobacterium* that induced the highest transient *GUS* expression was 0.07. The lowest transient *GUS* expressions were observed at high *Agrobacterium* concentrations of 0.8 or 0.2 in all maize lines except for inbred line E04 co-cultivated on YEP media whose lowest transient *GUS* expression was 5.56% at OD_{660} = 0.03. Analysis of variance revealed that there was a significant difference in transient *GUS* expression based on *Agrobacterium* concentration, with the highest mean transient *GUS* expression of 20.90% observed at OD_{660} = 0.07 and the least transient transformation of 9.17 % observed at OD_{660} = 0.8 ($F_{6, 47}$ = 7.65, P = 0.0002). Mean transient *GUS* expressions for OD's 0.2 and 0.03 were 12.22% and 10.42% respectively.

To determine that the observed transient *GUS* expression was not a mere expression of the gene in *Agrobacterium*, 20 T_0 plants regenerated from immature embryos co-cultivated in either YEP or MS media were screened through PCR for the expression of the 300bp bar selectable marker gene. From the 40 screened plants, Five plants regenerated from immature embryos co-cultivated in YEP media were positive while all the 20 plants regenerated from immature embryos co-cultivated in MS media were negative (Plate 4.4).

Table 4.4: Transient *GUS* expression in different maize inbred lines after co-cultivating on YEP and MS media.

CCM	**Line**	***Agrobacterium* concentration**			
		0.8 (100%)	**0.2 (75%)**	**0.07 (50%)**	**0.03 (25%)**
YEP	CML 216	16.67±3.33^{b}	20.00±0.00^{ab}	36.67±3.33^{a}	16.67±2.02^{b}
	CML 144	8.89±4.84^{a}	3.33±3.33^{a}	12.22±6.19^{a}	6.67±6.67^{a}
	E04	11.11±5.56^{a}	16.11±3.89^{a}	19.17±3.63^{a}	5.56±5.56^{a}
	A04	0.00±0.00^{b}	9.44±0.56^{ab}	15.56±2.94^{a}	12.78±3.64^{a}
MS	CML 216	6.67±1.33^{a}	10.00±3.77^{b}	23.33±6.67^{a}	6.67±1.67^{a}
	CML 144	3.33±3.33^{a}	3.33±3.33^{a}	13.33±6.67^{a}	10.00±5.77^{a}
	E04	0.00±0.00^{a}	6.67±6.67^{a}	16.67±3.33^{a}	3.33±3.33^{a}
	A04	6.67±3.33^{a}	10.00±5.77^{b}	23.33±6.67^{a}	6.67±6.67^{a}

Values with the same letter in the same row are not significantly different by Tukey's pair-wise comparison ($P \leq 0.05$). Means with ±SE

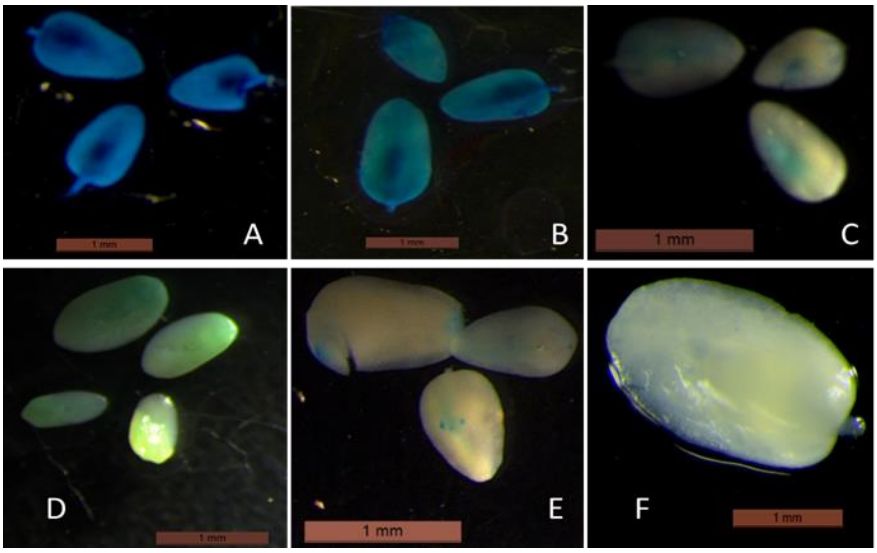

Plate 4.3: Immature maize embryos showing different GUS expression levels after co-cultivation in YEP and MS media.

(A) CML 144 co-cultivated in YEP media at 0.07 *Agrobacterium* concentration. (B) A04 embryos co-cultivated in YEP+PRO media at 0.07 *Agrobacterium* concentration. (C) E04 embryos co-cultivated in YEP+PRO media at 0.2 *Agrobacterium* concentration. (D) A04 embryos co-cultivated in MS media at 0.8 *Agrobacterium* concentration. (E) CML 216 embryos co-cultivated in MS media at 0.03 *Agrobacterium* concentration. (F) Control (YEP without *Agrobacterium*) negative for *GUS* staining.

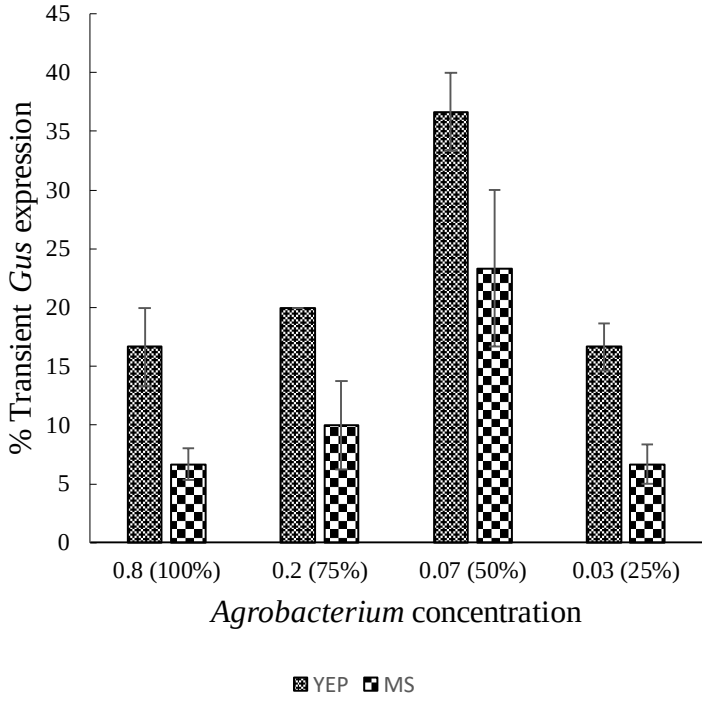

Figure 4.2: Transient transformation frequencies of CML 216 inbred line based on transient GUS expression using YEP and MS media.

Vertical bars represent standard errors.

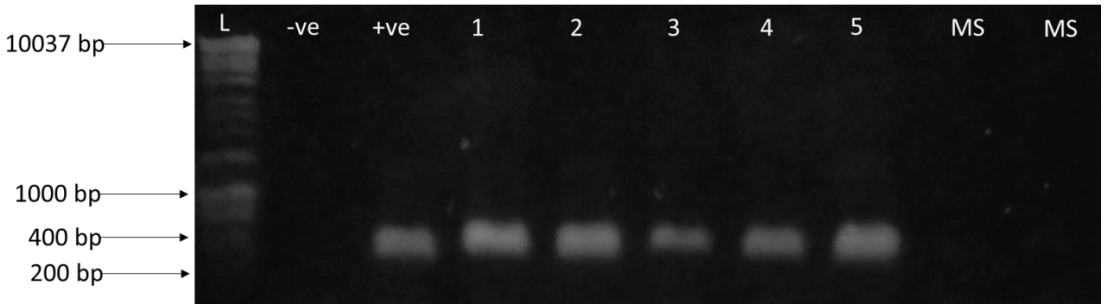

Plate 4.4: Gel electrophoresis of PCR using bar gene specific primers on T_0 plants.

Lines 1-5; putatively transgenic plants co-cultivated on YEP media. MS; plants co-cultivated on MS media negative for the transgene. Negative control was untransformed maize DNA and Positive control was pTF 102 plasmid DNA.

