# *AGROBACTERIUM TUMEFACIENS*-MEDIATED TRANSFORMATION OF THREE SWEET POTATO VARIETIES WITH *XEROPHYTA VISCOSA* PEROXIREDOXIN 2 GENE CONFERRING DROUGHT STRESS TOLERANCE


**By**

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**A thesis submitted in partial fulfilment of the requirement for the award of the degree of Master of Science (Biotechnology) in the school of Pure and Applied Sciences of Kenyatta University**

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## Declaration

I Phillip Mike Aonenji Gerald Kamwendo declare that 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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## Dedication

I dedicate this thesis to my two lovely children Pamela and Phillip Junior for their perseverance, patience, prayers and hope for better times to come as I pursue this goal.

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## Abbreviations

| | |
|---|---|
| ABA | Abscisic acid |
| ANOVA | Analysis of Variance |
| ASAL | Arid and Semi arid Land |
| ASARECA | Association for Strengthening Agricultural Research in Eastern and Central Africa |
| CaMV | Cauliflower Mosaic Virus |
| cDNA | Complementary De-oxyribo Nucleic Acid |
| CIP | International Potato Centre |
| DNA | De-oxyribo Nucleic Acid |
| dNTPs | Di Nitrogen Tris Phosphate |
| DREB/CBF | Dehydration-responsive element B/C-repeat |
| DRE/CRT | Dehydration-responsive element/C-repeat |
| EDTA | Ethylene diamine tetraacetic acid |
| EST | Elementary Sequencing Tags |
| FAO | Food and Agriculture Organization |
| $GA_3$ | Giberrellic Acid |
| GST | Glutathione-S-Transferase |
| HSP | Heat Shock Proteins |
| IAA | Indole-3- Acetic Acid |
| KSP36 | Kenyan Sweet Potato 36 |
| LEA | Late Embryogenesis Abundant proteins |
| LSD | Least Significant Difference |

| | |
|---|---|
| MAP | Mitogen Activated Protein |
| MAPK | Mitogen Activated Protein Kinase |
| MAPKK | Mitogen Activated Protein Kinase Kinase |
| MAPKKK | Mitogen Activated Protein Kinase Kinase Kinase |
| MS | Murashige and Skoog |
| PCD | Programmed Cell Death |
| PCR | Polymerase Chain Reaction |
| ROS | Reactive Oxygen Species |
| SADC | Southern Africa Development Community |
| SARRNET | Southern Africa Root and Tuber Research Network |
| SOD | Superoxide dismutase |
| TBE | Trisbase Boric Acid and EDTA |
| UV | Ultra Violet |
| XvPrx2 | *Xerophyta viscosa* peroxiredoxin 2 |

## Abstract

Sweet potato [*Ipomoea batatas* (L) Lam] is an important food crop and belongs to the family Convolvulaceae. It is a creeping herbaceous perennial vine that is cultivated as an annual crop in the world. Sweet potato is ranked fourth in terms of importance as staple food mostly in the developing world. Countries in Eastern and Southern Africa account for 76% of sweet potato production in Africa. Drought is one of the major abiotic constraints to production of many arable crops with yield losses estimated at 20% although values as high as 100% have been documented under severe conditions resulting into total crop failure. Although several strategies such as conventional breeding, crop introduction, hybridization have been employed in the past to improve drought tolerance, these efforts need to be complemented. Biotechnology offers alternative tools to the development of drought tolerant cultivars via techniques such as genetic engineering. In this study, three test cultivars of sweet potato namely KSP 36, PIPI and Zambezi were regenerated and transformed for drought tolerance via *Agrobacterium tumefaciens*-mediated transformation system using a drought tolerant *Xerophyta viscosa* peroxiredoxin 2 gene (*XvPrx2)* isolated from "the resurrection" plant *Xerophyta viscosa.* The three cultivars were subjected to transformation experiments where *Agrobacterium* strain EHA101 bearing a standard plasmid was used to infect the transgene into the selected leaf and stem explants in a factorial design. It was established that any time interval from 30 to 60 minutes is sufficient for infection as there were no significant differences ($p \leq 0.15$) in infection time regimes. Response to *Agrobacterium* infection, transformation and regeneration was genotype dependent. A total of 10 plantlets were regenerated from over 300 calli used giving regeneration frequencies (RF) of 0.02 for KSP 36, 0.04 for both Zambezi and PIPI. The survival rate of calli on selection was used to calculate the transformation frequencies (TF) which varied significantly ($p \leq 0.001$) among the three cultivars as follows KSP36 (0.26), PIPI (0.66) and Zambezi (0.75). Using 0.25mg/L zeatin and 1mg/L Indole-3-acetic acid (IAA) only promoted root formation but when kinetin (2mg/L) and IAA (1mg/L) were used shoot formation was promoted. Leaf explants recorded 87% calli formation whereas stems recorded less than 20%. There were significant differences in cultivar response to regeneration and transformation at $p \leq 0.001$ with Zambezi giving the best response. Seven putative transformants from Zambezi cultivar were identified to have been transformed through PCR giving a transformation frequency of 3.3% and transformation efficiency of 2.67%.

# CHAPTER ONE

# 1. INTRODUCTION

## 1.1 Background

Sweet potato [*Ipomoea batatas* (L) Lam] belongs to the family Convolvulaceae (Purseglove, 1968). It is a creeping herbaceous perennial vine that is cultivated as an annual crop in the world (Kay, 1973). It is a dicotyledonous plant producing both tuberous and fibrous roots (Austin, 1987). Its flowers are complete with a compound superior pistil, five separate stamens attached to the corolla with petals united into a trumpet or bell shaped corolla. It is a hexaploid with 90 chromosomes while other *Ipomoea* species have 30 chromosomes (Pursegrove, 1968). It produces tubers, stems and leaves which are utilized as human food and animal feed respectively (Kapande *et al.*, 1995; Mkumbira, 1995; Kay, 1973 and Kapinga et al., 1995).

Globally sweet potato ranks 7$^{th}$ in terms of importance as a food crop mostly in developed countries whereas in developing countries it is ranked 4$^{th}$ (CIP, 2007). It accounts for 95% of the world's food production. In Africa the area under sweet potato is estimated to be 1,714,000 hectares, with an annual production of 5.5 million tonnes (FAO, 1996; CIP, 2005). Countries in Eastern and Southern Africa account for 76% of sweet potato production in Africa with Uganda as the leading producer (FAO, 1996). Sweet potato is considered as a subsistence crop since it has very low input requirements compared with other crops and mainly grown as a food crop. The only major input is labour for land husbandry practices and finally harvesting. In large part of eastern, central

and southern Africa, small scale farmers with limited land, labour and capital mostly grow sweet potato (Teri *et al.,* 1995). The crop is one of the world's high yielding crops adapted to a wide range of agro-ecological zones of the world doing well in sub-optimal conditions compared to other important crops such as maize, rice, wheat, beans (Woolfe, 1992). The crop is often planted on marginal lands with limited water supply and grows during drought conditions but it is able to produce acceptable yields hence it serves as a food security crop (Opeke, 1991). Its yield potential under good rainfall conditions is more than 30t/ha. However, yield and quality of tubers are significantly reduced under drought conditions. Yield response to drought conditions also varies among cultivars. A number of the indigenous cultivars exhibit high resilience to drought conditions than the newly introduced yellow-orange fleshed cultivars which are highly recommended for their superior nutritional values in terms of vitamin A content (CIP, 2005).

Apart from being used as human food and animal feed, sweet potato has other uses as raw material for industrial products such as, starch, noodles, candy, desserts and flour and also as a source of income to farmers living near urban centres (Horton *et al.,* 1991; Kapande *et al.,* 1995; Kumwenda, 1995; Martin, 1988). The flour is used to make sweet potato porridge, doughnuts, short-cakes, biscuits, buns, bread, beverages and other products. The need to manipulate sweet potato genetically for drought tolerance for higher yields can not be over emphasized in order to meet the growing demand.

The *p*eroxiredoxin 2 gene (prx2) has been isolated from *Xerophyta viscosa,* a resurrection plant which is renowned for its ability to dehydrate up to 5% moisture content and

endures long periods of drought but upon the onset of the rains *X. viscosa* resumes its normal physiological functioning within 24 to 72 hours. This prx2 gene codes for peroxiredoxin enzyme which scavenges for reactive oxygen species (ROS) which plants produce when under biotic or abiotic stresses (Peters, 2003).

**1.2 Problem statement**

Drought is the major cause of decreased productivity and poor quality yields of most staple crops including sweet potato, leading to imbalances in terms of food supply and demand hence famine incidences (FAO, 1996). Food sufficiency in view of a global population growth rate of 3.0% is a daunting task particularly to developing countries (FAO, 1996; 1998). Interventions such as food importation and food aid have failed to eliminate this deficit as they are very expensive, unsustainable and create a dependency syndrome.

Overcoming drought problems in Arid and Semi Arid Lands (ASAL) of Africa is a promising goal to increasing crop productivity. This can be achieved through the development of drought tolerant crops including sweet potato cultivars among many other interventions. Efforts such as sweet potato hybridization, introduction of seeds and production of new clones by the International Potato Centre's (CIP) Regional office for the Sub-Saharan Africa in the SADC region (Mkumbira, 1995), have been employed but there is still no suitable drought tolerant cultivars available in the germplasm collection so far evaluated.

## 1.3 Justification

Changing agro-climatic conditions, frequent drought occurrences, degraded soil conditions and socio-economic instability have adversely affected field crop production in Africa (FAO, 1998). This has aggravated poverty levels and famine in cereal and root crops producing countries. For example, the Southern Africa Development Community (SADC) region experienced the worst ever drought conditions in recent years in 1991/92, 2001/02 in which sweet potato production decreased by 40%, compared with 60% for cassava and nearly 95% for maize (FAO, 2005). Consequently sweet potato is gaining in importance both as a food and a cash crop. The increasing demand for sweet potato is not being met by the current supply because of low, poor quality yields of sweet potato produced by smallholder farmers (Mkumbira, 1995). To increase production of sweet potato, there is an urgent need to explore genetic transformation of recommended sweet potato cultivars to drought tolerance. Convention breeding programmes have failed to address this problem fully. The use of biotechnological tools will complement efforts achieved through conventional breeding programmes. This study was conducted to transform selected sweet potato cultivars via *Agrobacterium*-mediated transformation with *XvPrx2* gene isolated from the resurrection plant *Xerophyta viscosa* that codes for peroxiredoxin which scavenges for ROS formed during biotic or abiotic oxidative stress.

## 1.4 Null Hypothesis

It is impossible to regenerate and transform with *XvPrx2* gene selected, high yielding sweet potato cultivars in Eastern and Southern Africa.

## 1.5 Research objectives

### 1.5.1 General objective

To optimize protocols for regeneration via somatic embryogenesis and *Agrobacterium*-mediated genetic transformation of selected regional sweet potato cultivars.

### 1.5.2 Specific objectives

i. To optimize tissue culture protocol for rapid regeneration of KSP36, PIPI and Zambezi cultivars.

ii. To transform KSP36, PIPI and Zambezi sweet potato cultivars with *XvPrx2* gene using an *Agrobacterium*-mediated transformation system.

## 1.6 Expected outputs

i. *In-vitro* reproducible regeneration systems developed for KSP36, PIPI and Zambezi applicable to other sweet potato cultivars covered by the Southern Africa Root and Tuber Research Network (SARRNET) and Association for Strengthening Agricultural Research in Eastern and Central Africa (ASARECA) regions.

ii. Routine *Agrobacterium*-mediated transformation systems developed for KSP36, PIPI and Zambezi applicable to other sweet potato cultivars for the SARRNET and ASARECA regions.

# CHAPTER TWO

# 2. LITERATURE REVIEW

## 2.1 Ecological requirements and distribution of sweet potato

Sweet potato is adapted to a wide range of climatic conditions and is known to produce high yields in sub-optimal conditions (Teri *et al.,* 1998). It is widely grown in tropical, subtropical and warm temperate areas throughout the world from 40° N to 32° S. The crop is adaptable to varying agricultural systems from intensive horticultural to subsistence farming with differing cultivar adaptability to soil and other conditions. Thus most small scale farmers grow sweet potato in marginal lands (Kay, 1973; Purseglove, 1968). The plant can do well in areas receiving rainfall between 500 to 1000 mm with low humidity as the crop reaches maturity. Sweet potato can tolerate considerable periods of drought but the yields are drastically reduced if the water shortage persists for 50-60 days after planting at the time when storage root initiation has begun (Kay, 1973; Purseglove, 1968).

Sweet potato does well in temperature regimes ranging from 10°C to 24°C for optimum growth but also in temperatures as high as 35°C. Abundant sunshine and warm nights with a minimum of cool cloudy weather are required. Temperature below 10°C retards growth and damages the crop (Kay, 1973; Purseglove, 1968). Altitudes from sea level to as high as 2100 M above sea level are known to sustain the growth of sweet potato (Kay, 1973).

Sandy loams reasonably high in organic matter, with permeable sub-soil are ideal for the cultivation of sweet potato. The crop is very sensitive to alkaline and saline conditions and requires good soil drainage. Best yields are produced on soils with a pH range of 5.6 – 6.6, acid soil deficient in calcium or magnesium (Kay, 1973, Janson and Roman, 1991). Short days with low light intensity promote the root development. Sweet potato requires a day length of 11.5 hours or less to promote flowering and at 13.5 hours flowering ceases but tuber yield appears to be unaffected (Kay, 1973).

## 2.2 Constraints to sweet potato production

Sweet potato production in SARRNET and many other African countries faces challenges from both abiotic and biotic stresses. The biotic constraints include viral diseases (Sweet potato featherly mottle virus, sweet potato mottle virus), weevils (Cyclas ssp.), vertebrate pests (moles, porcupines, monkeys etc.) and root knot nematodes (especially in Botswana) (Teri *et al.,* 1995). Abiotic constraints include salinity, infertility of the soil and most importantly, drought (Teri *et al.,* 1995). Other constraints include post-harvest losses due to lack of storage technologies or cultivars suitable for long storage and limited forms of utilization including processing and agronomic constraints such as late maturing, low yielding and drought susceptible cultivars (Teri *et al.,* 1995; Mbeza and Kwapata, 1995). In most African villages, crop production constraints such as environmental degradation, soil nutrient depletion, low fertilizer input, inadequate food processing amenities, poor roads to markets and general lack of information to make science based decisions that underlie farming methodologies and systems are prominent (Machuka, 2001).

