EVALUATION OF PROTOCOLS FOR REGENERATION OF SWEET POTATO (*Ipomoea batatas* (L.) Lam.) IN TISSUE CULTURE

BY

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DECLARATION AND RECOMMENDATION

DECLARATION

I declare that this thesis is my original work and that it has not been previously presented in this or any other University for an award of a Degree.

Judith N. Oggema

Signed ……

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RECOMMENDATION

This thesis has been submitted for examination with our approval as university supervisors.

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DEDICATION

I dedicate this thesis to my late parents: The Dr Matthias and Mrs Moreen Oggema,

whose remarkable parenting laid a firm education background in me

And to my twin Juliana and the others Annette, Seb, Angela, and Dominic

For your support and encouragement, what a wonderful team we all are!

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ABSTRACT

Tissue culture techniques have opened a new frontier in agricultural science for addressing food security and poverty issues. These techniques have involved development of suitable plant regeneration protocols that can reduce disease infestation by producing healthy plants rapidly and hence increase yields in Kenya. The current research focus in sweet potato (*Ipomoea batatas* (L.) Lam) has been the development of transgenic sweet potatoes, with ability to resist viral diseases of which suitable plant regeneration protocols are fundamental. A study was conducted at Kenya Agricultural Research Institute (K.A.R.I.) Njoro, to determine an efficient tissue culture protocol for rapid regeneration and multiplication of locally adapted sweet potato cultivars and thereafter compare growth and yield between the regenerated and conventionally propagated sweet potato cultivars under field conditions. In the laboratory experiment, leaf explants from sweet potato cultivars *Mugande, SPK004, Kemb10, Japon tresmesino* and *Zapallo* were tested on media supplemented with six concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D) (0, 0.5, 1.0, 2.0, 3.0 and 5.0 mg L^{-1}). The cultures were set as a Completely Randomised Design (CRD) in factorial arrangement replicated three times. Calli incidences were significantly ($P \leq 0.05$) higher at 0.5 mg L^{-1} and 1.0 mg L^{-1} 2,4-D levels. These concentrations were used to further study production of plants using the regeneration methods callus induction and somatic embryogenesis. The number of plants regenerated varied significantly ($P \leq 0.05$) with the regeneration method and the 2,4-D levels. The highest number of plants was obtained at 0.5mg L^{-1} 2,4-D levels. For both studies, *Zapallo* was most responsive in all variables measured followed by *SPK004* using callus induction method. A field experiment was set up as a Randomised Complete Block Design (RCBD) replicated three times, with the five cultivars germinated and raised under two regeneration methods. Under the field study, significant ($P \leq 0.05$) interaction was detected between the test cultivars and regeneration method. *SPK004* gave the highest score for the growth variables plant stand count and height, while for the number of branches and leaf area *Mugande* outperformed all cultivars. The highest marketable and total yield was recorded with *Zapallo*. Despite conventional propagation method giving higher growth rates the difference in yield between the propagation methods did not vary significantly ($P \leq 0.05$). Virus detection for Sweet Potato Feathery Mottle Virus (SPFMV) established that field plants had a higher virus titre compared to the tissue culture regenerated plants. From the study, regenerating *Zapallo* using callus induction method at 0.5 mg L^{-1} 2,4-D levels gave lower disease ratings and subsequently highest marketable number of tubers therefore callus induction is recommended as the most suitable regeneration protocol for the local sweet potato cultivars.

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ABBREVIATIONS

ABA	abscisic acid
ANOVA	analysis of variance
BAP	benzyl adenine purine
CI	callus induction
CRD	completely randomised design
2,4-D	2,4-dichlorophenoxyacetic acid, a synthetic auxin
ELISA	enzyme linked immuno-sorbent assay
FAO	food agricultural organisation
GA_3	gibberellic acid
IAA	indole acetic acid, naturally occurring auxin
IAEA	international agency of atomic energy
KCl	potassium chloride
kPa	kilopascals
mgL^{-1}	milligrams per litre
NAA	napthaleneacetic acid, a synthetic auxin
RCBD	randomised complete block design
SE	somatic embryogenesis
S.E	standard error
SPFMV	sweet potato feathery mottle virus

CHAPTER ONE

INTRODUCTION

1.1 Origin, Importance and Uses of Sweet Potato

The sweet potato (*Ipomoea batatas* (L.) Lam.) is a native American plant belonging to the family Convolvulaceae, order Polemoniales (Burden, 2005). Of more than 1000 species, only *Ipomoea batatas* is of major economic importance as food (Horton, 1994). Sweet potato is ranked seventh in importance worldwide after rice (*Oryza sativa),* wheat *(Triticum aestivum*), maize *(Zea mays),* potatoes (*Solanum tuberosum*), barley *(Hodeum vulgare)* and cassava (*Manihot esculentum*) (FAO, 2003). In Africa, extensive cultivation of sweet potato has been done for household consumption, practised in regions surrounding Lake Victoria in Uganda, Northwest Tanzania (Onueme, 1978), Central and Western Provinces in Kenya where the crop occupies a national status as a food security crop (Munga *et al.,* 2000).