4.5 Effect of supplementing YEP on transformability

4.5.1 Supplement YEP with 2,4-D

The effects of different supplements such as 2, 4-D, cysteine and proline acting either as individual additives or in combination with others were observed to affect transient *GUS* expression differently. The addition of 1.5 mg/l 2,4-D growth regulator to YEP co-cultivation media resulted in lower transient *GUS* expression than co-cultivating on YEP media without the growth regulator. Except for maize inbred line CML 144 which showed a slight increase in *GUS* expression from 7.78% on YEP media to 7.79% when co-cultivated on YEP+2,4-D, all other maize lines registered lower transient *GUS* expression values when co-cultivation was done on YEP+2,4-D compared to transient *GUS* expression observed when co-cultivation was done on YEP media. On YEP media, inbred lines; A04, CML 216 and E04 registered transient *GUS* expression values of 9.44%,19.17%, and 12.99% respectively, while on YEP+2,4-D these lines registered lower values of 3.89%,4.17% and 10.76% respectively (Table 4.5). However, co-cultivating immature maize embryos on YEP+2,4-D media resulted in higher transient *GUS* expression than co-cultivating on MS media. Except for inbred maize line CML 216 which showed a decrease in transient *GUS* expression to 4.17% from 9.17% when co-cultivated in YEP+2,4-D and MS respectively, all other inbred lines showed higher transient *GUS* expression when co-cultivated on YEP+2,4-D than when co-cultivation was done on MS media. Transient *GUS* expression on MS media for maize inbred line A04, CML 144 and E04 were 3.72%, 7.50%, and 6.67% respectively, compared to *GUS* expression levels of 3.89%, 7.79% and 10.79% on YEP+2,4-D media (Table 4.5). Analysis of variance, however, revealed that there was no significant difference in transient *GUS* expression in all the maize lines when co-cultivation was done on YEP+2,4-D media ($F_{3, 47}$ =0.60, P = 0.619).

Table 4.5: Effects of different media supplements on transient *GUS* expression on maize inbred lines.

Maize line	YEP	MS	YEP+24D	YEP+CYS	YEP+PRO	YEP+ALL
A04	9.44±2.03^{ab}	3.72±1.39^{a}	3.89±1.73^{a}	4.26±2.31^{a}	7.87±1.94^{b}	4.68±1.76^{a}
CML 144	7.78±2.51^{b}	7.50±2.50^{a}	7.79±2.37^{a}	3.75±2.03^{a}	10.25±3.04^{ab}	11.67±3.53^{a}
CML 216	19.17±3.79^{a}	9.17±2.60^{a}	4.17±1.49^{a}	5.83±2.29^{a}	19.17±4.68^{a}	9.17±3.36^{a}
E04	12.99±2.55^{ab}	6.67±2.56^{a}	10.76±5.53^{a}	1.39±1.39^{a}	17.64±5.27^{ab}	9.19±3.07^{a}
Means	12.34	6.76	6.65	3.81	13.73	8.68

Values with the same letter in the same column are not significantly different by Tukey's pair-wise comparison ($P \leq 0.05$). Means with ±SE

4.5.2 Supplementing YEP with Cysteine

The addition of cysteine to YEP co-cultivation media was also observed to impact transient *GUS* expression negatively. Lower transient *GUS* expression was observed after co-cultivation was done on YEP+CYS media compared to *GUS* expression observed when co-cultivation was done on YEP media without supplement in all lines. Transient *GUS* expression values of 4.26% (A04), 3.75% (CML 144), 5.83% (CML 216) and 1.39% (E04) were observed when co-cultivation was done on YEP+CYS media, relatively lower than 9.44% (A04), 7.78% (CML 144), 19.17% (CML 216) and 12.99% (E04) for the same lines co-cultivated on YEP media. Except for maize line A04 which showed higher transient *GUS* expression when co-cultivated on YEP+CYS (4.26%) compared to MS (3.72%), all other maize lines recorded lower transient *GUS* expression when co-cultivated on YEP+CYS media than when co-cultivation was done on MS media (Table 4.5). Analysis of variance also revealed that there was no significant difference in transient *GUS* expression when immature maize embryos of the different inbred lines were co-cultivated on YEP+CYS media ($F_{3, 47} = 0.97$, $P = 0.415$).

4.5.3 Supplementing YEP with Proline

The supplementation of YEP co-cultivation media with proline seemed to impact transient transformation positively in three maize inbred lines. Maize inbred line

CML 144 and E04 registered higher *GUS* expression values of 10.25% and 17.64% on YEP+PRO compared to 7.78% and 12.99% when co-cultivated on YEP media. Inbred line CML 216 maintained a transient *GUS* expression of 19.17% when co-cultivated on both YEP and YEP+PRO media. Maize line A04, however, showed lower transient *GUS* expression when co-cultivated on YEP+PRO media (7.87%) than when co-cultivation was done on YEP media (9.44%). All the transient *GUS* expression values observed from the four lines after co-cultivation on YEP+PRO media were higher than those observed when co-cultivation was done on MS media. There was however no significant difference in transient *GUS* expression within the four inbred maize lines when co-cultivation was done on YEP+PRO media ($F_{3, 47}$ = 1.55, P = 0.216).

4.5.4 Supplementing YEP with 2,4D, cysteine and proline

The combination of 2,4-D, cysteine and proline in YEP co-cultivation media was observed to result in lower transient *GUS* expression than that observed when using un-supplemented YEP media. Only inbred line CML 144 registered a higher transient *GUS* expression on YEP+ALL media (11.67%) than that observed after co-cultivating on YEP media (7.78%). Inbred lines A04, CML 216 and E04 registered lower transient *GUS* expression levels of 4.68%, 9.17%, and 9.19% respectively when co-cultivated on YEP+ALL media compared to 9.44%, 7.78% and 12.99% when co-cultivation was done on YEP media. However, the transient *GUS* expression observed when immature embryos were co-cultivated on YEP+ALL media was higher than that observed when co-cultivation was done on MS media in inbred lines A04, CML 144 and E04. Maize inbred line CML 216 exhibited the same transient *GUS* expression of 9.17% when co-cultivation was done on YEP+ALL media and MS media (Table 3.5). Analysis of variance revealed that there was no significant difference in transient *GUS* expression on all maize lines when co-cultivation was done on YEP+ALL media ($F_{3, 47}$ = 0.66, P = 0.583).

4.6 Response of the fourth leaf of tropical maize inbred lines to drought stress

The growth patterns of the fourth leaf of all the tropical maize inbred lines under unstressed/control conditions, mild drought stress and severe drought stress were similar. Differences were only observed in the time taken to achieve maximum leaf length by the different inbred lines and in the mature lengths of the plant's leaves. Under control conditions, inbred line CML 144 was the first to attain maximum leaf length by the 11th day post-emergence (DPE), followed by inbred line E04 which attained maximum leaf length by the 12th DPE. The fourth leaf of inbred line CML 216 achieved maximum length on the 13th DPE while the last line to attain maximum leaf length of the fourth leaf under control conditions was A04 on the 14th DPE (Appendix 9).

When mild/moderate drought stress was imposed, the maximum leaf length of inbred line CML 216 was attained on the 13th DPE but all the other lines experienced a delay in onset of maximum leaf length. CML 144 attained maximum leaf length on the 15th DPE while inbred line E04 and A04 attained maximum leaf length on the 16 DPE. When the maize inbred lines were subjected to severe drought stress, further delay in the attainment of the maximum length of the fourth leaf was observed. Maize inbred lines CML 144 attained maximum leaf length on the 18th DPE while CML 216, A04 and E04 attained maximum leaf lengths on the 20th DPE while (Appendix 9). ANOVA revealed that there was no significant difference in the mature leaf lengths of all maize lines under control conditions ($F_{3, 11}$ = 1.91, P = 0.2063) as well as the mature leaf lengths of all maize lines under severe drought stress ($F_{3, 11}$ = 3.48, P = 0.0702). However, under moderate stress, it was observed that there was a significant difference in the mature leaf lengths of the four maize inbred lines under study ($F_{3, 11}$ = 8.77, P= 0.0066).

Under control conditions, mature leaf lengths ranged from 43.94 cm for maize inbred line E04 to 35.42 cm for maize inbred line A04, with inbred lines CML 144 and

CML 216 registering intermediate mature leaf lengths of 41.00 cm and 38.83 cm respectively (Figure 3.3). Under mild drought stress, the longest mature leaf length of 43.17 cm was observed in inbred line E04, followed by CML 144 and A04 whose mature leaf length was 41.25 cm and 37.00 cm respectively. CML 216 under mild stress registered the smallest mature leaf length of 36.06 cm (Figure 3.4). The mature leaf lengths under severe drought stress were 31.28 cm for E04, 30.79 cm for inbred line A04, 30.38 cm for CML 144 and 27.94 cm for maize inbred line CML 216 (Figure 3.5). Irrespective of the maize line, ANOVA also revealed that there was a significant difference in the mature lengths of the fourth leaf ($F_{2, 35}$ = 39.18, P = < 0.0001) based on the level of stress excreted. Although there was no significant difference in the mature length of the fourth leaf under control and mild drought stress, mature leaf lengths at these stress levels were significantly different from those observed under severe drought stress (Table 3.6). Maize inbred lines CML 216 and E04 registered a reduction in mature leaf lengths when mild drought stress was exerted. However, it was observed in inbred lines CML 144 and A04 that exertion of mild drought stress led to an increase in mature leaf lengths of the fourth leaf. The increase or decrease in mature leaf lengths between control and severe drought seedlings were however not significantly different. Leaf folding and drooping were observed in seedlings subjected to moderate and severe drought stresses and the severity of the folding of leaves increased with the level of stress and the day temperature. Leaf lengths of maize seedlings are detailed in appendix 9.