## 2.3 Plant response to drought stress

Drought is the main abiotic constraint to production of many arable crops, and can cause an estimated yield loss of 20% and 100 % under severe conditions resulting in crop total failure (Edmeades *et al.,* 1994). Drought tolerance is defined as the ability of a given cultivar to show a relatively small yield reduction upon exposure to drought. This implies that breeding for stress is at the expense of yield. Drought resistance is a complex trait. Several important mechanisms are involved in drought tolerance i.e. drought escape via short life cycle and developmental plasticity, drought avoidance via enhanced water uptake and reduced water loss, drought tolerance via osmotic adjustment and antioxidant capacity and drought recovery via desiccation tolerance (Mackill *et al.,* 1999).

Under different abiotic and biotic stress conditions, plants generate/accumulate reactive oxygen species (ROS), including hydrogen peroxide ($H_2O_2$), superoxide anion and hydroxyl radicals which damage cellular structures (Inze *et al.,* 1995; Smirnoff, 1993; Sagadevan et al., 2002). Large amounts of $H_2O_2$ accumulation lead to programmed cell death (PCD). However, a relatively small amount of $H_2O_2$ is known to modify gene expression and results in enhanced plant stress responses. Accumulation of $H_2O_2$ can, in turn, induce the expression of detoxification and stress protection genes/enzymes such as heat shock proteins (HSPs), glutathione-S-transferases (GSTs), catalases, ascorbate/glutathione peroxidases, superoxide and pathogenesis-related proteins, thus protecting plants from stress damages (Kovtun *et al.,* 2000). The capacity of antoxidative defence system determines the fate of the cell and whether the cell continues to function

or suffers photo-oxidation (Foyer *et al.*,1994). HSPs have been reported to serve as molecular chaperones that participate in ATP-dependent protein unfolding or assembly/disassembly reactions and prevent protein denaturation during stress (Pelham, 1986). Correlations between expression of HSPs and thermo-tolerance have been found in maize, tomato, and creeping bent grass (Park *et al.,* 1996, Preczewski *et al.,* 2000, Ristic *et al.,* 1998). HSPs and GSTs play essential roles in detoxification and stabilization of damaged proteins, thereby assisting cell recovery from stresses (Slater *et al.,* 2003).

**2.4 Molecular insights into drought stress**

Drought stress induces various biochemical and physiological responses in plants. Plants respond to drought stress through dehydration or desiccation (Ramanjulu *et al.,* 2002). A number of genes have been described that respond to drought at transcriptional level (Bohnert *et al.,* 1995; Ingram and Bartels, 1996; Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997, 1999, 2000; Seki *et al.,* 2002). Stress-inducible genes have been used to improve the stress tolerance of plants by gene transfer (Holmberg and Bulow, 1998; Bajaj *et al.,* 1999). More than 50 drought responsive genes have been characterised, but possibly more than hundreds of other genes are involved (Seki *et al.,* 2001). For example Dehydration-responsive element/C-repeat (DRE/CRT) has been identified as an important *cis*-acting element in drought-, high salt-, and cold stress-responsive gene expression in an abscisic acid (ABA)-independent manner (Yamaguchi-Shinozaki, 2000; Shinwari et al., 1998; Takahashi et al., 2002).

Transcription factors (DREB/CBF) involved in DRE/CRT-responsive gene expression have been cloned (Gilmour *et al.,* 1998; Liu *et al.,* 1998). DREB1/CBFs are thought to function in cold-responsive gene expression, whereas DREB2s are involved in drought-responsive gene expression (Thomashaw, 1999). DREB1A target genes which include *rd29A/lti78/cor78, kin1, kin2/cor66; cor15a, rd17/cor47*, and *erd10* have been identified (Kasuga *et al.,* 1999, Jaglo-Ottossen et al., 1998). Most of these genes are induced by both drought and cold stress and contain the DRE or DRE-related CCGAC core motif in their promoters (Thomashaw, 1999).

Besides transcription factors, other genes that are known to confer drought stress tolerance in plants include genes encoding water channel proteins, membrane transporters, cytoplasmic and chloroplast proteinases mitogen activated protein (MAP) kinases (e.g. MAPKK, MAPKKK), osmoprotectant synthases, chaperonines (Kovtun *et al.,* 2000). Also late embryogenesis abundant (LEA) proteins and detoxification enzymes (e.g. superoxide dismutase-SOD) (Bruce *et al.,* 2002) do confer drought stress tolerance. In addition, heat shock proteins (Nieto-Sotelo *et al.,* 2002), a tobacco MAPKKK (Shou *et al.,* 2004b) and genes encoding the sugar trehalose (Penna, 2003) and glycine betaine (Quan *et al.,* 2004) have also been shown to confer thermo- and drought tolerance in transgenic maize and tobacco (Shou et al., 2004a). Many research groups have also isolated genes from desiccation tolerant plants such as the resurrection plant in South Africa, a desert moss that can slow its activity down to zero when water is scarce, and revive from almost complete dehydration (Gardner *et al.,* 2002). These genes include a peroxiredoxin *XvPer2* (Garwe *et al.,* 2003) which protects nucleic acids against oxidative

stress-induced injury and *XvSAP1* which encodes a stress inducible signalling membrane binding protein, (Mowla *et al.,* 2002) from the desiccation tolerant plant *Xerophyta viscosa* (Family Velloziaceae). ABA induced genes have also been identified from the model moss *Physcomitrella patens* through a pilot Elementary Sequencing Tags (EST) sequencing project (Machuka *et al.,* 1999).

The above advances demonstrate the promise of using genetic transformation as a complementary approach to address drought stress in sweet potato. Being an 'orphan' crop, there are so far limited reports of transgenic research that focus on drought tolerant sweet potato for deployment in Africa. The first transgenic sweet potato cultivars developed for virus resistance underwent trials in Kenya in 2001. Molecular characterisation of these plants has also been carried out and methodologies for *Agrobacterium*-mediated transformation developed for two cultivars, namely CPT560 and KSP36 (Wanja *et al.,* 2005; Njagi, 2004; Njagi *et al.,* 2004). The stage is set to identify and/or access and transfer such genes to Root and Tuber crops in SARRNET countries such as Malawi to enhance drought tolerance. The success in manipulating genes conferring desirable traits into economically viable cultivars would serve to reduce the escalating famine cases in most African and other developing countries.

**2.5 Sweet potato improvement for stress tolerance**

Most of Africa's land mass is classified as Arid and Semi Arid (ASAL) and is characterised by drought stress problems. Worse still global climatic change is on the increase and is expected to lead to higher temperatures hence greater evapo-transpiration

and increased drought incidences thereby creating more drought prone crop growing areas. However, development of drought tolerant cultivars through conventional breeding is time-, space- and labour-consuming (Flowers and Yeo, 1995) and also introduces a junk of other unwanted linked genes together with the gene expressing the trait of interest. Biotechnology offers alternative tools to drought tolerance development in plants, through genetic engineering as it is specific to the gene of interest (Machuka, 2003, Byerlee and Gregory, 1999). Efforts to embrace biotechnology in most of African countries are at an advanced stage as evidenced by establishment of BioSafety policies (Africabiolines, 2006). One way forward in solving the drought problem is to use genetic transformation techniques. To date, biotechnology is already being applied rapidly to solve biotic constraints in sweet potato, such as controlling insect and viral diseases and enhancing nutritional content (Garcia *et al.,* 1999; Njagi, 2004; Njagi *et al.,* 2004; Yamaguchi *et al.,* 2004; Lowe et al., 1994, Newell et al., 1995).

### 2.5.1 Constraints of sweet potato to conventional breeding

Sweet potato is unsuitable for conventional breeding programmes due to the following limitations: first the plant's nature makes it difficult to cross as there are few cultivars which flowers and those that flower do so for a very short period of time. Male sterility and self pollination of most flowers is another limitation that makes sweet potato impossible to be used in the breeding programmes (Woolfe, 1992; Purseglove, 1968). Different cultivars possess different numbers of chromosomes thus making them mutually incompatible. Its vegetative means of propagation makes it difficult to be used in the conventional breeding programmes (Kay, 1973; Wambungu, 2001). These

problems necessitate the need to use other strategies such as genetic engineering to fill the gap left by conventional breeding.

**2.6 Plant transformation**

There are two major DNA delivery systems that have gained wide application, these includes biolistic and *Agrobacterium*-mediated transformation. Transformation and recovery of fertile transgenic plants via particle bombardment was first reported by Gordon-Kamm *et al.,* (1990). In biolistic method, a plasmid or linearized DNA with the gene of interest is fixed on to tungsten or gold particles (micro-carriers). The micro-carriers are delivered to host cells at high speed so as to penetrate the nucleus of the plant cell using different methods such as mechanical impulse or macroprojectile, magnetic or electrostatic forces, gene gun and electric discharge (Bendahmane *et al.,* 1999). This technology has been used to transform various maize target tissues (Gordon-Kamm *et al.,* 1990), including immature zygotic embryos from inbred lines (Koziel *et al.,* 1993; Dunder *et al.,* 1995; Brettschneider *et al.,* 1997) and HII germplasm (Songstad *et al.,* 1996; Pareddy *et al.,* 1997).

However, due to demerits associated with biolistic method such as high copy numbers and inability to transfer larger DNA segments; this method is losing its popularity paving way for the most recent and promising technology for transformation referred to as *Agrobacterium tumefaciens*-mediated transformation. This method uses a bacterial plasmid as a vector (Frame *et al.,* 2002). This method of transformation is the most widely used to introduce foreign genes into plant cells (Ishida *et al.,* 1996). This gene

delivery system results in a greater proportion of stable, low copy number transgenic events than does the biolistic gun (Ishida *et al.,* 1996, Zhao *et al.,* 1998). It offers the possibility of transferring larger DNA segments into recipient cells (Hamilton *et al.,* 1996); is highly efficient (Ishida *et al.,* 1996, Zhao *et al.,* 1998) and it possesses broad host range.

*Agrobacterium tumefaciens* is a soil bacterium that contains a Ti plasmid (tumour-inducing) which naturally infects dicotyledonous plant cells, making the bacteria an excellent vector for the transfer of foreign DNA (Hajdukiewicz et al., 1994). By removing the tumour inducing genes and replacing them with the genes of interest, efficient transformation can occur (Gelvin, 2000). In plants, transformation involving this natural gene transfer mechanism of *Agrobacterium*, transgenic plants and progeny from important monocot crops such as maize (Ishida *et al.,* 1996) have been obtained through *Agrobacterium*-mediated transformation. Principally, the bacteria can transfer a piece of its plasmid DNA into infected plant cells where it integrates into the nuclear genome and expresses its own genes, whose products disrupt the hormonal balance within the plant cells and induces their proliferation to form tumours (Zupan and Zambryski, 1995).

The transfer-DNA (T-DNA) is located on a large plasmid referred to as Ti plasmid which also contains other functional parts for virulence (*vir)*, conjugation (*con)* and the origin of its own replication (*ori*) (Hooykaas and Schilperoort, 1992). Any gene located in the T-DNA region in principal can be transferred into plant cells. Only the 25 base pair direct repeats at the right and left borders are necessary (Zupan and Zambryski, 1995). During

transformation, a modified T-DNA vector is constructed in which the desired DNA fragment is inserted between the T-DNA border regions. This vector is transferred into *Agrobacterium* and virulence gene products actively recognize, excise, transport and integrate the T-DNA region into the host plant genome (Hooykaas and Schilperoort, 1992). The efficient DNA delivery systems such as *Agrobacterium*-mediated transformation have facilitated comprehensive genetic engineering of crops.

## 2.7 Marker Genes

The use of marker genes in a transformation process aims to give selective advantage to the transformed cells, allowing them to grow faster and better and to kill all the untransformed ones. In general the selectable/marker gene is introduced into the plant along with the gene of interest. Sometimes the marker gene is the gene of interest that will express an agronomic characteristic, such as herbicide resistance (Aragao and Brasileiro, 2002). In this study *bar* gene was used as a selectable marker for the transformed cells. The *bar* gene was isolated from *Streptomyces hygroscopicus*. This gene codes for Phosphinothricin-N-Acetyltransferase enzyme (PAT, EC 2.3.1.-) (Murakami *et. al.*, 1986). The PAT enzyme inactivates herbicides with phosphinothrin (PPT) as an active compound such as Basta™, Liberty™ and Herbiace™. The herbicide is detoxified through the acetylation of the PPT amino free group using acetyl Coenzyme A as a cofactor that prevents PPT binding glutamine synthatase enzyme (GS). The success stories on the use of *bar* gene as selectable marker are mostly reported in cereals such as maize, rice, sorghum, wheat, barley oats and rye (Vasil, 1994; Vain *et al.*, 1995; Vain, 2007).

## 2.8 Source of the drought tolerant gene-*Xerophyta viscosa* and mode of action

***Xerophyta viscosa*** (Baker) [Family: Veloziaceae] is unusual and tough plant. It is specific to Africa and is found on mountain top habitats such as Cathedral Peak in the Drakensberg Mountains which stretches across Lesotho and South Africa. The plant is so unique in that it is able to survive long periods without water, but when it rains again the plants re-hydrates completely and remarkably resumes full metabolic functions within 24-72 hrs depending on the species (Figure 1). The resurrection plant is known to dehydrate up to 5% moisture content. The plant survives the long periods of drought and when the rains come it "resurrects" (Peters, 2003).