Lately, sweet potato has received increased attention because the crop can adapt to a wide range of environmental conditions and grow on fertile as well as marginal soils with limited fertility and inadequate moisture (Bioethics, 2004). The vine provides good ground cover due to its spreading characteristic that enables the crop to take advantage of incoming radiation as well as suppress weed growth and competition. This crop can be cultivated without pesticides and fertilizers as it is sown with vine cuttings rather than seeds (CGIAR, 2006). Planting and harvest periods are more flexible than those of crops such as cereals and legumes (Anonymous, 2003). This root crop is important in areas where maize crop has failed in Kenya due to the frequent unreliable rainfall (KARI, 2000).

Sweet potato contains an abundance of calories important in the human diet. It has abundant vitamin A, ascorbic acid, amino acid lysine, starch, water, iron, and calcium. According to Woolfe (1992), the sweet potato tuber contains 1.7 % protein, 0.4 % lipids, 26.2 % carbohydrates, 3.9 % dietary fibre, 32 % calcium, 47 % phosphorous, 0.7 % iron, 114 kilo calories energy and 71 % water per 100g dry matter. Leaves and tubers of this crop are consumed as fresh vegetables and snack foods while vines are used as fodder for livestock (Purseglove, 1977).

The value of sweet potatoes may be increased by processing into products such as jam, dried crisps, chips, sweets, candy, sauce, paste and non-alcoholic beverages. It may also be dried and ground into flour for baking purposes and also used as a weaning food for children. Market awareness of the orange-fleshed varieties such as *Zapallo* and *Japon tremesino* is increasing (CVPG, 2003) as they are rich in β - carotene, a nutrient effective in preventing certain types of cancers (Prakash, 1994).

1.2 Production Constraints of Sweet Potato

Despite sweet potato having a high potential, yield in Kenya has been declining over the years resulting in low production. The average yield in Africa is 8,965 kg ha^{-1} on 2.5 million ha of harvested area (FAO, 2003). According to CAB (2004), Kenya's annual production equates to 520,000 mt on 58,000 ha of harvested area. However, the average sweet potato yield stands at 4,558 kg ha^{-1} which is less than half of the world's yield of 14,062 kg ha^{-1}. This has been attributed mainly to Sweet Potato Virus Diseases (SPVD) (Aritua *et al.,* 1998, Gibson *et al.,* 2000) and attack by pests mainly weevils, *Cylas spp*. It is necessary to consider reducing this gap in sweet potato production, which has fallen behind total human population growth rate.

The sweet potato weevil (*Cylas sp.)* is the most destructive, with the most devastating species being *C. formicularis, C. puncticollis* and *C. bunneus.* These pests attack the crop during the dry spell, destroy tubers by feeding on roots, vines, leaves and deposit terpenes that make sweet potato inedible. Terpenes are unsaturated hydrocarbons with low solubility characteristics and hence low palatability (Leningher, 1991). The damage caused by *Cylas spp* can be as high as 60 % - 97 % (Mullen, 1984; Mwaniki, 2000), hence reducing the quality and marketable yield of sweet potato.

The greatest danger is the susceptibility of sweet potato to viral diseases which have caused substantial yield reduction. As much as 80 % yield loses due to viral infections can be experienced (Wambugu, 1995; Odame *et al*., 2002; Bioethics, 2004). The SPVD are transmitted through planting vines taken from infected plants or through virulent insect vectors mainly whitefly (*Bermisia tabaci*) and aphid (*Aphis gossypi*) which transmit Sweet Potato Feathery Mottle Virus (SPFMV) and Sweet Potato Chlorotic Stunt Virus (SPCSV) respectively (Aritua *et*

al., 1998). The greatest challenge is with SPFMV because symptoms of infected plants are transient and farmers are unable to recognize them (Wambugu, 1995). In East Africa, the viral diseases were first reported in Uganda in the 1940's. A crop that initially amounted to 30,000 kg ha^{-1} per annum was reduced to 4,000 kg ha^{-1} (Aritua *et al.,* 1999). Virus affected plants become stunted with small-distorted leaves, often narrow strap-like and crinkled with a chlorotic mosaic-vein clearing (Gibson *et al.,* 2000).