Table 4.6: Mature leaf lengths in cm of the fourth leaf under different drought stress levels.

Maize line	Leaf length Control	Leaf length Mild stress	Leaf length Severe stress
CML216	38.83±3.18^{a}	36.06±1.66^{c}	27.94±0.63^{a}
CML144	41.00±0.99^{a}	41.25±0.69^{ab}	30.38±0.92^{a}
A04	35.42±0.23^{a}	37.00±0.50^{bc}	30.79±0.33^{a}
E04	43.94±3.99^{a}	43.17±1.34^{a}	31.28±1.08^{a}

Values with the same letter in the same column are not significantly different by Tukey's pair-wise comparison ($P \leq 0.05$). Means with ±SE

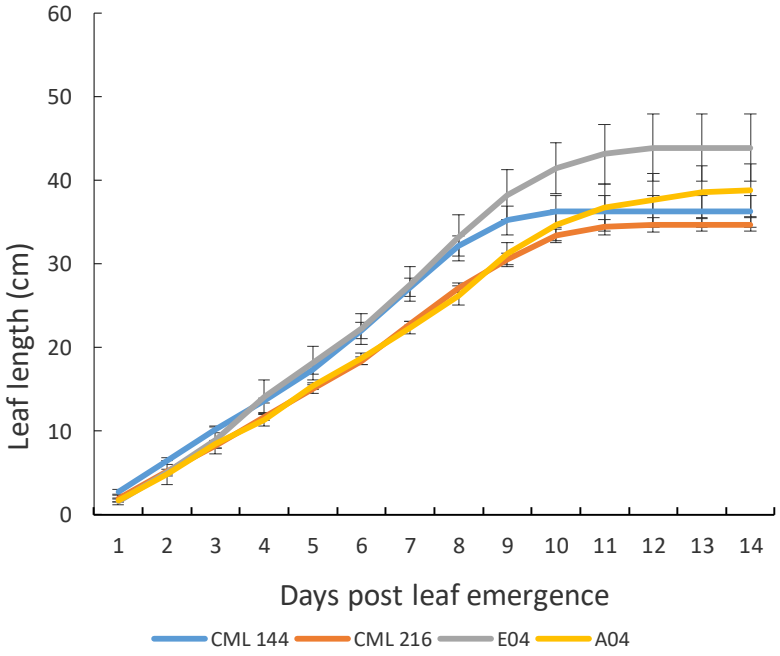

Figure 4.3: Leaf lengths of the four inbred lines under control conditions.

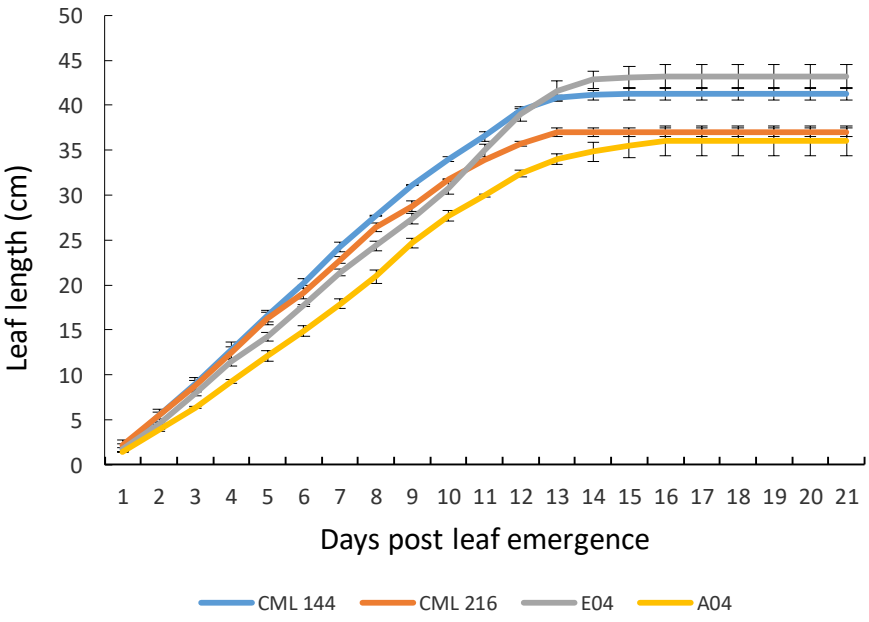

Figure 4.4: Leaf lengths of the four inbred lines under moderate stress conditions.

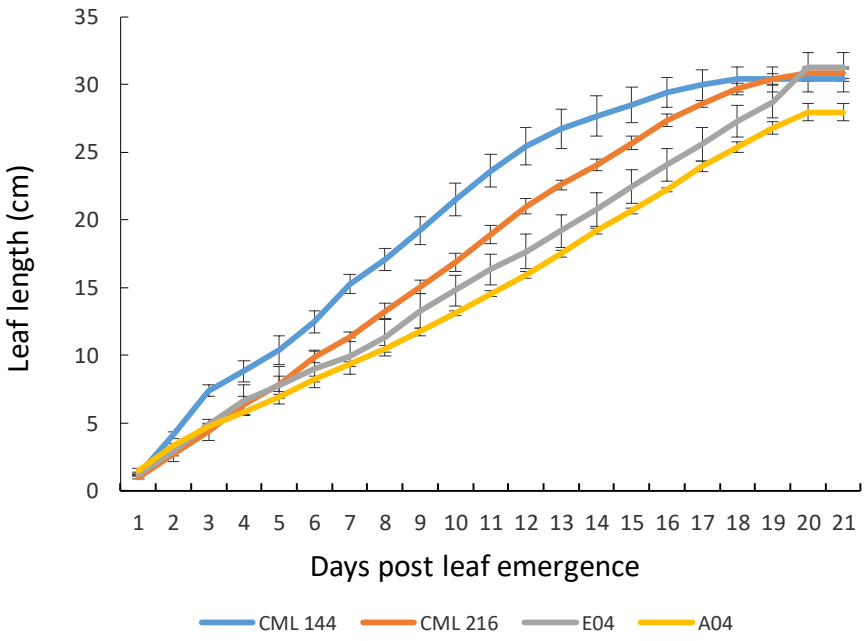

Figure 4.5: Leaf lengths of the four inbred lines under severe stress conditions.

4.7 Relative growth rate of the fourth leaf of the maize seedlings

Under all drought conditions, growth rate followed a similar trend. All inbred lines showed accelerated growth up to a certain maximum after which the rate of growth started to decelerate. Maize plantlets which were not subjected to drought stress had a relatively higher growth rate than those subjected to moderate and severe drought stress and achieved maximum growth early. Under control conditions, the onset of maximum growth rate ranged from 7th to 10th DPE. CML144 was the first to achieve maximum growth rate by the 7th DPE (5.3cm/day), followed by E04 (5.6 cm/day) and CML 216 (4.5 cm/day) on the 8th DPE while A04 achieved maximum growth rate (5cm/day) at the 10th DPE. Analysis of variance revealed that there was a

significant difference in the growth rate of the fourth leaf based on the different maize lines under control conditions ($F_{3, 11}$ = 8.98, P = 0.0034). The highest mean growth rate was observed in inbred line E04 (2.02 cm/day), followed by inbred lines A04 (1.77 cm/day), CML 144 (1.60 cm/day) and CML 216 (1.56 cm/day) (Table 4.7).

After subjecting seedlings to mild stress, the rate of growth reduced. Although maximum growth rate was achieved at the same time as that of the controls, the rate of growth at that time was relatively lower. CML 144 had a maximum growth rate of 4.04 cm/day, while inbred lines E04 and CML 216 had maximum growth rates of 4.11 cm/day. Maize line A04 at moderate drought stress had a maximum growth rate of 3.72 cm/day (Appendix 10). Again, analysis of variance revealed that subjecting maize seedlings to moderate drought stress impacted a significant difference in the growth rate of the fourth leaf based on the different maize inbred lines ($F_{3, 11}$ = 9.12, P = 0.0021). High mean growth rate were observed in lines E04 (1.97 cm/day) and CML 144 (1.86 cm/day), while inbred lines CML 216 and A04 had the least growth rates of 1.66 cm/day and 1.65 cm/day respectively.

Subjecting maize seedlings to severe drought stress caused a marked reduction in the rate of growth in all lines. Maximum growth set in later except for CML 144 whose maximum growth rate was observed at the 7th DPE but reduced steadily up to the 19th DPE when growth stopped. CML 216 showed maximum growth rate at the 12th DPE (2.08 cm/day) while A04 showed maximum growth rate at the 13th DPE (1.89 cm/day) (Appendix 10). All maize lines under severe stress showed a slow and prolonged growth rate compared to moderate drought and control conditions. Analysis of variance revealed that there was no significant difference in the mean growth rate of the fourth leaf of maize seedlings based on the different maize lines. Mean growth rates ranged from 1.43 cm/day for inbred line E04 to 1.26 cm/day for line A04. Inbred lines CML 216 and CML 144 had intermediate growth rates of 1.42

cm/day and 1.39 cm/day when subjected to severe drought stress. Detailed growth rates of seedlings per day are provided in appendix 10.