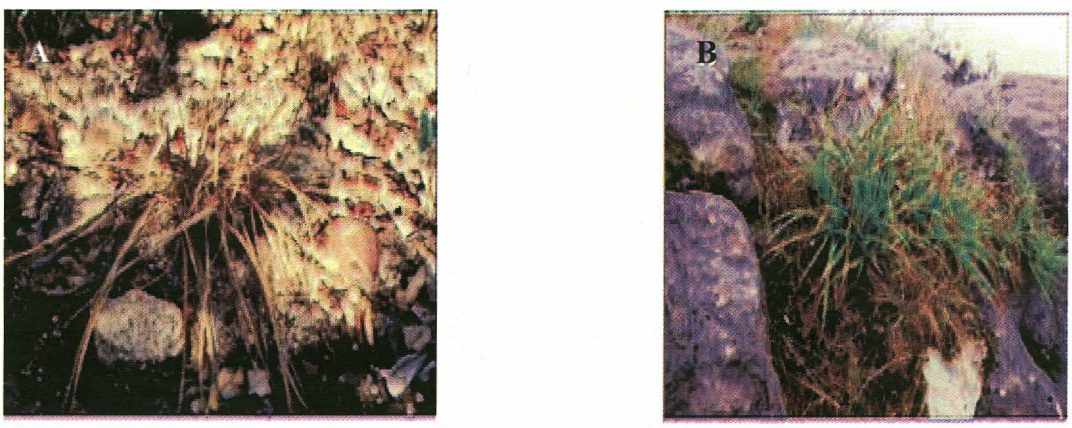



**Figure1: Xerophyta viscosa.**
**A**: Dehydrated up to 5% moisture content in its natural environment. **B**: Re-hydrated *Xerophyta viscosa* plant Note: The plant is known to resuming normal metabolic functions within 24 to 72 hrs depending on the plant species.

**Source:** Peters, S. (2003) *Science for Africa,* University of Cape Town, South Africa.

*XvPrx2* gene codes for antioxidant peroxiredoxin which scavenges for the reactive oxygen species (ROS) produced when the plant is stressed through biotic or abiotic causes (Garwe *et al.,* 2003). The ROS produced include hydrogen peroxides ($H_2O_2$), hydroxyl radicals and superoxide anions (Inze and Van Montagu, 1995; Smirnoff, 1993). These are broken down into alcohol, water and oxygen by the *XvPrx2* gene there by reducing/preventing the nucleic acid and cell membrane damage. These two (nucleic acid and cell membrane) when damaged are the ones responsible for the death of the plant cells hence the whole plant and not the lack of water as it had been believed for a long time (Mowla *et al.,* 2002).

# CHAPTER THREE

# 3. MATERIALS AND METHODS

## 3.1 Source of explants

Sweet potato cultivars were obtained from International Potato Centre (CIP) at Kenya Agricultural Research Institute (KARI) after virus indexing and were grown in plastic pots under screen house conditions at Kenyatta University. Three cultivars namely KSP36, PIPI and Zambezi were used in this study. Leaf and stem explants from healthy looking plants 2-5 weeks old (Figure 2) were used in the regeneration and transformation experiments. The leaf explants were cut into a size of 5 mm X 10 mm while the stem explants were cut between 5 mm to 10 mm exclusive of the nodes. Leaves from the $2^{nd}$ up to the $8^{th}$ node and stem internodes from the $2^{nd}$ node to the base starting from the apex were used.

## 3.2 Surface sterilization

Stem cuttings obtained from the potted sweet potato in the green house were used for *in vitro* propagation. The stem cuttings 8 to 10 nodes long were surface sterilized by first washing them under running water in order to remove the loose dirt on the surface. This step was followed by soaking them in 70% ethanol for 3 minutes; this was done in the preparation area. The explants were then further soaked in 2.5% sodium hypochloride (JIK$^{TM}$) with 2 to 3 drops of Tween 20 added for 20 minutes with occasional shaking under sterile conditions in the laminar floor (Njagi, 2004).

### 3.3 Propagation of selected sweet potato cultivars

Procedures for tissue culture and regeneration described by Njagi (2004) were followed using stem segments 4-6 nodes long as initial explants and at least 2 nodes were inserted into the media for rooting purposes. Cultures were incubated at 27±2$^0$C and a photoperiod of 16 hrs provided by cool white fluorescent lamps, with a light intensity of 1000-2000 Lux. The cultures were maintained in the growth room as *in vitro* source of "clean" sterile explants (leaves and stems) for regeneration and transformation experiments (Figure 2).

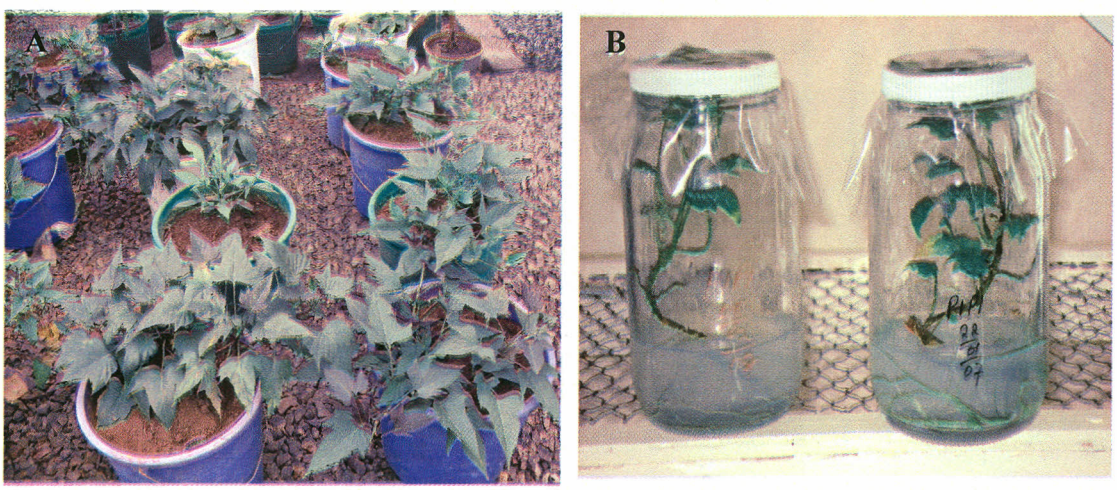



**Figure 2: Stock plants of KSP36, PIPI and Zambezi establishment.**
**A:** Screen house establishment of sweet potato in plastic pots. **B:** *In vitro* propagation of sweet potato in the growth room in growth bottles. These were the source of explants for the regeneration and transformation experiments.

### 3.3.1 Media preparation

Nutrient media containing Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962, 1974) supplemented with agar 8 g/L and sucrose 30 g/L formed the basic sweet potato media (BSP media) in this study. Sucrose level requirements for culture developmental stages were varied accordingly. Before autoclaving at 15 kilopascals at 121°C, the media pH was adjusted to 5.8 with 1N NaOH and 1N HCl. Growth regulators such as 0.2 mg/L 2, 4-D, 0.5 mg/L BAP, 2mg/L kinetin, 15 mg/L giberrellic acid, 0.3 mg/L abscisic acid, 1 mg/L indole acetic acid (IAA) and 3 mg/L zeatin were added into media in different concentrations and combinations, depending on the developmental stages of the explants.

## 3.4 Transformation of Sweet potato

*Agrobacterium*-mediated transformation of sweet potato was done as described by Njagi (2004) at the plant transformation laboratory in Kenyatta University. The transfer DNA was mobilized in *Agrobacterium* strain EHA101 with a standard binary vector containing the gene of interest (*XvPrx2)* driven by *Xerophyta viscosa peroxiredoxin sap* (*XvPsap)* promoter. The gene construct bearing the *XvPrx2* gene also harboured the *bar* gene as a marker gene encoding bialaphos resistance for positive selection of transformed tissues in culture (Figure 3)

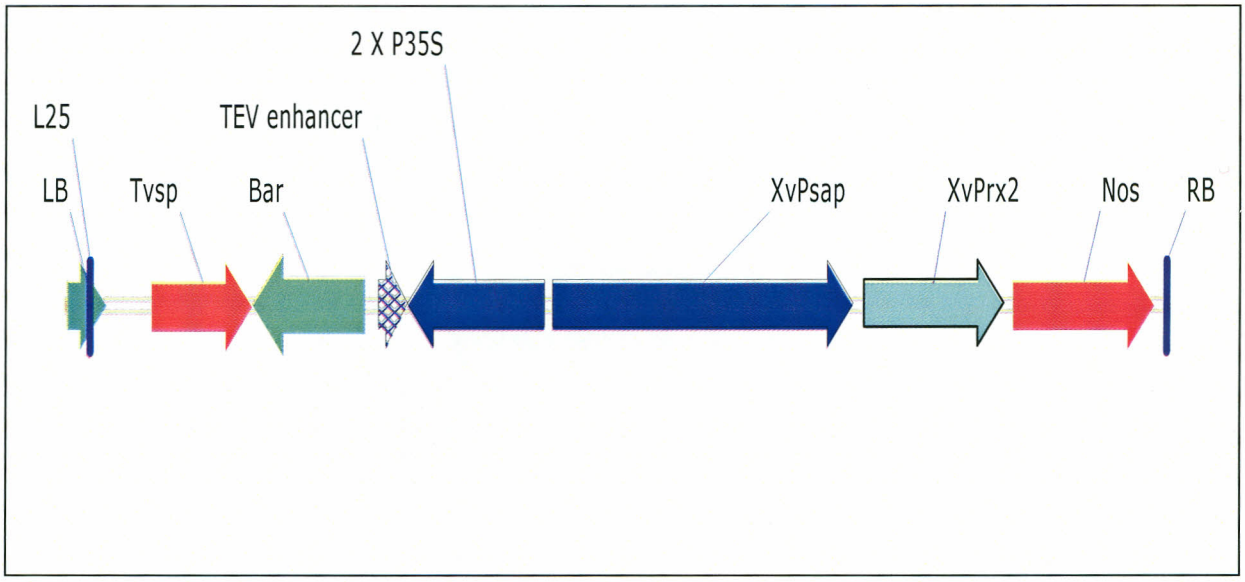



**Figure 3: The transfer-DNA in the gene construct**
Bearing *Xvprx2* gene driven by *XvPsap* and terminated by *nos*. The construct also harbours *bar* gene driven by *p35S* between the Left and Right Boarders of the plasmid.

This gene construct was constructed by Richard Okoth Odour under the supervision of Professor Jennifer Thompson of the University of Cape Town in South Africa. Through the collaborative initiative between Kenyatta University and University of Cape Town, this gene was made available for use in this study.

### 3.4.1 *Agrobacterium tumefaciens* maintenance and explants infection

*Agrobacterium tumefaciens* carrying the *XvPrx2* gene was maintained on yeast extract peptone (YEP) media (yeast extract 15 mg/L, peptone 10 mg/L, sodium chloride 5 mg/L and bacterial agar 10 mg/L) and then post autoclave, ultrafilter sterilized chloramphenicol 50 mg/L, kanamycin 50 mg/L and spectinomycin 50 mg/L were added. These were refreshed every three weeks for rejuvenation of the *A. tumefaciens* cultures (Figure 4).

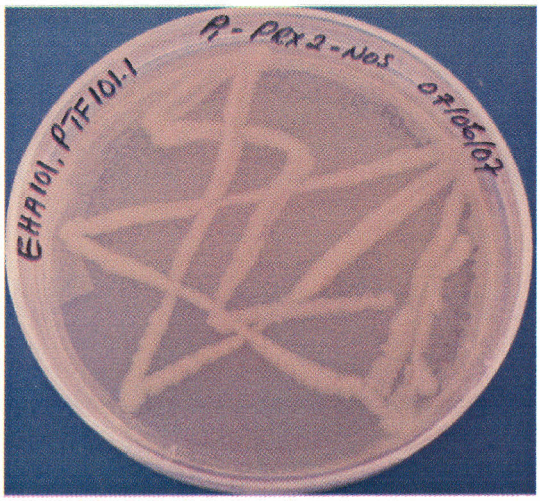



**Figure 4: *A. tumefaciens* bearing *XvPrx2* gene maintenance on YEP media.**
The media was refreshed at a 3 week interval in order to rejuvenate the *Agrobacterium.*

Two scoops of *A. tumefaciens* from the solid YEP plate grown for three days were added into liquid YEP in 50ml falcon tubes for over night shaking at 28 °C. This was followed by centrifugation at 14000 rpm for ten minutes and the supernatant was discarded and the sediments were suspended in co-culture media (BSP media minus agar). The optical density of the suspension was adjusted to 0.5 using a spectrophotometer at 260 nm wave length.

Poked leaf and stem explants were put on pre-culture media [BSP media supplemented with acetosyringone and galacturonic acid (inducers)] for 2-3 days. The explants were infected with the *A. tumefaciens* suspension by means of immersion of explants for time regimes of 30, 45 and 60 minutes. The explants were blotted by placing them on the Whatman filter paper to remove the excess liquid and then put on fresh pre-culture media for 3 days co-culture period at 21°C. After which the explants were washed in ant-

bacterial wash (basic SP media minus agar but supplemented with carbenicilin 500 mg/L and cefotaxime 100 mg/L) to kill the excess *Agrobacteria* then blotted on Whatman filter paper and cultured onto the callus induction media.

### 3.4.2 Callus induction

Calli were induced by placing the explants onto the callus induction media which consisted of basic sweet potato media supplemented with 2, 4-D (1 mg/L) and BAP (0.5 mg/L). These are responsible for calli initiation and maintenance respectively. Post autoclave, carbenicilin (500 mg/L) and cefotaxime (100 mg/L) were added to the media to control the recurrence of the *Agrobacterium* and bialaphos (1.5 mg/L) was also added into the callus induction media for the selection of the transformed calli. Cultures were incubated in the dark at 28°C for a period of 4-8 weeks or more depending on sweet potato cultivar. Sub-culturing was done at 3 week interval.

### 3.4.3 Selection for putative transformed calli

Two selection pressures were used to select for transformed calli (0.2 mg/L and 0.5 mg/L bialaphos for selection I and II, respectively). The selection media was the same as callus induction media where bialaphos was added depending on the selection stage. Explants were maintained on selection 1 for a period of 3 to 4 weeks then transferred onto selection II for another 2-3 weeks depending on the calli development. Selection II pressure was maintained in subsequent stages to eliminate the possibility of escapes (non

transformed cells). Proliferating calli that were large in size were subdivided into smaller sizes to ensure firm contact with the media. Only whitish friable calli (embryogenic) were transferred onto the next stage of development.