Clean planting material has been produced in tissue culture and virus indexed by enzyme linked immuno sorbent assay (ELISA). ELISA is a sensitive serological test that detects pathogens (Mukasa *et al*., 2003). Double antibody sandwich ELISA (DAS-ELISA) with antibodies from Agdia inc® (Elkhart In) is a common SPFMV confirmation test (Jericho and Thompson, 2000). Due to the variation in different hosts there is need to standardize the antibody and the antigen to determine the combination of the antibody and antigen that would give a significant difference in mean absorbance values between the antigen and the buffer during ELISA tests. Despite the detection, free exchange of planting stock among communities continues to encourage the spread of infected material (Munga *et al.,* 2000), enhancing the need for healthy plant material to establish stock.

Research in Kenya has been directed to the development of sweet potato cultivars resistant to SPFMV (Odame *et al*., 2000). A transgenic sweet potato resistant to SPFMV has been developed by genetic engineering using a virus coat protein (*cp*) gene (Wambugu, 1995). Transgenic sweet potato cultivars have undergone gene transformation to enable them to resist viral attack thereby potentially increasing yields of sweet potato roots and foliage (Odame *et al*., 2002). The transformation procedures have been genotype-dependant and often difficult to regenerate (Lowe *et al*., 1995). In addition, the transformation procedures to produce transgenic sweet potatoes have been done in exotic cultivars which have been unable to withstand the local environmental conditions as well as viral challenge in the field (Odame *et al*., 2000). To develop transgenic plants there is a need to transform local sweet potato cultivars popular among farmers and adapted to the local environmental conditions for different regions in Kenya.

1.3 Statement of the Problem

The success in production of the transgenic sweet potato is dependent on the reliability and efficiency of the regeneration protocol used. However there is inadequate knowledge on a suitable tissue culture protocol that would be efficient and reliable in regenerating rapidly the locally adapted sweet potato cultivars. Methods developed to produce sweet potato using tissue culture have resulted in low frequencies of shoots and consequently low plant regeneration. This calls for development of appropriate biotechnology packages that will include a suitable cultivar and tissue culture regeneration protocol to reduce SPVD severity and hence produce healthy sweet potato plants *in vitro* that will improve availability of sweet potato planting materials.

1.4 Objective of Study

1.4.1 General objective

The general objective of the study was to develop a suitable tissue culture protocol for the regeneration of locally adapted, high yielding sweet potato (*Ipomoea batatas* (L.) Lam.) cultivars.

1.4.2 Specific objectives

The specific objectives were to determine:

1. The best *in vitro* regeneration method for multiplying sweet potato cultivars *Mugande, SPK004, Kemb10, Japon tresmesino* and *Zapallo*,
2. The interaction effects of the *in vitro* methods and genotypes on plant regeneration,
3. The effects of genotype on *ex vitro* survival and growth rates of regenerated plants and
4. The effect of propagation method on growth and yield of sweet potato.

1.5 Hypotheses

1. The *in vitro* regeneration method is dependent of genotype.
2. There is an interaction between the sweet potato cultivars used and regeneration method on plant regeneration.
3. There are genotypic differences in *ex vitro* survival and growth rates of plants regenerated to mature whole plants.

4. There is an interaction between cultivar and method of propagation on growth and yield of sweet potato.

1.6 Research Justification

The shortage of healthy, disease-free planting material in sweet potato crop production as well as the current weather uncertainties in Kenya has not guaranteed sufficient harvest to meet the demands of the ever-growing human population. Different management strategies have been attempted to minimize yield losses such as the use of resistant cultivars, crop rotation, natural enemies and insecticides, which have all been ineffective due to the varying cropping patterns, expensive and concealing nature of the vectors. Chemical control of pests and diseases has proved expensive, a health hazard to the users and not safe to handle as a result not able to check the declining production. Conventional breeding methods have resulted in limited improvement in sweet potato due to the biological complexities mainly self incompatibility and male sterility associated with the crop.

Plant regeneration in tissue culture has important applications in crop improvement. It involves rapid micropropagation, virus elimination, plant transformation, embryo rescues, and anther culture. The success in production using plant regeneration is dependent on an efficient regeneration protocol used in obtaining viable, true to type plants in sweet potato. Local varieties with suitable agronomic characteristics and popular to farmers offer the best material to be used for crop improvement. There is need to embark on a suitable regeneration protocol that will rapidly propagate locally adapted sweet potato cultivars and avail to farmers.

Though still in infancy stage, the Kenyan government has recognized the potential of embracing appropriate biotechnology packages to help improve the declining production of major food and cash crop hence increasing food production through yield improvement. There is therefore need to produce and multiply virus free sweet potato planting materials in sufficient quantity for farmers of which biotechnology tools are seen as a new frontier that will open up new avenues to boost and solve agriculture production problems.