Table 4.7: Mean growth rates of the different maize inbred lines at different stress levels in cm/day.

Maize line	Growth Rate Control	Growth Rate Moderate Stress	Growth Rate Severe Stress
A04	1.77 ± 0.24^{ab}	1.65 ± 0.17^{b}	1.26 ± 0.06^{a}
CML 144	1.60 ± 0.26^{b}	1.86 ± 0.22^{ab}	1.39 ± 0.13^{a}
CML 216	1.56 ± 0.22^{b}	1.66 ± 0.20^{b}	1.42 ± 0.09^{a}
E04	2.02 ± 0.29^{a}	1.97 ± 0.21^{a}	1.43 ± 0.08^{a}

Values with the same letter in the same column are not significantly different by Tukey's pair-wise comparison ($P \leq 0.05$). Means with ±SE

4.8 Drought impact on fresh and dry weights of seedlings

Drought stress was observed to impact the fresh and dry weights of maize seedlings differently. When maize seedlings were not subjected to drought stress, it was observed that CML 144 seedlings had the highest fresh weight (16.39g), followed by A04 (15.70g) and E04 (15.57g) while CML 216 had the least fresh weight (11.97g). These fresh weight measurements were taken 30 days post emergence of the fourth leaf. Analysis of variance revealed that the fresh weights of inbred lines CML 144, A04 and E04 were significantly different from that of inbred line CML 216 ($F_{3, 35}$ = 7.14, P = 0.0008). After subjection to moderate drought stress, ANOVA also revealed that there was a significant difference in the fresh weights of seedlings ($F_{3, 35}$ = 12.17, P = < 0.0001) based on the different maize lines. Maize inbred line A04

registered the highest fresh weight after subjection to moderate drought stress (12.56g), followed by E04 (11.68g) and CML 144 (10.49g) while CML 216 had the least fresh weight (8.08g).

After maize seedlings were subjected to severe drought stress, ANOVA also revealed that there was a significant difference in the fresh weights based on the different maize lines ($F_{3, 35}$ = 24.73, P = < 0.0001). The highest fresh weights of maize seedlings under severe stress were observed in maize lines E04 (6.78g), A04 (5.67g) and CML 144 (5.37g), with inbred line CML 216 registering the least fresh weight (1.78g) under severe drought stress (Table 4.8). Irrespective of the maize line, ANOVA also revealed that there was a significant difference in the fresh weights of maize seedlings based on the different drought stress levels ($F_{2, 107}$ = 130.52, P = < 0.0001). The highest fresh weight was observed in seedlings under control conditions (14.90g), followed by those subjected to moderate drought stress (10.70g) and the least registered by seedlings subjected to severe drought stress (4.89g).

After seedlings were dried, it was observed that there was no significant difference in the dry weights of seedlings which had not been subjected to drought stress. Dry weights of unstressed maize seedlings ranged from 1.79g for inbred line A04 to 1.48g in CML 216. However, subjecting maize seedlings to moderate and severe drought had a significant impact in their dry weights. Maize seedlings subjected to moderate drought had dry weights ranging from 1.67g in A04 to 0.96g in E04 while CML 144 and CML 216 registered dry weights of 1.34g and 1.03g respectively. Analysis of variance revealed that there was a significant difference in the dry weights of the different maize lines after subjection to moderate drought ($F_{3, 35}$ = 15.96, P = < 0.0001).

Very low dry weights were registered when maize seedlings subjected to severe stress. Inbred lines A04 and CML 144 registered dry weights of 0.79g and 0.70g respectively while E04 and CML 216 registered dry weights of 0.42g and 0.33g

respectively (Table 4.9). ANOVA revealed that based on the stress level and irrespective of the inbred line, there was a significant difference in the observed dry weights of the maize seedlings ($F_{5, 107} = 63.61$, $P = < 0.0001$). The mean dry weight under control conditions was 1.65g, while that of moderate drought stress was 1.25g and 0.56g under severe drought stress (Table 4.10).

Table 4.8: Fresh weights in grams of 4-week old seedlings after subjection to different drought conditions.

Maize line	Fresh weight Control	Fresh weight Mild Stress	Fresh weight Severe Stress
A04	15.70±0.99[a]	12.56±0.45[a]	5.67±0.27[a]
CML 144	16.39±0.88[a]	10.49±0.59[a]	5.37±0.32[a]
CML 216	11.97±0.62[b]	8.08±0.15[b]	1.78±0.74[b]
E04	15.57±0.29[a]	11.68±0.82[a]	6.78±0.19[a]

Values with the same letter in the same column are not significantly different by Tukey's pair-wise comparison ($P \leq 0.05$). Means with ±SE

Table 4.9: Dry weights in grams of 4 week old seedlings after subjecting them to different drought conditions.

Maize line	Dry weight Control	Dry weight Mild Stress	Dry weight Severe stress
A04	1.79±0.13[a]	1.67±0.08[a]	0.79±0.04[a]
CML 144	1.77±0.20[a]	1.34±0.09[b]	0.70±0.03[a]
CML 216	1.48±0.10[a]	1.03±0.09[bc]	0.33±0.04[b]
E04	1.58±0.09[a]	0.96±0.05[c]	0.42±0.03[b]

Values with the same letter in the same column are not significantly different by Tukey's pair-wise comparison ($P \leq 0.05$). Means with ±SE

Table 4.10: Fresh and dry weights of maize seedlings in grams of 4 week old seedlings based on the different drought levels.

Drought level	Fresh weight	Dry weight
Control	14.90±0.46[a]	1.65±0.07[a]
Moderate	10.70±0.39[b]	1.25±0.07[b]
Severe	4.89±0.38[c]	0.56±0.04[c]

Values with the same letter in the same column are not significantly different by Tukey's pair-wise comparison ($P \leq 0.05$). Means with ±SE

4.9 Relative expression of *PARP2* gene under drought stress

As determined by Agarose gel electrophoresis on the isolated maize RNA, non-degraded RNA was obtained based on observation of the 28s and 18s bands (Plate 4.5). After cDNA synthesis of the obtained RNA, RT-PCR using gene specific primers designed from *PARP2* exon gene regions revealed that there were differences in expression of the gene under different drought conditions in the different maize inbred lines under study. Based on the bands observed on 1.5% agarose gel, it was possible to amplify the *PARP2* gene in all the inbred line samples obtained from severe drought subjects. Amplification of the *PARP2* gene from samples obtained from moderate drought stress in all the four maize inbred lines was also possible. In lines CML144, E04 and A04, relative expression of the gene was only detected under moderate and severe drought conditions (Plate 4.6). Contrary to expectations, RT-PCR revealed amplification of the gene under unstressed conditions for inbred line CML 216 in one of the samples (Plate 4.7) in addition to expression at moderate and severe stress conditions. All other inbred lines did not exhibit amplification of the gene at control conditions.

By comparison, it was observed that expression of the gene under moderate stress was relatively lower than that observed at severe drought stress based on the brightness of the observed bands. The RT-PCR performed using the *PARP2* gene primers from converted cDNA from drought stressed and unstressed plants produced an amplification of the expected band size of 1038 bp.

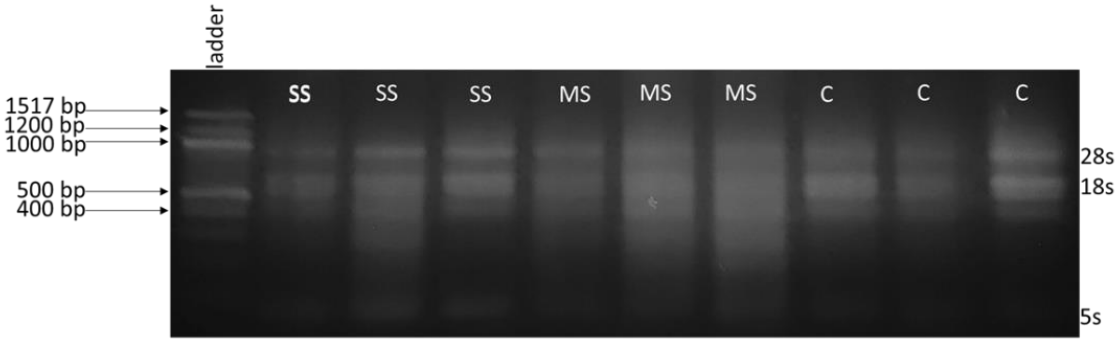

Plate 4.5: Agarose gel electrophoresis of maize total RNA, showing a high concentration of RNA.