### 3.4.4 Initiation of somatic embryos

The selected calli were transferred onto calli maturation/embryo initiation media which consisted of autoclaved basic sweet potato media. After autoclaving the media was supplemented with carbenicilin (250 mg/L), cefotaxine (100 mg/L), abscic acid (ABA) (1 mg/ml) and bialaphos (1.5 mg/L). Basic acid was used for the initiation of embryo development whereas carbenicilin and cefotaxine were added to eliminate *Agrobacterium* upon recurring and bialaphos was added to eliminate the escapes (non- transformed calli). The cultures were maintained in the dark at 28°C in the dark room. The calli were maintained on this media for exactly two weeks.

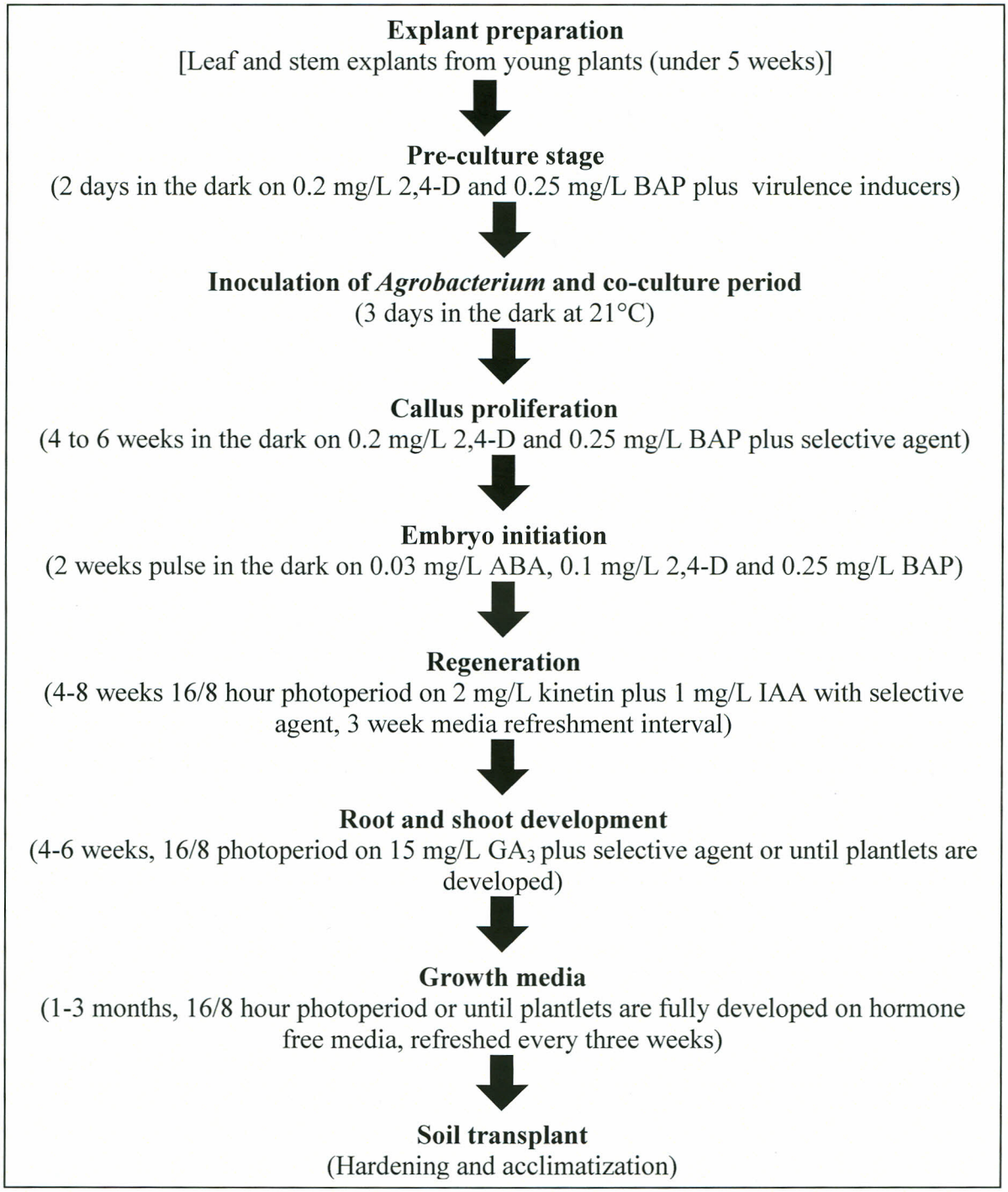
### 3.4.5 Regeneration of transformed calli

Somatic embryos were transferred onto regeneration media where embryos matured and later germinated into complete plantlets. The media consisted of autoclaved BSP media supplemented with kinetin (2 mg/L), Indole-3- acetic acid (1 mg/L), cefotaxine (100 mg/L), carbenicilin (500 mg/L) and bialaphos (1.5 mg/L). Calli were kept on this media as long as it took to form green colouration and for the embryo to germinate. The media were refreshed every three weeks. On average the calli remained on this media for 2-3 months in a 16/8 hours photoperiod regime at 28 °C.

### 3.4.6 Rooting and shooting of the regenerants

The greening calli showing primordial leaves and roots were transferred onto rooting and shooting media which consisted of autoclaved BSP media with more sucrose (60 mg/L) supplemented with giberrellic acid (15 mg/L) plus carbenicilin (500 mg/L), cefotaxine (100 mg/L) and half- strength bialaphos (0.75 mg/L) after autoclaving. The regenerants were maintained on this media with frequent refreshing of media every three weeks until the plantlets were big enough to be transferred into the small plastic pots containing sterile peat moss for hardening in the glass house. Incubation conditions were 16/8 hours light/dark at 28°C. The schematic presentation of the regeneration and transformation process is shown in Figure 5.

**Figure 5: Schematic presentation of regeneration and transformation Process**




**Source: Njagi (2004).**

### 3.4.7 Hardening-off of the regenerants

The grown plantlets were transferred into small pots filled with sterile peat moss covered with a plastic bag to regulate the humidity and temperature for 2 weeks at room temperature in the glass house. Water was added using hand sprayer in mist form just enough to support the plant. Plastic bags were removed for the plantlets to get acclimatized to the green house environment. The plantlets were transferred into sterile soil mixed with manure at the ratio 3:1 after the acclimatization process. Watering regimes were reduced to twice a week applying through the soil.

## 3.5 Molecular analysis

Molecular analysis work was carried out to establish whether the gene transferred through the *Agrobacterium*-mediated transformation had been integrated in the transgenic sweet potato genome. Polymerase Chain Reaction (PCR) screening and electrophoresis were used for identification and confirmation of putative transformants.

### 3.5.1 DNA Extraction

DNA for molecular analysis was isolated from transgenic and non transgenic (control) plants following a partially modified protocol described by Saghai-Maroof *et al.,* (1984) which stipulates stages to follow for the isolation of DNA from small amounts of plant tissues. Three hundred to 400 mg of leaf tissue from each sample were ground in liquid nitrogen to a fine powder which was transferred into 15 ml polypropylene centrifuge

tubes and 9.0 ml of warm CTAB (cetyltrimethyl ammonium bromide) extraction buffer at 65°C and 2 µl of B-mercaptoethanol were added to each tube. The tubes were incubated at 65°C for 60-90 minutes. The tubes were then placed at room temperature to cool for 4-5 minutes and 4.5 ml of chlorofoam: octanol (24:1) was added to each tube. The tubes were vortexed briefly and gently to avoid shearing of DNA and then inverted several times. Then tubes were span at 13 000-15 000 rpm for 5-10 minutes in a table –top centrifuge at room temperature. The aqueous top layer was transferred to a new-labelled 15 ml tubes and 4.5 ml of chlorofoam: octanol (24:1) was added to each tube and rocked gently for 5-10 min and then centrifuged for 10 min at 13000-15000 rpm at room temperature. The top aqueous layer was pipetted into 15 ml new tubes containing 30 µl of 10 mg/ml Rnase A (pre-boiled), mixed gently and incubated for 30 min at room temperature. Six millilitres of Isopropanol (2-propanol) was added and mixed by very gentle inversion which was followed by centrifugation at 13000-15000 rpm to precipitate the DNA. The DNA was placed in 5 ml plastic tubes containing 1 ml of Tris EDTA (TE) and let to stand for overnight to dissolve the DNA at room temperature. From the dissolved DNA 500 µl were pipetted and placed into 1.5 eppindorf tubes where 0.1 x volume of ammonium acetate ($NH_4O$ Ac) was added and mixed well then 2.5 x volume of absolute ethanol was added and incubated in the freezer for 20 minutes. The product was then centrifuged for 10 min and supernatant decanted, the DNA was blotted on filter paper to dry completely.

### 3.5.1.1 DNA washing

One ml of 70% ethanol (EtOH) was added to each tube and span at 14000 rpm for 30 minutes to precipitate the DNA. The supernatant was then aspirated. One ml of EtOH 95% was added to each tube for another DNA wash followed by spinning at 14000 rpm for 30 minutes. The supernatant was then poured off and the tubes air-dried for 15 minutes. Then 150 µl of distilled water were added to each tube to dissolve the DNA. Two micro litres of 10mg/ml (DNAse-free) RNAse A was added to each tube. The tubes were vortexed briefly and gently and incubated at 37°C for 1 hour and samples were then stored at 4 °C until they were analysed.

### 3.5.1.2 DNA preparation from *Agrobacterium* cultures

Two micro litres of a 48 hrs culture of the *Agrobacterium* in YEP medium were scooped and suspended in 50µl of sterile distilled water. The preparation was incubated at 95°C for 10 minutes to produce a bacterial lysate. The preparation was span at 14 000 rpm for 5 minutes and the supernatant harvested. This bacterial lysate was used in PCR reactions as positive control.

### 3.5.2 Polymerase chain reaction assay

Polymerase chain reactions (PCR) was performed in 0.2 ml tubes in 25 ml final reaction volume consisting of 2.5 µl of X10 PCR buffer, 1.5 µl of 25 mM of $MgCl_2$, 2.5 µl of 2.5 mM dNTPs, 2.5 µl *bar* primers, 0.2 µl *Taq* polymerase, 1.0 µl of DNA template and

16.3 ml double distilled water. Amplification of DNA sequences using *bar* 1 (20 mer) forward primer 5′ GCATCTACCATGAGCCCAGA 3′ and *bar 2* (18 mer) reverse primer 5' TGCCTCCAGGGAATTCAG 3' of *bar* gene was carried out with the following parameters: initial denaturation at 94°C for 5 minutes, followed by 35 amplification cycles (denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, extension for 1.5 minutes at 72°C) and a final extension at 72°C for 7 minutes. Bands were resolved on a 0.8% agarose gel buffered in 0.5x TBE buffer.

### 3.6 Data management

The experiments involving three sweet potato cultivars, gene construct and two types of explant used were setup as a factorial experiment with two factors design and three replications and etri dishes were used as replicates. The main factors were sweet potato cultivars and type of explants. Data collected include the survival rate from two levels of selection (0.2 mg/L and 0.5 mg/L of bialaphos), transformation efficiency, regenerants survival and the rate of regeneration. A comparison in response to transformation among the three cultivars, type of explant used and response was also analyzed. The data was analyzed by analysis of variance (ANOVA) using Genstat2 Discovery edition for windows. Means were separated at 5% significance level using the least significant difference (LSD). The transformation efficiency (TE) of the putative transformants of sweet potato explants was determined by dividing the number of confirmed putative transformants by the number of initial infected/inoculated explants expressed as a percentage. Transformation frequency was determined by the division of putative transformants by the initial number of explants infected expressed as a percentage.

# CHAPTER FOUR

# 4. RESULTS

## 4.1 *In vitro* culture of sweet potato

All the three selected sweet potato cultivars were cultured and maintained under *in vitro* conditions in the laboratory with no contamination. Elimination of external contaminants such as fungus and bacteria is necessary in tissue culture as they affect the growth of cultures negatively (Wanja, 2005). All the cultivars survived the surface sterilization process with a mean survival rate of 95% (Table1). Provision of all the necessary nutritional and environmental requirements in the growth room is of prime importance for the successful establishment of sweet potato *in vitro.*

**Table 1: Survival rate of *in vitro* explant cultures of the sweet potato cultivars.**

| Cultivar | Number of Explants cultured | Number of explants survived | Survival rate in culture (%) |
|---|---|---|---|
| KSP 36 | 200 | 190 | 95 |
| PIPI | 200 | 192 | 96 |
| ZAMBEZI | 200 | 189 | 94.5 |
| Total | 600 | 571 | 95 Mean |

Sweet potato stem cuttings were grown *in vitro*, the effect of temperature on the growth of cultures was investigated for a period of 3 weeks (Table 2). It was established from this investigation that temperatures rise above 28°C caused necrosis of the explants there

by affecting the supply of explants for regeneration and transformation experiments (Figure 6). A temperature rise of between 3 to 7°C from the recommended 28°C in this study resulted in a mean drop of 56.5% (Table 2) in explant survival rate.

**Table 2: Effect of temperature on growth of sweet potato in vitro**

| Cultivar | Number surviving at 28°C per 200 explants (A) | Survival rate (%) (B) | Number surviving at 31-35°C per 200 explants (C) | Survival rate (%) (D) | Survival rate differential (%) (E) (E=B-D) |
|---|---|---|---|---|---|
| **KSP 36** | 190 | 95 | 80 | 40 | 55 |
| **PIPI** | 192 | 96 | 90 | 45 | 51 |
| **ZAMBEZI** | 189 | 94.5 | 62 | 31 | 63.5 |
| **Mean** | **190** | **95** | **77** | **38.5** | **56.5** |

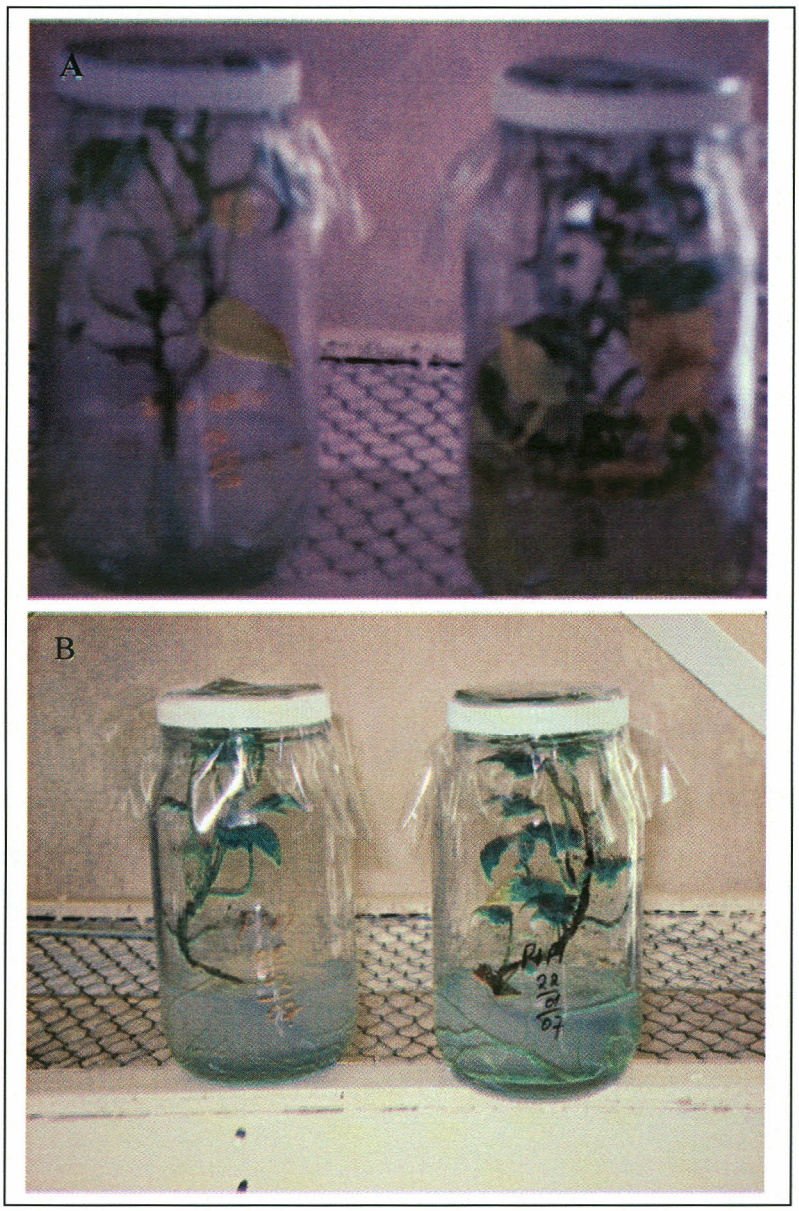



**Figure 6: The effect of temperature on the *in vitro* grown sweet potato.**
**A:** Temperature affected plants at 30°C showing necrotic leaves. This affected the availability of leaf and stem explants for regeneration and transformation experiments.
**B**: Healthy growing plants at 28°C

### 4.2 Regenerability of selected sweet potato cultivars

In order to find the right concentration of 2, 4-D for calli induction and regeneration different levels of 2, 4-D were tested on KSP36, PIPI and Zambezi under i*n vitro* conditions. Five levels of 2, 4-D (0 mg/L, 1 mg/L, 2 mg/L, 3 mg/L and 4 mg/L) and a constant concentration of BAP (0.5 mg/L) were used to find the optimum concentration to be used for callus induction in regeneration of transformants (Table 3). It was established from this study that concentration of 2 mg/L 2,4-D was optimum (Figure 7) to induce callus which eventually resulted in development of embryogenic callus. Concentrations less than 2 mg/L 2,4-D (0-1.9) induce embryogenic calli at a lower rate than at 2mg/L 2,4-D, concentrations above 2mg/L did not increase the calli formation percentage. At concentrations greater than 3mg/L there was no calli formation. There were no significant differences ($p>0.05$) in explants used in calli formation as both leaf and stem explants formed calli. However, there were differences in cultivar response with KSP36 performing poorly than PIPI and Zambezi as very few calli were developed. Also shown here is the genotypic dependence response among the three cultivars to 2,4-D in terms of callus formation with PIPI, Zambezi and KSP36 producing 57%, 41% and 38% of calli respectively, at a concentration of 2 mg/L of 2,4-D (Figure 7). A total of 10 plantlets (2 from KSP36 and 4 each from Zambezi and PIPI) were regenerated from the three cultivars giving regeneration frequencies of 2% for KSP36 and 4% for both PIPI and Zambezi with an overall mean RF of 3% (Table 3).

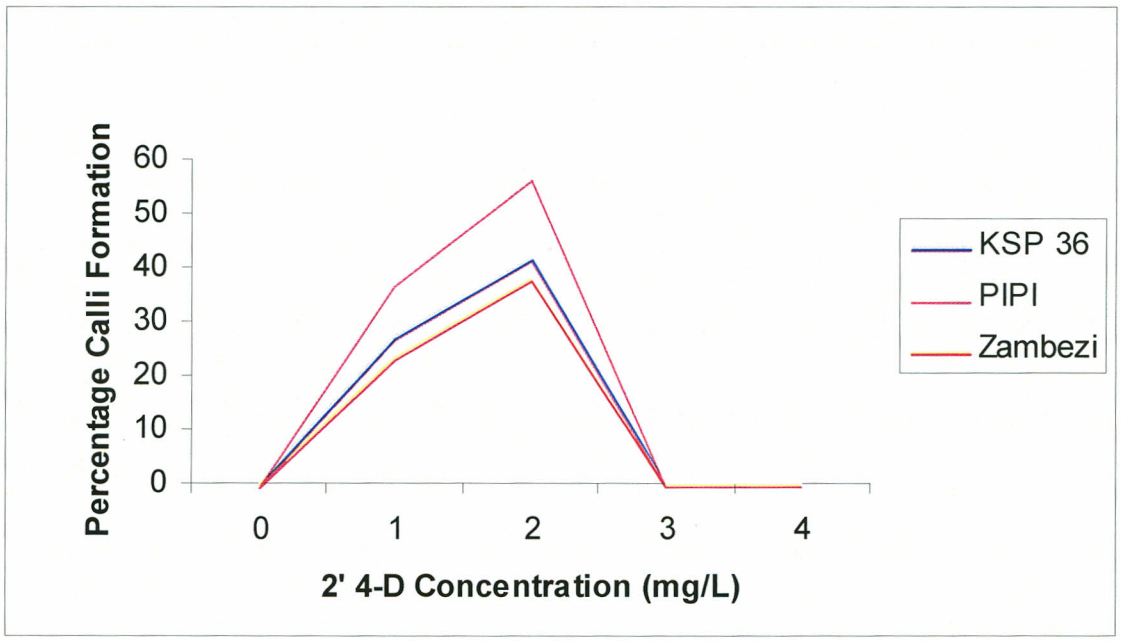


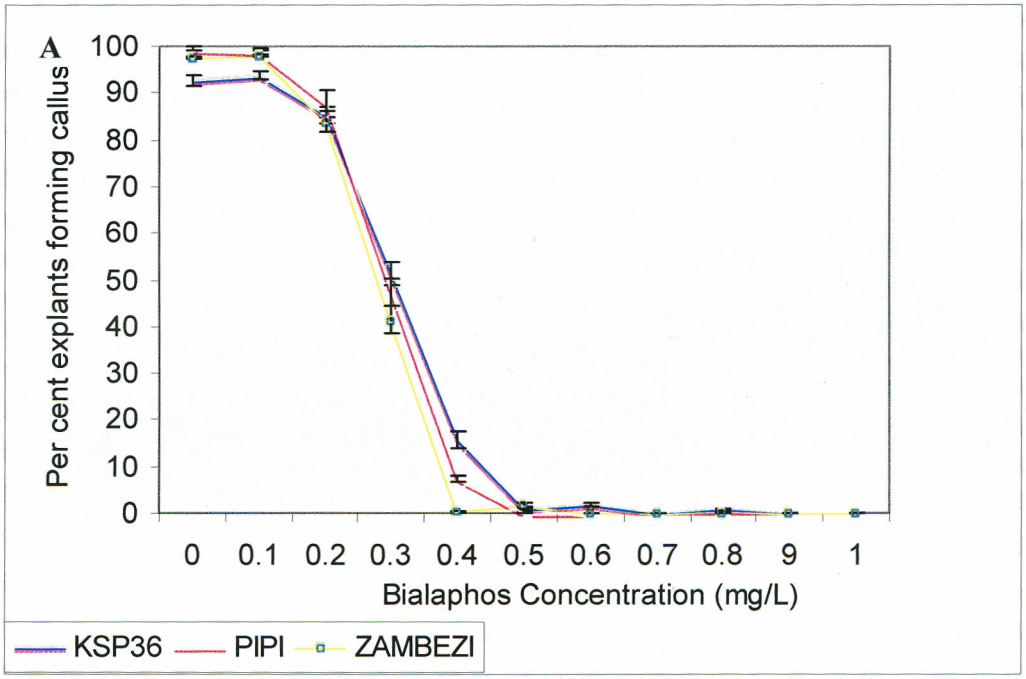

**Figure 7: 2, 4-D concentration optimization curve for calli induction.**

**Table 3: Effects of optimum 2,4–D concentration on calli formation**

| Cultivar | Type of explant | Number of explants | Number forming calli | Number of embryogenic calli | No. of regenerants | Regeneration Frequency (%) |
|---|---|---|---|---|---|---|
| KSP 36 | Leaf | 50 | 10 | 2 | 2 | 2 |
| | Stem | 50 | 7 | 0 | 0 | |
| PIPI | Leaf | 50 | 45 | 2 | 2 | 4 |
| | Stem | 50 | 23 | 2 | 2 | |
| ZAMBEZI | Leaf | 50 | 32 | 3 | 3 | 4 |
| | Stem | 50 | 15 | 1 | 1 | |
| **Total** | | **300** | **132** | **10** | **10** | **Mean= 3** |

### 4.3 Optimization of bialaphos concentration for selection of transformants

The construct for the *XvPrx2* gene also harbours the *bar* gene as a marker gene which was used to select for putative transformed calli and plantlets. An experiment to establish the optimum concentration of bialaphos to be used in selection of transformed cells was conducted with varying levels (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.8 and 1 mg/L) using leaf and stem explants in 3 replicates. It was determined (Figure 8) that a concentration of 0.5 mg/L of bialaphos was optimum to select for transformed calli. Non transformed explants from the three cultivars started to die when the concentration of bialaphos reached 0.5 mg/L. Concentrations above 0.5 mg/L bialaphos killed all the explants. At 0.2 mg/L bialaphos marked the initial decline (Figure 8) in numbers of explants surviving selection pressure and as a result it was taken as the first selection pressure. The three cultivars responded differently to bialaphos selection with KSP36 being the most sensitive than the other two cultivars while the explants (leaf and stem) response was the same (Figure 9).



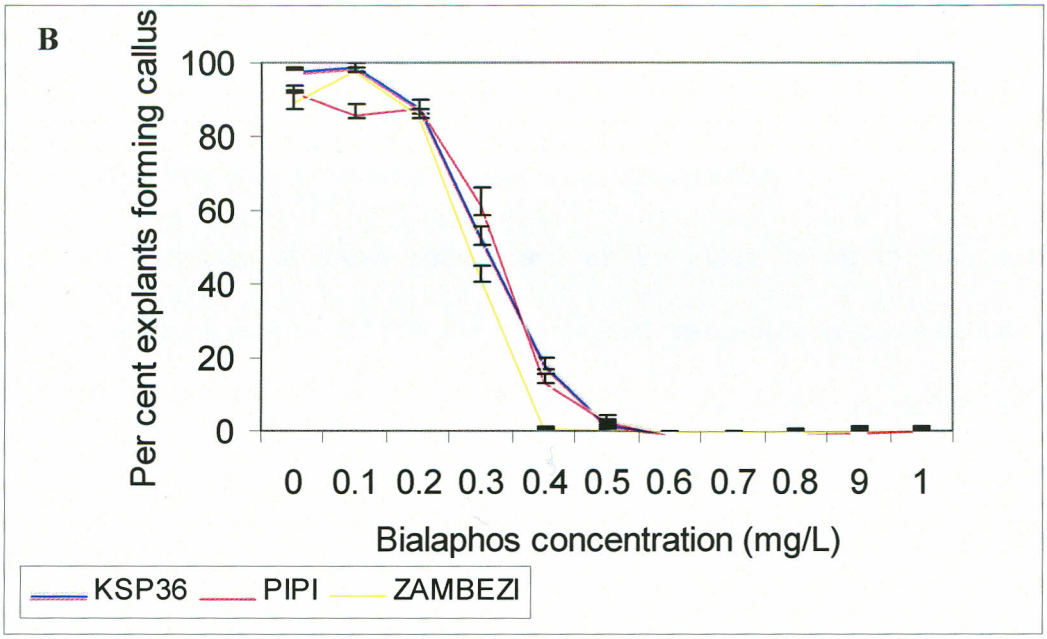




**Figure 8: Bialaphos titration curve.**
**A:** Stems and **B:** Leaves

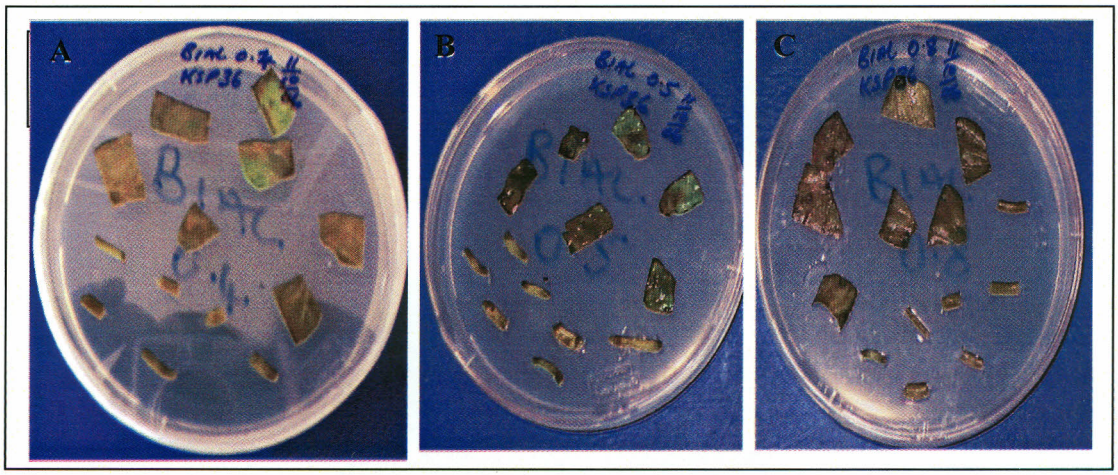



**Figure 9: Response of leaf and stem explants to different bialaphos concentration.** **A**: KSP36 leaf and stem Explants on 0.4 mg/L bialaphos. **B**: KSP36 Leaf and stem Explants on 0.5 mg/L bialaphos. **C:** KSP36 Leaf and stem Explants on 0.8 mg/L bialaphos. Notice the necrotic areas on the explants. The media used was callus induction media and similar results were obtained with the other two cultivars.