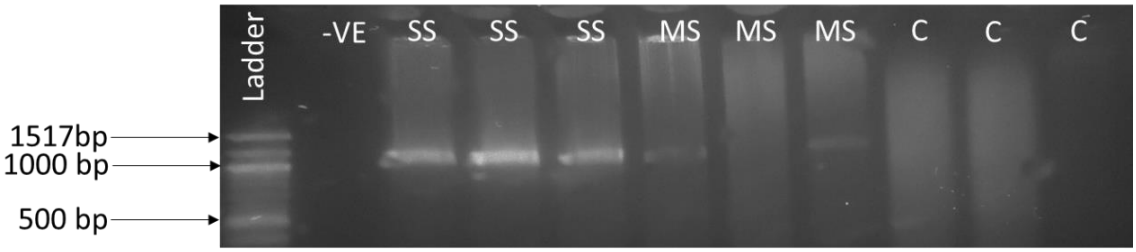

Plate 4.6: Agarose gel electrophoresis of the PCR products obtained after amplification of cDNA form maize inbred line E04 using *PARP2* primers.

SS samples were from plants under severe drought stress, MS samples were from plants subjected to moderate stress while C samples were from plants which were not subjected to drought stress.

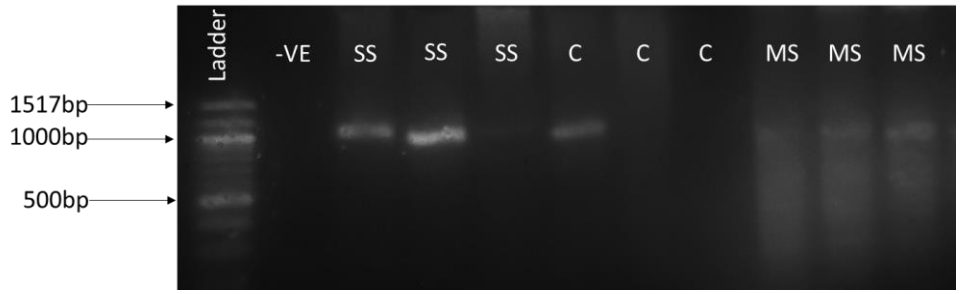

Plate 4.7: Agarose gel electrophoresis of the PCR products obtained after amplification of cDNA form maize inbred line CML 216 using *PARP2* primers.

SS samples were from plants under severe drought stress, MS samples were from plants subjected to moderate stress while C samples were from plants which were not subjected to drought stress.

CHAPTER FIVE

5.0 DISCUSSION CONCLUSIONS AND RECOMMENDATIONS

5.1 YEP as an alternative co-cultivation media

In any *Agrobacterium*-mediated transformation system, developing optical conditions for the transfer of a gene of interest within the T-DNA is a critical step. Therefore, the choice of co-cultivation media for transformation is important. It was generally observed in this study that the use of YEP media, as well as YEP+PRO and YEP+ALL for co-cultivation, resulted in a higher transient transformation than co-cultivation on MS media. This could be because YEP media supports the growth of *A. tumefaciens* hence ensures a constant availability of actively dividing *A. tumefaciens* cells in the media which were responsible for the observed increased transient transformation using YEP media. Increase in transient transformation when using YEP+PRO could be attributed to the effect of proline in the media since proline increases the delivery of T-DNA to *A. tumefaciens*, stimulates the growth of *A. tumefaciens* and supports the development of plant cells.

Increase in transient transformation is consistent with the findings of Cao et al. (2014) who documented that the addition of proline in MS based co-cultivation media increased transient *GUS* expression and eventually the transformation efficiency in maize. It was also observed by Carvalho et al., (2004) that the addition of proline and asparagine to co-cultivation media increases transient *GUS* expression in sorghum. Findings from the present study contrast those of Frame et al. (2006) and Vega et al. (2008) who observed that the use of cysteine in MS based and N6 based co-cultivation media increased the rate of transformation. In this study, it was observed that addition of cysteine and 2,4-D in YEP co-cultivation media led to a decrease in transient transformation. It is thought that co-cultivation media supplements like cysteine and 2,4-D do not directly affect the ability of *A. tumefaciens* to infect explants and transfer the *A. tumefaciens* T-DNA. However, sometimes, these substances may increase the frequency of transformed cells by

directly affecting the explant or the interaction between the explant and *A. tumefaciens* during co-cultivation. The observation that these substances increase transformation efficiency as observed by other authors could, therefore, be due to their interaction with the media used. The media in these cases favour the explant and these substances act as nutritional supplements to the explant but not to *A. tumefaciens*.

5.2 Effect of *A. tumefaciens* concentration on transformation.

A. tumefaciens concentration of OD_{660}=0.07 resulted in the best transient transformation when using YEP media for co-cultivation in all the four maize inbred lines under study. A number of reports have documented differences in transient *GUS* expressions using different *A. tumefaciens* cell densities/concentrations in maize (Frame et al., 2006; Huang & Wei, 2005; Ishida et al., 2007; Zhao et al., 2001). All of these studies report optimum *A. tumefaciens* densities of OD_{600}=0.3-1 for infection. Although they involved the use of MS, LS or N6 media for co-cultivation, a similar trend was observed using YEP media since transient transformation increased up to a certain point beyond which it started reducing. In the present study however, the optimum *A. tumefaciens* cell concentration was OD_{660}=0.07, way lower than what has been documented in other studies. The effectiveness of low *A. tumefaciens* cell densities in this study could be because YEP media favourably supports the growth of *A. tumefaciens* compared to MS and N6 media hence starting with a low number of infection cells results in a faster growth rate. It was reported by Gurlitz et al. (1987) that each plant cell binds a certain finite number of *A. tumefaciens* cells beyond which the plant cell will lose viability and the transformation efficiency reduces. Therefore, when using YEP co-cultivation media which is known to favourably support the growth of *A. tumefaciens*, the optimum number/ratio of *A. tumefaciens* cells to explant cell is reached faster hence when starting with an initial high infection number of bacterial cells (OD_{660}=0.8), most of the explant cells lose viability, resulting in a much reduced transient *GUS* expression by the third day of co-cultivation. An appropriate initial bacterial infection

(OD_{660}=0.07) and subsequent YEP co-cultivation ensured an optimum *A. tumefaciens* to explant ratio and therefore a high transient *GUS* expression.

Similar studies investigating effects of *A. tumefaciens* cell densities on transformation have been reported in other plants. In wheat, different optimum *A. tumefaciens* concentrations have been documented. Amoah et al. (2001) reported an optimum *A. tumefaciens* cell density of OD_{600}=1.5 for transient *GUS* expression using tiller explants, while Rashid et al. (2010) recorded optimum *A. tumefaciens* concentrations of OD_{600}=0.5 for transient *GUS* expression using embryo explants. In tomato, optimum *A. tumefaciens* cell densities of OD_{600}=1.0 have been reported using cotyledonary explants and MS media for co-cultivation (Gao et al., 2009). An optimum *A. tumefaciens* cell density of OD_{600}=1.5 has been reported in *Anthurium andraeanum* transformation using leaf explants and MS media for co-cultivation. Kim et al. (2009) reported an optimum *A. tumefaciens* cell density of OD_{600}=0.6–0.9 to infiltrate *Arabidopsis* leaves and express the *GUS* gene. In sugar cane an optimum *A. tumefaciens* cell density of OD_{600}=0.4-0.8 was reported by Priya et al. (2010) for transient *GUS* expression on callus. In oil palm, an optimum *A. tumefaciens* cell density of OD_{600}=0.8 has been recommended for transformation based on transient *GUS* expression (Yenchon & Te-chato, 2012). The observed differences in the optimum concentrations of *A. tumefaciens* for transformation suggests that effective transformation depends on many factors such as the explant of choice, the co-cultivation media and the time of infection.

5.3 Genotype dependent response to *Agrobacterium* mediated transformation

Genotype-dependent response was observed with respect to transient transformation, callus formation and regeneration frequency in this study. Generally, E04 was the best line with respect to callus formation (73.03%) while CML 216 was the best line for transformation and regeneration. Genotype-dependent response is not only

characteristic of maize but has also been reported with other cereals and many other crops as documented by Karami, (2009).

All the four maize inbred lines in the study responded differently to *A. tumefaciens* infection after co-cultivation on different media regimes. Inbred line CML 216 showed similarities in transient *GUS* expression when co-cultivated in (YEP, YEP+PRO) and (YEP+ALL, MS). A188 and Hi-II are temperate inbred maize lines which have been used for tissue culture and are efficiently transformed by *A. tumefaciens* but still exhibit genotype dependent response (Frame et al., 2006; Ishida et al., 1996; Zhao et al., 2001). This is also the case with the tropical inbred lines which have been used in other studies (Michelmore, 2008; Ombori et al., 2013b; Rasha et al., 2013). More recently, Cao et al. (2014) working on 14 maize inbred lines reported genotype dependence of *GUS* expression based on their differences in response to *A. tumefaciens* infection. All these inbred lines used by Cao et al., display poor agronomic characteristics and have been mostly used in basic studies to test trait genes but none has been used on a commercial scale.