### 4.4 Varietal response to transformation and regeneration

The three cultivars of sweet potato used in this study varied in their response to *Agrobacterium* infection. KSP36 did not respond favourably as compared to PIPI and Zambezi (Figure 10). Explants from KSP36 manifested higher mortality rate than the other 2 cultivars. Lowest callus formation was obtained from KSP36 as most of the explants got necrotic soon after putting them on callus induction media for 1 week. PIPI and Zambezi explants remained green after the same 1 week period proceeded into callus formation. The same trend was also observed in the regeneration experiments where KSP36 gave the least regeneration frequency of 0.02 compared to 0.04 for both PIPI and Zambezi (Table 3).

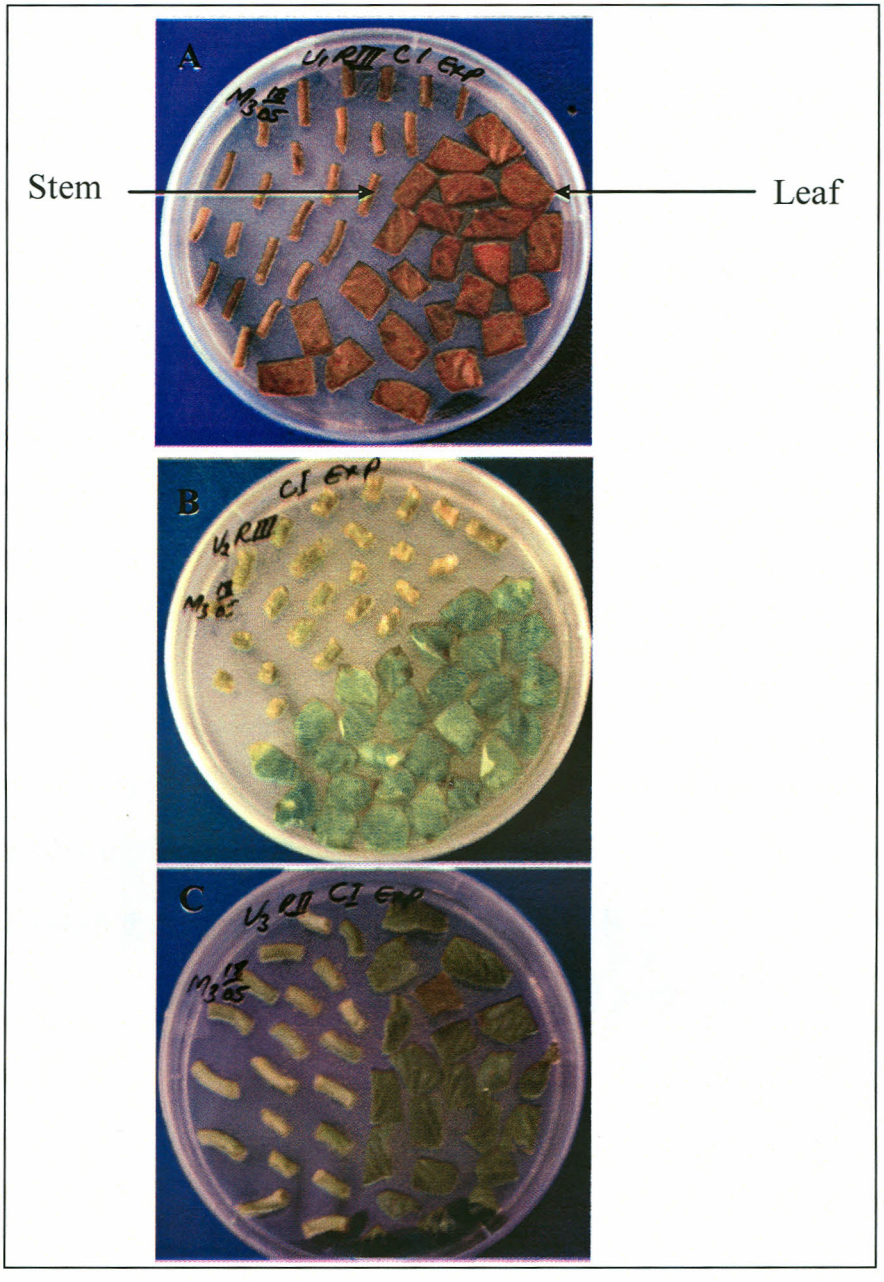



**Figure 10: Varietal response to *Agrobacterium* infection.**
**A**: KSP 36 leaf and stem explants. **B:** PIPI leaf and stem explants and **C:** Zambezi leaf and stem explants. Notice how KSP 36 responded, all the explants died. All the explants were of the same age (4 weeks old) and on callus induction media after 2 weeks.

### 4.4.1 Response of explants to transformation and regeneration

To evaluate the response of explant type used to *Agrobacterium* infection (Table 4), leaf and stem explants were evaluated for their transformability. Leaf explants responded favourably to *Agrobacterium* infection than did stem explants. There were significant differences ($p \leq 0.001$) in callus formation among the three cultivars with PIPI being the best (83.3% leaf and 35% stems) Zambezi produced 70% leaf and 25% stems. KSP36 produced the lowest calli formation percentages for both the leaf (25%) and stem (3.3%). The overall mean for leaf callus formation was 59.4% whereas that for stems was 21.1%. The same trend was observed in subsequent regeneration. All the regenerants sprout from calli originating from leaf explants.

Calli from the three cultivars were tested for their response to the selection pressures to check the effectiveness of the set selection pressures (Table 5). It was observed that KSP36 did not respond favourably to the selection agent. It was very sensitive to bialaphos since most of the calli from KSP36 did not proceed to the next stages in the regeneration process. There were significant differences ($p \leq 0.001$) in transformation frequency among the three cultivars with KSP36 having 26.7% compared to 75.5% and 66.5% for PIPI and Zambezi respectively.

**Table 4: Response of explants to infection**

| Cultivar | Number of leaf explants infected | Number of stem explants infected | Number of leaf explants forming calli | Number of stem explants forming calli | % Calli formation leaf | % Calli formation stem |
|---|---|---|---|---|---|---|
| KSP 36 | 300 | 300 | 75 | 10 | 25 | 3.3 |
| PIPI | 300 | 300 | 250 | 105 | 83.3 | 35 |
| ZAMBEZI | 300 | 300 | 210 | 75 | 70 | 25 |
| Mean | 300 | 300 | 178 | 63 | 59.4 | 21.1 |

**Table 5: Transformation frequency**

| Cultivar | Number of explants infected | Number of explants forming calli | Transformation Frequency (%) |
|---|---|---|---|
| KSP 36 | 300 | 80 | 26.7 |
| PIPI | 300 | 226 | 75.5 |
| ZAMBEZI | 300 | 199 | 66.5 |

### 4.5: Regeneration of putative transformants

PIPI and Zambezi Sweet potato cultivars renowned for their high nutritive value (high Vitamin A content) and high yielding were selected for drought tolerance transformation in this study. Ten putative transformants from Zambezi were regenerated (Table 6) giving a transformation frequency of 3.33% and transformation efficiency of 2.33%. Putative transformed calli from KSP36 and PIPI produced roots without any shoots at the same

growth regulator concentration and combination thus showing genotype dependent response of transformed calli to regeneration.

**Table 6: Transformation efficiency for putative transformants sweet potato**

| Cultivar | No. of explants infected | No. of regenerants | No. of confirmed transformants | Transformation Frequency (%) | Transformation Efficiency (%) |
|---|---|---|---|---|---|
| KSP 36 | 300 | 0 | 0 | 0 | 0 |
| PIPI | 300 | 0 | 0 | 0 | 0 |
| ZAMBEZI | 300 | 10 | 7 | 3.33 | 2.33 |

### 4.5.1 Pictorial stages for production of transgenic plants

Transformants were identified by initially selection for putative transformants in bialaphos as a selective agent. Calli which had been transformed remained live whereas all non-transformed calli or sections of calli that were not transformed died due to the effect of bialaphos (Figure11).

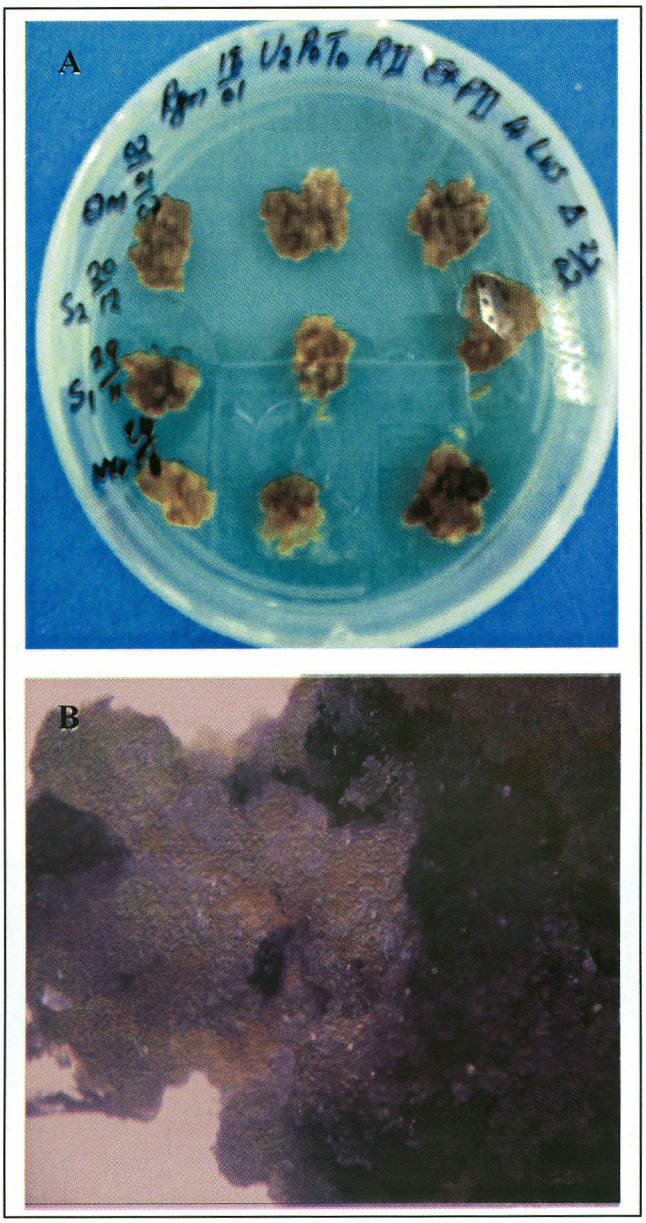



**Figure 11: Callus response to bialaphos selection.**
**A**: Untransformed calli dying during selection. **B:** Untransformed section of callus dying. Notice the black/brown area representing dead tissue due to bialaphos selection.

Production of transgenic plants started with calli production (Figure 12A). The calli were placed on embryo initiation media after 4 to 8 weeks and the embryos formed were oval or round with cream colouration, some were translucent while others were pale green

(Figure 12B). Most of these embryos just formed roots without giving any shoot. Majority of the calli that showed dark green pigmentation and compactness produced neither a root nor a shoot, they remained dormant and some just died in the course of time in culture. Those that were light green or purplish in colour and friable enlarged and gave some roots and shoots. Plant regeneration on ABA containing media was via somatic embryogenesis and the cultures remained on this media for 2 weeks as it was experienced that any extended exposure time lead to the death of the cultures and those surviving had difficulties in regenerating. Figures 12A-D summarizes the pictorial stages that were followed to produce transgenic plants including acclimatization process.