Genotype dependent response in transformation has also been reported in other plants as a bottleneck to *A. tumefaciens* mediated transformation. In barley, genotype dependent response has reduced most transformation experiments to using a few genotypes like the Golden Promise spring variety, since other genotypes do not yield any transformation events (Harwood et al., 2009; Harwood 2012). In wheat, although most genotypes can be regenerated *in vitro*, the bulk of genetic transformations is done using biolistic methods since most genotypes are unsusceptible to *A. tumefaciens* (Harwood, 2012; Varshney & Altpeter, 2001). Other plants which exhibit genotype dependent transformation include; pepper, melon, soy beans and peanuts (Steinitz et al., 1999; Galperin et al., 2003; Donaldson & Simmonds, 2000; 2000 Krishna *et al*., 2015).

5.4 Effects of 2,4-D on callus formation and regeneration

In this study, it was observed that the optimum 2,4-D concentration for callus formation was 1.5 mg/l. In other studies, the auxin 2,4-D has commonly been used to induce embryogenic calli from maize in ranges of 2-3 mg/l (Abebe et al., 2008; Binott et al., 2008; Oduor et al., 2006). Abebe et al. (2008) and Odour et al. (2006) reported optimum 2,4-D concentrations of 2 mg/l are required to form embryogenic calli from tropical maize immature embryos while Jia et al. (2008) used 3 mg/l to form embryogenic calli from mature embryos of tropical maize genotypes. Consistent with their findings, this study observed optimum formation of embryogenic calli at 2,4-D concentrations of 1.5 mg/l after pre-culturing our embryos on YEP and MS co-cultivation media. This therefore suggests that YEP media does not significantly affect the callus forming ability and subsequent regeneration ability of tropical maize. Although unlike embryos rested in MS media for 3 days, some embryos pre-cultured on YEP co-cultivation media did not form any calli, suggesting that YEP media might have a slightly inhibitory effect on the cell signalling pathway and to some extend disrupts the regeneration process of immature maize embryos.

5.5 *PARP* genes and drought stress

It has been established from this study that drought stress affects certain aspects of maize growth and development. Aspects such as leaf length, growth rate, fresh weight and dry weight were observed to be lower significantly in plants subjected to severe drought stress compared to those which were not subjected to drought. Although it was observed that different maize inbred lines respond differently to drought stress, the final outcome of drought damage in all maize lines is similar. This is in agreement with other studies which have shown that drought stress hinders the growth and development of maize. Khan et al. (2001) observed that components of growth in maize variety YHS 202 that were affected by drought include height, leaf area index, root structure, biomass, fresh weight, dry weight and diameter of the

stem. Aslam et al. (2015) reported cases of leaf folding and drooping in maize plants subjected to drought stress levels of 50% and 25% of field capacity, a phenomenon which was also observed in our study. This phenomenon is thought to be genetically controlled as a strategy by the plants to withstand drought and is controlled by several genes among them *ZMNF-YB2* in maize (Entringer et al., 2014).

In this study, it was observed that the rate of growth in all the maize lines was higher in plants that were not subjected to drought stress than those subjected to moderate and severe drought stress. The observed reduction in rate of growth as a result of onset of drought stress can be attributed to two factors; turgor loss in expanding cells and metabolic regulations in the plant. Loss of turgor in expanding cells leads to inhibition of cell division while regulation in metabolism due to onset of drought is an adaptive mechanism by the plant to restrict increase in size of transpiring leaf area under drought (Blum, 2005). Reduction in fresh weight as well as dry weights of plants as drought progressed as observed in this study could be attributed to several factors. These include; leaf rolling and drooping under drought stress, leaf wilting which leads to blocking of stomata and reduced gas exchange in plants experiencing drought stress. All these factors lead to reduction in the plant leaf area exposed to sunlight hence reduced photosynthesis. This eventually causes a reduction in the plants biomass as a result of reduced food accumulation and carbon assimilation (Chaves et al., 2002).

The role of poly(ADP-ribose) polymerase (PARP) in stress tolerance and energy homeostasis in plants has been outlined and it is now evident that this protein is involved in the plant's ability to tolerate or succumb to DNA damage. Ionising radiations induce the expression of *PARP1* gene in plants while accumulation of toxic metals like cadmium as well as dehydration trigger the expression of *PARP2* (De Block et al., 2005). Dehydration stresses that can trigger expression of *PARP2* gene in plants range from biotic to abiotic stresses. These include insect damage,

fungal and bacterial pathogens, cold stress, light stress, mechanical damage and drought stress (O'Kane et al., 1996). This explains why there was an expression of the *PARP2* gene under conditions where drought was absent in inbred line CML 216. This suggests that other forms of stress had set in to trigger the expression of the gene. It has been shown that *PARP2* gene in plants is the most important of all *PARP* genes in enabling the plant respond to DNA damage and induce immune response (Song et al., 2015). As observed in this study, dehydration stress at moderate and severe drought stress triggered the expression of this gene in maize.

From this study, it was observed that the maize line E04, which had the longest leaves under control and moderate drought stress had the lowest expression of *PARP2* gene. It is postulated that maize line E04 was able to maintain energy homeostasis by reducing the breakdown of NAD^+, hence conserving energy. This could explain why E04 plants had the longest leaves compared to other plants and suggests an inverse relationship between *PARP* gene expression and drought survival as well as good expression of physiological traits. When plants experience drought stress, there is an increase in expression of *PARP* genes, which leads to a rapid breakdown of the NAD^+ pool. As a result, re-synthesis of NAD^+ is stimulated, leading to use of three to five molecules of ATP for every molecule of NAD^+ synthesized. This eventually leads to depletion of ATP and onset of apoptosis (De Block et al., 2005). To counter this damaging effect of the expression of *PARP* genes, chemical inhibitors and genetic mutations have been employed. Inhibition of expression of *PARP* genes leads to over-expression of other genes that respond to stimuli, abiotic stresses, JA, ABA, lipids and secondary metabolites. Therefore, it has been shown that *PARP* mutants can tolerate abiotic stresses unlike other plants expressing *PARP* genes (Schulz et al., 2014).

5.6 Conclusion

Maize inbred lines CML 144, CML 216, A04 and E04 are among the important maize lines which are used in Kenya to study regeneration and transformation. In this study:

1. It was not possible to for maize inbred line embryos to form calli entirely on YEP media. However, pre-culturing these embryos on YEP co-cultivation media for three days before callus induction on MS based CIM does not affect their callus formation ability and regenerability.

2. The plant growth regulator 2,4-D at concentration of 1.5 mg/l was observed to be optimum for callus formation in maize inbred lines and is not affected by the co-cultivation media used.

3. A better co-cultivation media based on Yeast Extract Peptone has been reported. All the tropical maize inbred lines involved in the study reported a higher transient transformation on YEP as well as YEP+PRO and YEP+ALL media compared to co-cultivation on MS media.

4. The use of low *Agrobacterium* cell densities (OD_{660} = 0.7) at co-cultivation was observed to increase transient transformation.

5. The expression of *PARP2* gene is directly proportional to the level of drought experienced by maize plants. At high levels of drought stress, there is high expression of the *PARP2* gene while at low levels of drought stress, the gene is expressed at low levels or not expressed at all.

Based on the above findings, all the null hypotheses are rejected. This is because YEP media was observed to affect the callus formation ability of maize inbred lines and enhanced the transformation efficiency of immature maize embryos. Additionally, the expression of *PARP2* gene differed under different drought stress conditions.

5.7 Recommendations

Since YEP media does not affect the callus formation ability of tropical maize embryos and positively impacts their transient *GUS* expression, the following recommendations are made.

1. The protocol of transforming immature maize embryos using *Agrobacterium* densities of OD_{660} = 0.7 and YEP media at co-cultivation as identified in this study is suitable and therefore highly recommended for the transformation of recalcitrant tropical maize inbred lines.

2. Although the relationship between *PARP2* gene expression and drought was determined. It is recommended that real time qPCR technique be used to determine the precise levels of gene expression at the different levels of drought stress.

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APPENDICES

Appendix 1: Media for *Agrobacterium tumefaciens* culture

1. YEP solid

- Yeast extract peptone 5 g/L
- Peptone 10 g/L
- NaCl 5 g/L
- Bactoagar 15 g/L
- Kanamycin 100 mg/l
- Spectinomycin 100 mg/L

2. YEP liquid

- Yeast extract peptone 5 g/L
- Peptone 10 g/L
- NaCl 5 g/L

Appendix 2: Murashige And Skoog (MS) basal media composition

MACRONUTRIENTS	MICRONUTRIENTS
NH_4NO_3 (1650 mg/L)	H_3BO_4 (6.2 mg/L)
$CaCl_2.2H_2O$ (332.02 mg/L)	$CoCl_2.6H_2O$(0.025 mg/L)
$MgSO_4.7H_2O$ (180.54 mg/L)	$CuSO_4.5H_2O$ (0.025 mg/L)
KNO_3 (1900 mg/L)	FeNaEDTA (36.7 mg/L)
KH_2PO_4 (170 mg/L)	$FeSO_4.7H_2O$ (27.8 mg/L)
	$MnSO_4.H_2O$ (16.9 mg/L)
	Kl (0.83 mg/L)
	$Na_2MoO_4.2H_2O$ (0.25mg/L)
	$ZnSO_4.H_2O$ (8.6 mg/L)

Appendix 3: Preparation of antibiotic and media supplement stock solutions

Kanamycin (100 mg/ml)

Five grams of kanamycin mono sulfate salt (Duchefa, Haarlem Netherlands) was dissolved in 10 ml of 1N NaOH and topped up to 50 ml with sdH_2O. The solution was aliquoted into 2 ml tubes and stored at -20 ºC. To obtain a final kanamycin concentration of 100 mg/l in the medium, 1ml of the100 mg/ml Kanamycin stock was added to 1 liter of media.