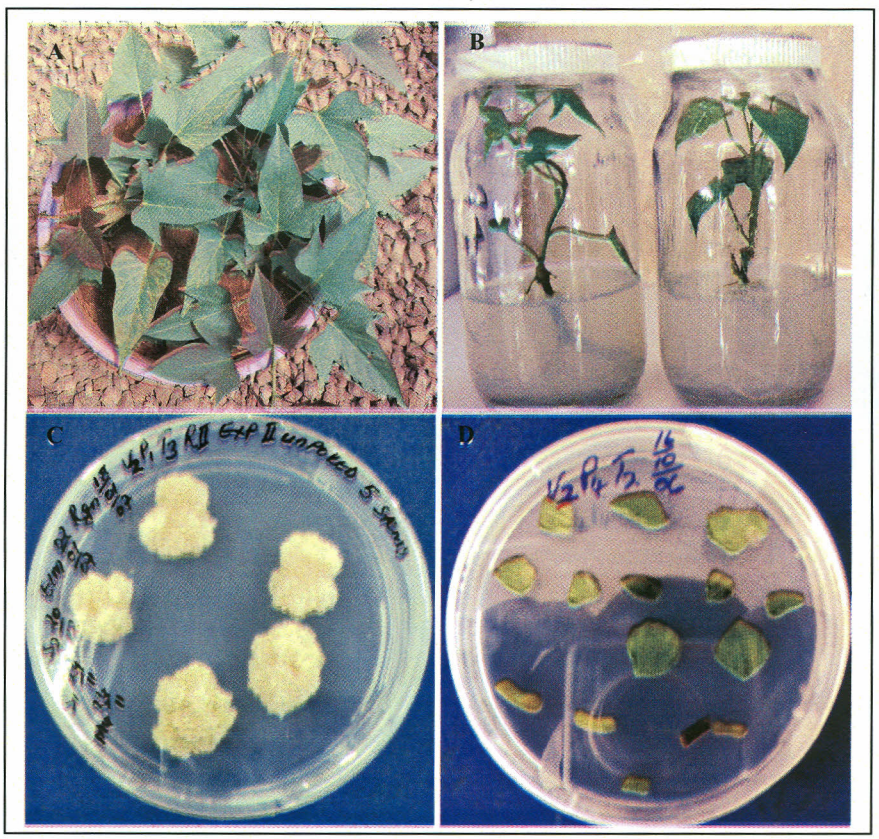



**Figure 12A: Stages in production of transgenic sweet potato - Callus production.**
**A**: Stock plant establishment in the screen house. **B:** *In vitro* micropopagated plants in the growth chamber. **C:** Leaf and stem explants on callus induction media. **D:** Callus formation


**Figure 12B: Transgenic sweet potato - Embryo initiation and regeneration.**
**A**: Embryo formation and initiation of green colouration. **B:** Root and shoot initiation. **C:** Development of a shoot showing primordial leaves. **D:** Fully developed shoot.

Matured calli changed colour from cream white to green and started showing primordial leaves and root tips. The calli developed root tips first before the shoot and the developed roots were cut to avoid growth imbalance due to the inherent concentration of the auxins produced by the roots as they develop. Calli induction took 4 -8 weeks at most but on average it took 6 weeks. Seven months were required for shoot formation from callus and

4-12 weeks for root formation. It was observed that it is faster to initiate roots than shoots in sweet potato (Figure 12C). The three cultivars of sweet potato used in this study responded differently in terms of callus formation, transformation and regeneration. PIPI and KSP36 performed poorly mainly in regeneration whereas Zambezi performed better having produced 10 putative transformants. On average there were 7 roots per callus obtained.

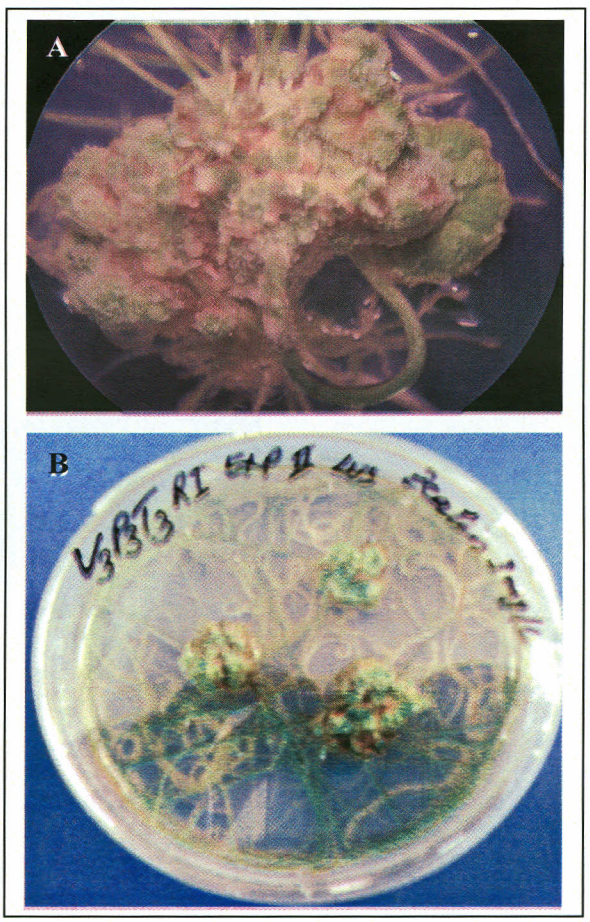



**Figure 12C: Root initiation.**
**A:** Root development from a single callus. **B:** Root proliferation without any shoot.

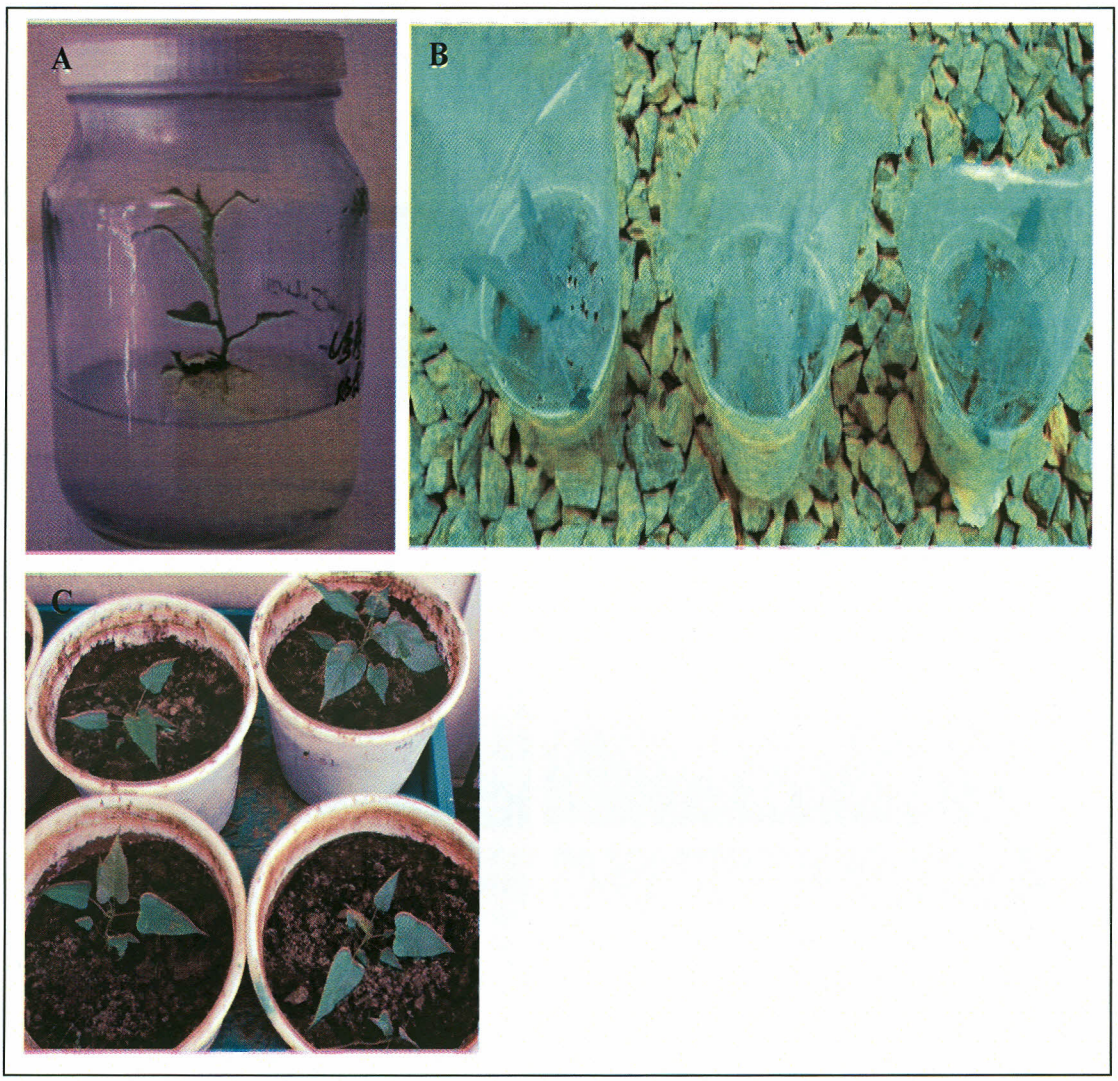



**Figure 12D: Transgenic sweet potato plant – Establishment of regenerants.**
**A:** Regenerants planted in a hormone free MS media. **B:** Acclimatization process of the regenerants to the green house condition. **C:** Acclimatized regenerants in the green house

### 4.5.2 Abnormal development in sweet potato regeneration.

Some variations were observed in the regenerated sweet potato. These included aerial roots, bald shoots, hard green calli, calli developing whitish sections had occurred in all the three cultivars. Figure 13 outlines the type of abnormalities observed.

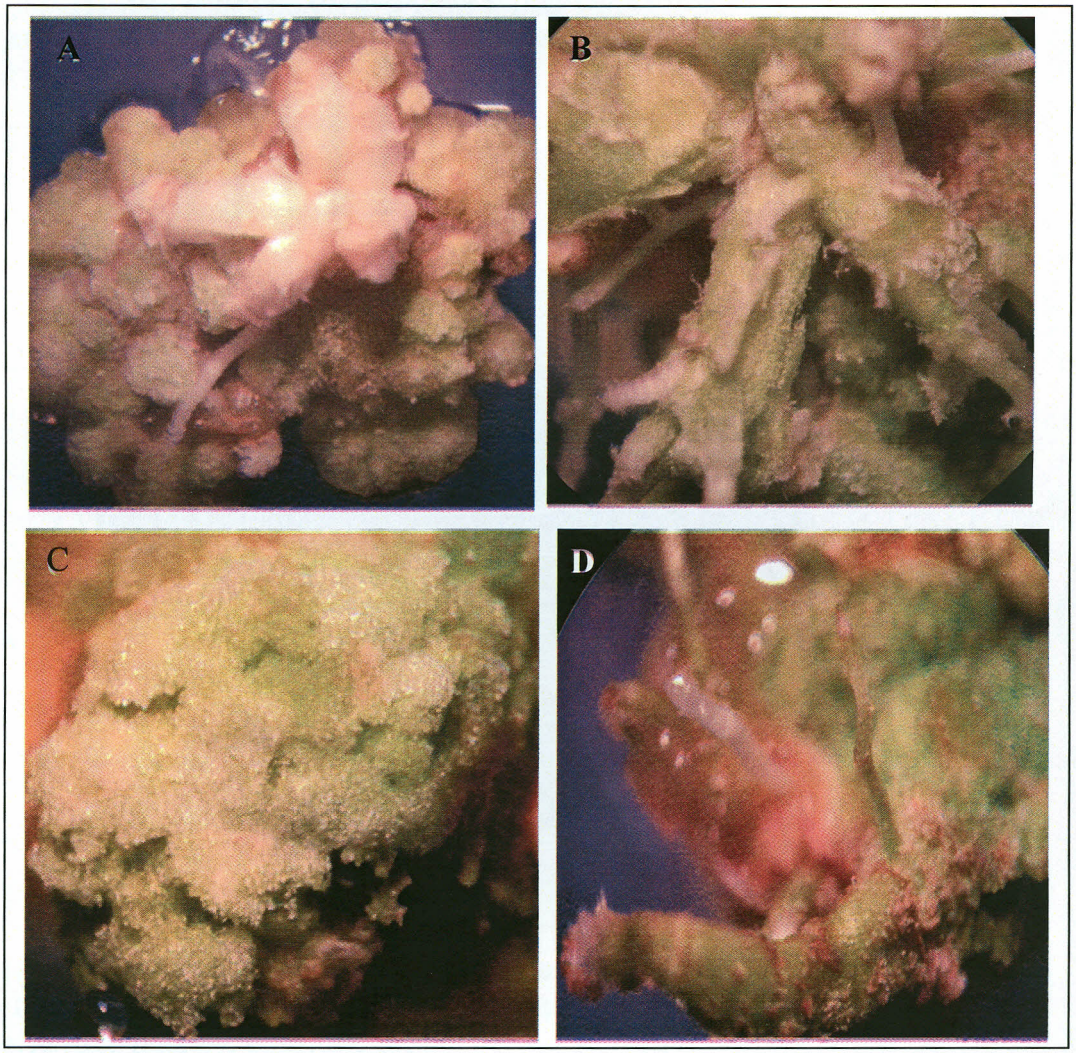



**Figure 13: Abnormal development in sweet potato regeneration.**
**A**: Calli developing whitish top. **B:** Multiple rooting. **C:** Hard greening calli with whitish sections. **D:** Bald headed shooting with aerial roots

## 4.6: Gel electrophoresis and PCR results.

DNA extracted from the putative transformants was assayed for the gene of interest using *bar* primers since the gene construct had the bar gene as a marker gene. There was amplification of the *bar* fragment. PCR products indicated the presence of the *bar* gene in the samples. Genomic DNA was present in all the samples (Figure 14) and seven samples were confirmed to be transformants by PCR assay (Figure 15).