Spectinomycin stock (100 mg/ml)

Five grams of spectinomycin (Phytotechnology laboratories, Shawnee mission, KS, USA) was dissolved in 50 ml of sdH2O, aliquoted into 2 ml tubes and stored at -20 ºC. One milliliter of the 100 mg/ml spectinomycin stock was added to 1 litre of media to make a final concentration of spectinomycin in the media, 100 mg/l.

Cefotaxime stock (500mg/ml)

Ten grams of cefotaxime (Sigma-Aldrich and Merck KGaA, Darmstadt, Germany) was dissolved in 20 ml of sdH_2O and aliquoted in 2 ml Eppendorf tubes then stored at -20 ºC. One ml of the 500 mg/L stock was added to 1L of CIM to obtain a final concentration of 500 mg/L.

Carbenicillin stock (250 mg/ml)

Five grams of Carbenicillin (Sigma-Aldrich and Merck KGaA, Darmstadt, Germany) was dissolved in 10 ml of ethanol and topped up to 20 ml with sdH_2O and aliquoted in 2 ml Eppendorf tubes then stored at -20 ºC. To 1L of RM, one ml of the 250 mg/ml stock was added to attain a final concentration of 250 mg/L.

2,4-D stock (1.5 mg/ml)

0.075 grams of Sodium 2,4-dichlorophenoxyacetate monohydrate (Sigma-Aldrich and Merck KGaA, Darmstadt, Germany) was dissolved in 10 ml of dimethyl sulfoxide (DMSO) and topped up to 50 ml with sdH_2O. The solution was then aliquoted into 2 ml Eppendorf tubes and stored at -20 ºC. One and two millilitres of the 1.5 stock solution were added in 1 litre of CCM to attain final concentrations of 1.5mg/L and 3mg/L respectively.

Appendix 4: Multivariate ANOVA for callus formation frequency

Class Level Information

Class	Levels	Values
GENOTYPE	4	A04 CML 144 CML 216 E04
CO_CULT_	2	MS YEP
TREATMENT	3	24D 0mg/l 24D 1.5mg/l 24D 3.0Mg/l

	DF	sum of squares	Mean Square	F-Value	Pr > F
TREATMENT	2	42.49385	21.24693	894.83	<.001
Residual	69	1.63834	0.02374		
Total	71	44.13219			

	DF	Sum of squares	Mean Square	F-Value	Pr > F
GENOTYPE	3	0.0978	0.0326	0.05	0.985
Residual	68	44.0344	0.6476		
Total	71	44.1322			

Appendix 5: ANOVA for transient GUS assays

A) ANOVA for the effect of genotype and *Agrobacterium* concentration on transient *GUS* expression using YEP media

	DF	sum of squares	Mean Square	F-Value	Pr > F
GENOTYPE	3	0.37875	0.12625	2.23	0.09
Residual	44	2.49080	0.05661		
Total	47	2.86955			

B) ANOVA for the effect of genotype on transient *GUS* expression using YEP+24-D media

	DF	sum of squares	Mean Square	F-Value	Pr > F
GENOTYPE	3	0.11961	0.03987	0.60	0.61
Residual	44	2.93018	0.06660		
Total	47	3.04979			

C) ANOVA for the effect of genotype on transient *GUS* expression using YEP+CYS media

	DF	sum of squares	Mean Square	F-Value	Pr > F
GENOTYPE	3	0.12428	0.04143	0.97	0.41
Residual	44	1.87796	0.04268		
Total	47	2.00223			

D) ANOVA for the effect of genotype on transient *GUS* expression using YEP+ALL media

	DF	sum of squares	Mean Square	F-Value	Pr > F
GENOTYPE	3	0.13508	0.04503	0.66	0.58
Residual	44	3.01488	0.06852		
Total	47	3.14996			

E) ANOVA for the effect of genotype on transient *GUS* expression using YEP+PRO media

	DF	sum of squares	Mean Square	F-Value	Pr > F
GENOTYPE	3	0.39530	0.13177	1.55	0.216
Residual	44	3.75130	0.08526		
Total	47	4.14660			

F) ANOVA for the effect of genotype and *Agrobacterium* concentration on transient *GUS* expression using MS media

	DF	sum of squares	Mean Square	F-Value	Pr > F
GENOTYPE	3	0.10402	0.03467	0.63	0.60
Residual	44	2.43116	0.05525		
Total	47	2.53517			

Appendix 6: ANOVA for the effect of drought stress level on mature leaf lengths

A) ANOVA for the effect of control stress level on mature leaf lengths

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	3	145.5152597	48.5050866	2.14	0.1740
Error	8	181.7474593	22.7184324		
Corrected Total	11	327.2627190			

B) ANOVA for the effect of moderate drought on mature leaf lengths

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	3	103.6549556	34.5516519	8.77	0.0066
Error	8	31.5287204	3.9410900		
Corrected Total	11	135.1836759			

Source	DF	Sum of	Mean Square	F-Value	Pr > F
Model	3	19.76279630	6.58759877	3.48	0.0702

C) ANOVA for the effect of severe drought on mature leaf lengths

Error	8	15.13064630	1.89133079
Corrected Total	11	34.89344260	

Appendix 7: ANOVA for the effect of drought stress on the fresh weight of seedlings

A) ANOVA for the effect of drought on the fresh weight of seedlings at control conditions

Source	DF	Sum of Squares	Mean Square	F-Value	Pr >F
Model	3	107.1500000	35.7166667	7.14	0.0008
Error	32	159.9688889	4.9990278		
Corrected Total	35	267.1188889			

B) ANOVA for the effect of drought on the fresh weight of seedlings at moderate drought

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	3	126.7186111	42.2395370	24.73	<.0001
Error	32	54.6511111	1.7078472		
Corrected Total	35	181.3697222			

Sum of

Source	DF	Squares	Mean Square	F-Value	Pr > F
Model	3	101.8777778	33.9592593	12.17	<.0001
Error	32	89.2622222	2.7894444		
Correcte Total	35	191.1400000			

C) ANOVA for the effect of drought on the fresh weight of seedlings at severe drought

Appendix 8: ANOVA for the effect of drought on the dry weight of seedlings

A) ANOVA for the effect of drought at control conditions on the dry weight of seedlings

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	3	0.59829722	0.19943241	1.15	0.3439
Error	32	5.54797778	0.17337431		
Corrected Total	35	6.14627500			

B) ANOVA for the effect of moderate drought on the dry weight of seedlings

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	3	2.83534444	0.94511481	15.96	<.0001
Error	32	1.89484444	0.05921389		
Corrected Total	35	4.73018889			

C) ANOVA for the effect of severe drought on the dry weight of seedlings

Source	DF	Sum of Squares	Mean Square	F-Value	Pr > F
Model	3	1.29863056	0.43287685	44.53	<.0001
Error	32	0.31106667	0.00972083		
Corrected Total	35	1.60969722			

Appendix 9: Leaf lengths of maize seedlings at different days under control, Moderate and severe stress conditions in cm

DAY	E04			A04			CML 144			CML 216		
	Control	Mild S.	Severe S.	Control	Mild S.	Severe S.	Control	Mild S.	Severe S.	Control	Mild S.	Severe S.
D 1	1.61±0.39	1.72±0.20	1.22±0.15	1.67±0.17	1.39±0.06	1.44±0.20	2.75±0.26	1.87±0.43	1.21±0.04	1.92±0.44	2.13±0.62	0.96±0.11
D 2	5.06±1.42	4.56±0.11	2.89±0.31	4.83±0.17	3.83±0.09	3.33±0.19	6.38±0.38	5.50±0.72	4.08±0.23	5.17±0.29	5.46±0.36	2.67±0.55
D 3	8.89±1.65	7.94±0.31	4.89±0.39	8.44±0.45	6.39±0.11	4.72±0.11	10.21±0.44	9.04±0.65	7.38±0.44	8.21±0.22	8.75±0.69	4.38±0.64
D 4	14.17±1.95	11.44±0.43	6.67±1.13	11.33±0.76	9.28±0.24	5.78±0.15	13.67±0.29	12.71±0.91	8.83±0.79	11.67±0.34	12.46±0.67	6.38±0.57
D 5	18.17±2.01	14.23±0.47	7.78±1.37	15.39±0.40	12.11±0.55	6.95±0.15	17.38±0.51	16.58±0.65	10.38±1.04	15.04±0.48	16.29±0.67	7.88±0.56
D 6	22.22±1.81	17.72±0.15	9.00±1.36	18.67±0.69	14.89±0.56	8.22±0.22	22.04±0.99	20.21±0.55	12.46±0.83	18.42±0.51	19.21±0.76	9.88±0.44
D 7	27.61±2.11	21.44±0.39	9.94±1.33	22.39±0.72	17.89±0.53	9.34±0.17	27.25±1.11	24.25±0.54	15.25±0.69	22.88±0.25	22.75±0.38	11.33±0.36
D 8	33.17±2.75	24.39±0.53	11.33±1.36	26.22±1.12	20.94±0.73	10.50±0.25	32.17±1.21	27.71±0.08	17.08±0.79	27.13±0.59	26.42±0.48	13.25±0.62
D 9	38.28±3.00	27.39±0.63	13.28±1.25	31.22±1.33	24.67±0.51	11.72±0.31	35.25±1.73	31.17±0.04	19.21±1.05	30.50±0.75	28.75±0.56	15.04±0.49
D 10	41.50±3.04	30.83±0.67	14.78±1.12	34.67±1.84	27.72±0.59	13.11±0.20	36.29±1.88	34.00±0.26	21.50±1.23	33.38±0.79	31.75±0.07	16.88±0.69
D 11	43.17±3.59	34.94±0.71	16.33±1.13	36.78±2.79	29.94±0.20	14.53±0.22	36.33±1.92	36.54±0.56	23.63±1.23	34.42±0.93	33.96±0.04	18.92±0.68
D 12	43.94±3.99	39.06±0.78	17.67±1.26	37.72±3.13	32.39±0.36	15.94±0.24	36.33±1.92	39.50±0.14	25.46±1.37	34.71±0.82	35.71±0.23	21.00±0.57
D 13	43.94±3.99	41.61±1.11	19.17±1.20	38.61±3.19	34.00±0.60	17.50±0.25	36.33±1.92	40.83±0.33	26.71±1.48	34.75±0.78	37.00±0.50	22.58±0.37
D 14	43.94±3.99	42.83±1.00	20.78±1.24	38.83±3.18	34.83±1.04	19.17±0.19	36.33±1.92	41.13±0.56	27.67±1.52	34.75±0.78	37.00±0.50	24.04±0.42
D 15	43.94±3.99	43.06±1.23	22.44±1.24	38.83±3.18	35.56±1.40	20.67±0.19	36.33±1.92	41.25±0.69	28.50±1.32	34.75±0.78	37.00±0.50	25.67±0.49
D 16	43.94±3.99	43.17±1.34	24.06±1.19	38.83±3.18	36.06±1.66	22.22±0.15	36.33±1.92	41.25±0.69	29.42±1.12	34.75±0.78	37.00±0.50	27.33±0.47
D 17	43.94±3.99	43.17±1.34	25.61±1.24	38.83±3.18	36.06±1.66	23.95±0.36	36.33±1.92	41.25±0.69	29.96±1.13	34.75±0.78	37.00±0.50	28.54±0.25
D 18	43.94±3.99	43.17±1.34	27.28±1.19	38.83±3.18	36.06±1.66	25.39±0.40	36.33±1.92	41.25±0.69	30.38±0.92	34.75±0.78	37.00±0.50	29.67±0.41
D 19	43.94±3.99	43.17±1.34	28.72±1.23	38.83±3.18	36.06±1.66	26.78±0.48	36.33±1.92	41.25±0.69	30.38±0.92	34.75±0.78	37.00±0.50	30.38±0.38
D 20	43.94±3.99	43.17±1.34	31.28±1.08	38.83±3.18	36.06±1.66	27.94±0.63	36.33±1.92	41.25±0.69	30.38±0.92	34.75±0.78	37.00±0.50	30.79±0.33
D 21	43.94±3.99	43.17±1.34	31.28±1.08	38.83±3.18	36.06±1.66	27.94±0.63	36.33±1.92	41.25±0.69	30.38±0.92	34.75±0.78	37.00±0.50	30.79±0.33

Xxxx – Maximum growth

Appendix 10: Growth rates of the different inbred maize lines under control, moderate and severe stress conditions in cm/day

DAY	E04			A04			CML 144			CML 216		
	Control	Mild S.	Severe S.	Control	Mild S.	Severe S.	Control	Mild S.	Severe S.	Control	Mild S.	Severe S.
D 2	3.45±1.04	2.79±0.53	0.95±0.15	2.11±1.06	2.44±0.06	1.06±0.06	3.46±0.15	3.33±0.15	1.46±0.36	2.88±0.34	2.92±0.22	1.46±0.11
D 3	3.83±0.29	2.83±0.10	1.11±0.24	2.89±0.31	2.56±0.06	1.11±0.06	3.63±0.31	3.54±0.11	1.55±0.49	3.05±0.12	3.29±0.37	1.50±0.07
D 4	4.00±0.17	3.39±0.21	1.22±0.06	3.17±0.17	2.78±0.15	1.17±0.00	3.71±0.40	3.67±0.34	2.08±0.22	3.25±0.19	3.33±0.25	1.71±0.47
D 5	4.06±0.20	3.49±0.34	1.33±0.25	3.28±0.80	2.83±0.33	1.17±0.10	3.83±0.22	3.63±0.43	2.79±0.33	3.38±0.25	3.54±0.59	1.71±0.11
D 6	5.28±0.34	3.50±0.25	1.39±0.11	3.61±0.53	2.89±0.15	1.22±0.06	4.67±0.49	3.88±0.32	2.88±0.25	3.38±0.07	3.67±0.11	1.79±0.25
D 7	5.39±0.39	3.72±0.29	1.45±0.15	3.72±0.15	3.00±0.25	1.28±0.15	5.21±0.15	4.04±0.15	3.29±0.62	3.46±0.18	3.71±0.15	1.83±0.25
D 8	5.56±0.64	4.11±0.06	1.50±0.17	3.83±0.69	3.06±0.22	1.39±0.11	4.92±0.49	3.46±0.48	2.29±0.21	4.46±0.33	3.83±0.11	1.92±0.36
D 9	5.11±0.47	4.11±0.80	1.50±0.17	4.06±0.43	3.72±0.24	1.39±0.11	3.08±0.96	3.46±0.11	2.13±0.26	4.25±0.36	3.00±0.63	2.00±0.13
D 10	3.22±1.24	3.45±0.40	1.56±0.11	5.00±0.33	3.06±0.11	1.50±0.19	1.04±0.40	2.96±0.42	2.13±0.13	3.38±0.31	2.33±0.21	2.00±0.07
D 11	1.67±1.35	3.00±0.10	1.61±0.06	3.44±1.12	2.44±0.34	1.42±0.12	0.04±0.04	2.83±0.27	1.83±0.25	1.04±0.25	2.21±0.11	2.04±0.11
D 12	0.78±0.70	2.94±0.29	1.61±0.06	0.94±0.47	2.22±0.39	1.41±0.05	0.00±0.00	2.54±0.36	1.83±0.15	0.29±0.11	1.75±0.26	2.08±0.27
D 13	0.00±0.00	2.56±0.66	1.67±0.29	0.89±0.59	1.61±0.29	1.89±0.06	0.00±0.00	1.33±0.48	1.25±0.13	0.04±0.04	1.29±0.40	1.67±0.17
D 14	0.00±0.00	1.22±0.48	1.67±0.00	0.22±0.22	0.83±0.44	1.56±0.06	0.00±0.00	0.29±0.23	0.96±0.15	0.00±0.00	0.00±0.00	1.63±0.07
D 15	0.00±0.00	0.22±0.22	1.67±0.17	0.00±0.00	0.72±0.36	1.72±0.24	0.00±0.00	0.13±0.13	0.97±0.22	0.00±0.00	0.00±0.00	1.58±0.46
D 16	0.00±0.00	0.11±0.11	2.56±0.31	0.00±0.00	0.50±0.25	1.67±0.17	0.00±0.00	0.00±0.00	0.83±0.30	0.00±0.00	0.00±0.00	1.46±0.24
D 17	0.00±0.00	0.00±0.00	2.00±0.29	0.00±0.00	0.00±0.00	1.56±0.06	0.00±0.00	0.00±0.00	0.54±0.04	0.00±0.00	0.00±0.00	1.21±0.42
D 18	0.00±0.00	0.00±0.00	1.95±0.36	0.00±0.00	0.00±0.00	1.44±0.06	0.00±0.00	0.00±0.00	0.42±0.23	0.00±0.00	0.00±0.00	1.13±0.40
D 19	0.00±0.00	0.00±0.00	1.78±0.78	0.00±0.00	0.00±0.00	1.39±0.11	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.71±0.04
D 20	0.00±0.00	0.00±0.00	1.56±0.06	0.00±0.00	0.00±0.00	1.17±0.17	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.42±0.08
D 21	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00