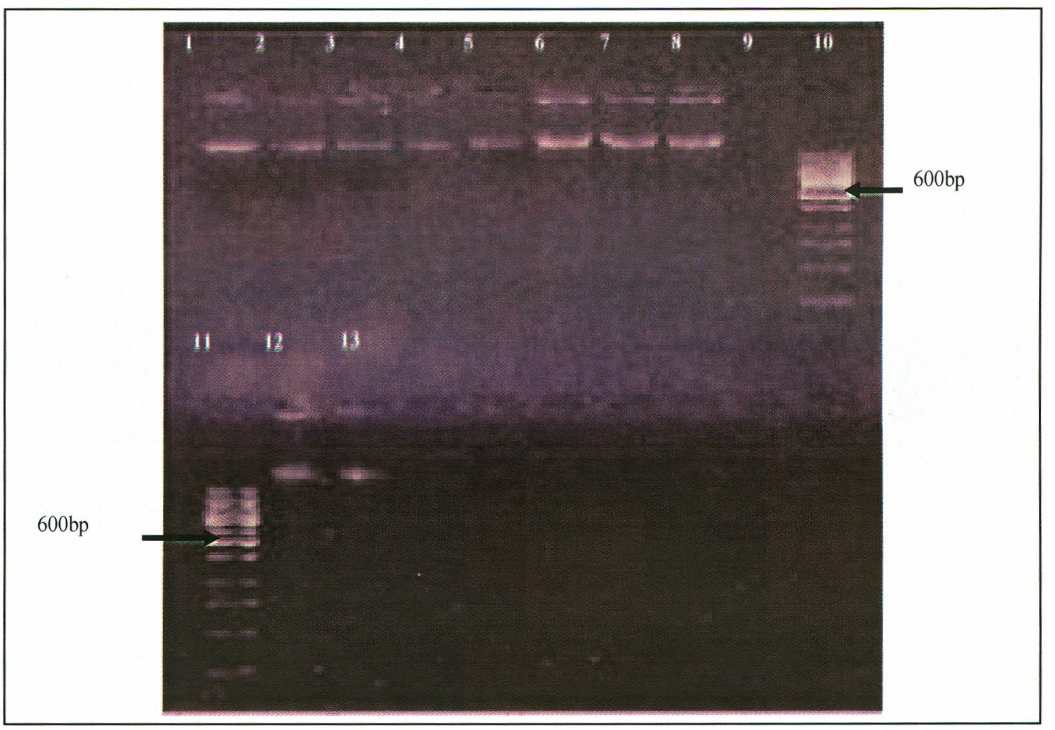



**Figure 14: Gel electrophoresis showing Genomic DNA.**
Lane 1 and 13-100bp molecular weight marker; Lane 2-pcr water; Lane 3-12 –DNA extracted from putative transformants.

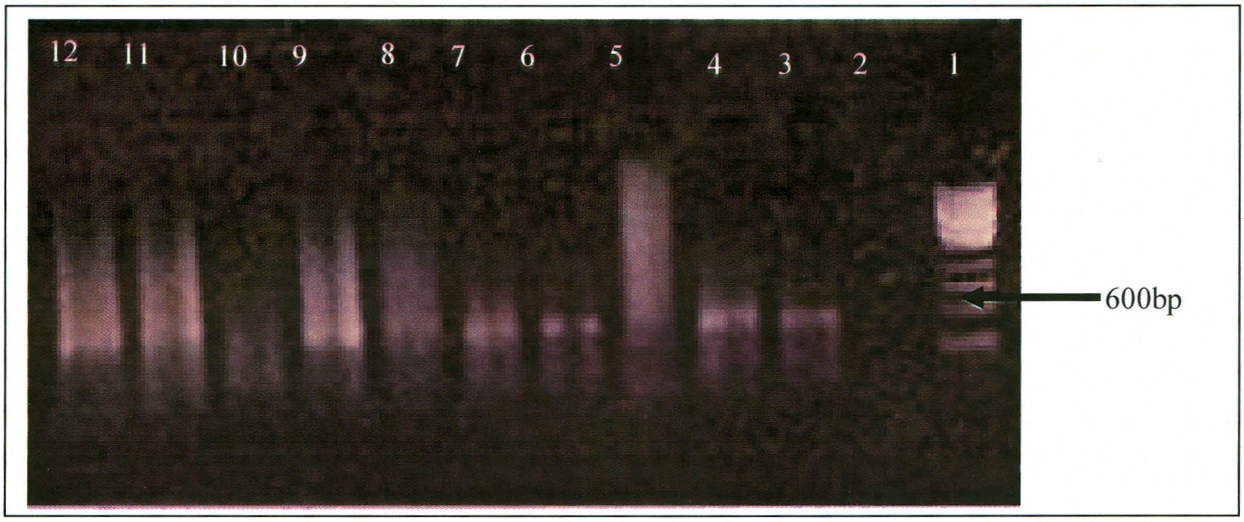



**Figure 15: PCR amplification of DNA Results.**
Seven of the 10 DNA samples used in this gel electrophoresis showed the presence of the *bar* gene with a characteristic 600bp bands (Lanes 4, 6, 7, 8, 9, 11, and 12) which is absent in the controls (Lane 2, 5 and 10) Lane 1 is 100bp molecular weight marker; Lane 2 is PCR water; Lane 3 is PTF1010.1 plasmid (Positive control); Lanes 5 and 10 DNA from non –transformed Zambezi plants (Negative control); Lanes 4, 6, 7, 8, 9, 11, and 12 – DNA from transformed Zambezi plants

# CHAPTER FIVE

# 5. DISCUSSION, CONCLUSION AND RECOMMENDATIONS

## 5.1 Discussion

Three sweet potato cultivars; KSP36, PIPI and Zambezi were established successfully in the growth room indicating the possibility of establishing sweet potato cultivars for *in vitro* propagation. This step is very vital in transformation and regeneration of sweet potato work. In this study a survival rate of 95% of explants was achieved. Temperature plays a critical role for the survival of cultures in the growth room and need to be optimal. Both high and low temperatures adversely affect the growth of cultures (Kay, 1973; Jarret and Gawel., 1991). In this study it was established that survival of sweet potato cultures *in vitro* is temperature dependent. Temperatures above the optimal (28°C) for most crops, in the growth room resulted in the necrosis of sweet potato plants in the present study. However in this study the effect of lower temperature on the growth of the crop was not observed but Kay, (1973); Purseglove, (1968) indicated that growth and performance of sweet potato is retarded at temperatures lower than 10°C. Jarret and Gawel., (1991) reported growth reduction of up to 50% in sweet potato *in vitro* cultures at temperature reduction of from 21.1 to 15.6 °C.

In this study it was established that 2, 4 –D concentrations between 1 to 2 mg /L were ideal to initiate callus formation which eventually led to the formation of embryogenic calli. However, Njagi (2004) reported that concentrations between 0.2 and 1 mg/L 2, 4-D were ideal to initiate embryogenic calli in KSP 36 and CPT 560 cultivars. Despite the differences the results in this study are in agreement with the findings of other previous

investigators in the embryogenic callus initiation in sweet potato who used 2,4-D concentrations between 0.1 to 3 mg/L (Jarret and Austin, 1984; Chee and Cantliffe , 1988; Lui *et al.,* 1993). The difference in concentration could be attributed to the differences in the cultivar used in those studies to the ones used in this study as response to growth regulators in sweet potato is genotype dependent (Aloufa, 2002). Embryogenic calli initiation is very important for the successful regeneration of sweet potato. Higher concentrations led to the death of the explants hence no calli formation.

*Agrobacterium*–mediated transformation requires the construction of the gene construct and that the cells acquiring the transfer DNA be identified, hence the use of marker genes (Vain et al., 1995, Frame *et al.,* 2002). In this study, the gene construct used harboured a *bar* gene as a selection marker. Determination of the optimum concentration of bialaphos to be used for selection of putative transformants of sweet potato was necessary because this was the first time that bialaphos was used as a selective agent as far as published literature is concerned. A concentration of 0.5 mg/L bialaphos was established through bialaphos killing curve as the optimal concentration for sweet potato. Successful reports of using bialaphos as a selective agent have been documented mainly on cereals (Vain et al., 1995; Vain, 2007) and so it was important to establish the best concentration in sweet potato. In selection of putative transformed calli for maize concentrations of 1.5 to 3.0 mg/L bialaphos have been used (Frame *et al.,* 2002). Response to bialaphos as a selective agent varied significantly among the three selected sweet potato cultivars. It was observed that KSP36 was the most sensitive to bialaphos compared to PIPI and Zambezi as most of its explants died on callus induction media where bialaphos was added. In

some cases where a few explants formed calli, the calli did not regenerate into plantlets as bialaphos was maintained on all subsequent stages hence these too died.

Most of the calli that were induced did not produce embryos as it was expected, majority of them died on regeneration media stage, an indication of efficiency of bialaphos selection for putative transformants. Bialaphos concentration maintained in the subsequent stages of regeneration process ensured that untransformed calli did not survive. This study however did not investigate the effects of exposure time to bialaphos on the regeneration of sweet potato. However, long exposure to bialaphos of maize embryogenic calli prohibited regeneration of the calli into plantlets (Vain et al., 1995; Vain, 2007). Njagi (2004) and Wanja (2005) reported the successful use of kanamycin, paramomycin and hygromycin as selective agents for transformed sweet potato. The work by Mburu (2007) on using phosphomannose as a selective agent for selection of putative plants open up the opportunities for using other selective agents with minimal requirements for their clearance for use in the production of transgenic plants. This opens the whole wide window of hope in addressing the biosafety concerns regarding transgenics in the use of antibiotics as selective agents (Machuka, 2004).

Leaf and stem explants were compared for their regeneration and transformability. It was established from this study that leaf explants responded better to both regeneration and transformation at $p \leq 0.001$. The difference in response to calli formation between the leaf and stem explants can be attributed to difference in their morphological structure and stages of maturity. The stem explants when cut and poked produced latex which hindered

the effective infection by the *Agrobacterium*, the shape of the stem (roundness) also may have contributed to the failure to form calli as opposed to the flat shape of the leaf which ensured that the large surface area of the leaf is in contact with the media.

Using of 0.25mg/L zeatin and 1mg/L IAA in this study only promoted rooting in KSP36, PIPI and Zambezi giving on average 7 roots per calli. However, Njagi (2004) reported that using the same combination induced both shoot and root proliferation and successfully regenerated KSP36 and CPT560. This discrepancy regarding KSP36 could be due to the fact that sweet potatoes are heterogeneous and genetically unstable and that mutations in sweet potato happen at higher rate than other crops (Aloufa, 2002). Shoot proliferation was only achieved when 2 mg/L Kinetin in combination with 1 mg/L IAA were used. Successful use of kinetin in regeneration of sweet potato has been reported at concentrations ranging form 0.2 to 2 mg/L (Herman *et al.,* 2002, Dong, 1987). It was easier to initiate roots from calli than it was for shoots. It took 8 weeks for callus to initiate roots while for shoot it required up to 7 months. Gunckle *et al.,* (1972) reported that it had taken 9 month to produce a single shoot and had indicated that regeneration and transformation of sweet potato are genotype dependent which is in agreement with Herman *et al.,* (2002) and Aloufa, (2002).

The effect of time of exposure to *Agrobacterium* infection was studied to establish the optimum time for effective infection of sweet potato explants. In this study three time regimes were used 30, 45 and 60 minutes and there were no significant differences ($p \leq 0.15$). However, infection times of 40 and 60 minutes and 24 hrs in the dark have

been recommended by previous investigators (Njagi, 2004; Wanja, 2005; Luo *et al.,* 2006).

The successful event of transformation depends on the successful suppression of the *Agrobacterium* from recurring in the subsequent stages of regeneration. Regeneration of putative transformants is highly compromised with frequent *Agrobacterium* recurrence (Hankoua *et al.,* 2006). To prohibit *Agrobacterium* from recurring requires the use of the right antibiotics which will be efficient in checking the growth of the *Agrobacterium* on the growth media for the calli. The *Agrobacterium* strain EHA101 used in this study was not fully controlled by the antibiotic used namely carbanicillin and cefotaxime as it was observed in the rate of recurrence. Hankoua *et al.,* (2006) reported that *Agrobacterium* strain EHA101 is resistant to carbanicillin and cefotaxime and the use of other antibiotics such as Timetin and Moxalactam is recommended. However this study did not compare the performance of other *Agrobacteria* strains such as LBA4404 against the same antibiotics. Mburu, (2007) reported differing performances of *Agrobacterium* strains in terms of transformation frequencies and efficiencies. The poor recovery of regenerants in this study from putative transformed calli could therefore be attributed to the *Agrobacterium* recurrence and this is in agreement with what Opabode (2006) and Hood et al., (1986) reported on the factors affecting transformation efficiency. Genotypic differences among the cultivars contributed to the poor recovery of regenerants and their subsequent response to growth regulator concentrations and combination used in this study.

Transformation frequency of 3.33% and efficiency of 2.33% were obtained in this study from Zambezi cultivar only after confirmation of putative transformants with polymerase chain reaction. The other 2 cultivars, KSP36 and PIPI did not regenerate even though Njagi (2004) reported that KSP 36 is both transformable and regenerable *in vitro.* The regeneration efficiency in sweet potato is genotype dependent and ranges from 0 to 85 % in tested cultivars (Gosukonda et al., 1995) and most importantly to note is that sweet potato cultivars are recalcitrant to regeneration or respond at low frequencies of less than 20% (Al-Mazrooci *et al.,* 1997; Luo *et al.,* 2006). Transformation efficiency is dependent on the regenerability of transformed calli.

A total of 10 regenerants were regenerated from this study but only 7 regenerants were confirmed to have been transformed with the gene of interest *XvPrx2* gene through PCR. This implies that no matter how efficient the selective agent might be, chances of having escapes regenerating can not be overruled. There was amplification of the *bar* gene which was present in the gene construct as a marker gene. This gave an indication that *XvPrx2* gene was also present since the entry of transfer DNA starts from the right border where the *XvPrx2* gene was located in the gene construct.

**5.2 Conclusion**

This study has shown that sweet potato can be transformed using *Agrobacterium tumefaciens* –mediated system for drought tolerance and that the transformed calli can be regenerated. *XvPrx*2 gene can be inserted into sweet potato genome to impart drought tolerance as is the case with the *X. viscosa* plant. Different cultivars of sweet potato will

respond differently to regeneration protocols, transformation and selective agents for transformants implying that there is genotype dependent response. To the best of my knowledge, this is the first study to use *XvP*rx2 gene to transform the selected sweet potato cultivars for drought tolerance in Eastern and Southern Africa region.

### 5.3 Recommendations

Based on the results from this study, the following recommendations are being proposed,

- Use of different types of growth regulators in varying ratios and combinations is encouraged in order to overcome genotype dependent response for successful regeneration of different sweet potato cultivars.
- Screening for the better selective agents for transformed calli of sweet potato as cultivars in this study responded differently to bialaphos used.
- The transformants regenerated in this study need to be assessed for their drought tolerance to confirm the expression of *XvPrx*2 gene.
- Southern hybridization to confirm stable integration of *XvPrx2* gene into the sweet potato genome.
- A study to establish time span in terms of generations taken to loose the expressivity of the gene is also recommended.
- A research to asses the effect of the gene inserted on the yielding and nutritive value of the selected cultivars is recommended.

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