CHAPTER TWO
LITERATURE REVIEW

2.1 Crop Improvement in Sweet Potato

Sweet potato is largely propagated by stem cuttings and seed (Prakash and Varadarajan, 1992). The crop may also be grown using tubers though germination is poor unless cured and treated well in concentrated sulphuric acid (H_2SO_4) (CVPG, 2003). Sexual multiplication is mainly reserved for use in breeding work for production of new cultivars (Sihachackr *et al.,* 1997).

The intervention by humans to improve this crop has been an ongoing process from the time of first domestication. Artificial selection and natural hybridization of sweet potato has resulted in the existence of a very large number of cultivars (Gichuki *et al.,* 1999). These cultivars have a wide genetic diversity which encompasses differences in morphological features such as colour, shape of roots and leaves, depth of rooting, maturity, pest and disease resistance and level of anthocyanin (Gichuki *et al.,* 2003). In East Africa, sweet potato has been found to have unique characteristics both morphologically and agronomically with different genetic markers being used to characterize the crop (Gichuki *et al.,* 1999). Sweet potato crop can be cultivated up to three times in a year with proper agronomic practices. Its phenological period lasts 3 - 5 months after planting depending on the cultivar. Processed products are perishable but last up to 4 months if stored well (Munga *et al.*, 2000).

It is necessary to improve sweet potato due to the high male sterility and incompatibility that exist within the cultivar. These biological complexities have resulted in very limited improvement of *I. batatas* by classical breeding (Dhir *et al*., 1998). Biotechnological tools that apply somatic hybridization and gene transfer processes are attractive in sweet potato as they enable direct introduction of desirable genes from other sources into preadapted cultivars by using *in vitro* methods (Prakash, 1994). Therefore interest is directed towards genetic improvement of sweet potato using *in vitro* methods (Ducreux and Sihachakr, 1987). In recent years, it has been learned that mutations also occur during *in vitro* culture of plant material and there are claims that such mutations could also be used by plant breeders in their never-ending task of genetic improvement of crop plants (Mulwa and Mwanza, 2006). In addition with *in vitro* regeneration it is now appropriate to examine "somaclonal variation" and gauge its potential

value in relation to the extensive experience already accumulated on plant mutants obtained from radiation and chemical-mutagen application. In cases where somaclonal variation is undesirable, such as for long-term *in vitro* germplasm storage in clonal propagation, one must find suitable methods to avoid it (FAO/IAEA, 2003). Hence an efficient method to regenerate sweet potato in tissue culture is essential to allow somatic embryos to be effectively induced and maintained in as wide a range of cultivars as possible with less genetic variations as possible (Prakash, 1994).

2.2 Micropropagation of Sweet Potato in Tissue Culture

Micropropagation of sweet potato began in the 1960's in Japan with the sole aim of maintaining germplasm (Torres, 1989). Today, the International Potato Centre in Peru has the global mandate for sweet potato research with nearly 4,000 accessions in its gene bank (CAB, 2004). In Kenya, improvement of sweet potato began in the late 70's (Wambugu, 1995). Tissue culture techniques introduced a new phase into agricultural research as a means of rapid mass propagation and also for conserving elite and rare cultivars threatened by extinction (Torres, 1989; Boxus, 2002). Advanced culture techniques later introduced to shorten breeding cycles and produce somatic hybrids included anther culture, cell suspension cultures and protoplast fusion (Lyndsey and Alderson, 1986; Brink *et al*., 2000). These techniques made it possible to study the mechanism by which cells differentiate.

Plant regeneration is the ability to form whole plants from a totally undifferentiated cell mass (callus) by manipulation of the growth regulators during culture in media (Torres, 1989). Plant regeneration in tissue culture involves use of *in vitro* cultivation of plant tissue, organs or single cells under aseptic conditions to form whole new plants (Yoshiaki, 2004). Regeneration can occur directly from the organs or somatic embryos from the dividing cells or by the dividing cells reverting to undifferentiated cell mass known as callus (Merkle *et al.,* 1995). Plant regeneration improves crops by exploiting somaclonal variation, gene transfers and somatic hybridization, all requiring regeneration in tissue culture (Sihachakr *et al.,* 1997). Regeneration of plants in tissue culture involves rapid multiplication of clean material, by initial collection of plant material from disease-free plants and use of single nodal cuttings to propagate plants in cell cultures (Otani *et al.,* 1998), which form callus and suspension cultures (Kumar, 1993).

According to Carswells and Locy (1984), plant regeneration varies with variety, explant source and method used for regeneration. Plant regeneration protocols use different guidelines to establish explants in aseptic culture media. When small pieces of plant tissue are put in a sterile culture on a solid medium the cells proliferate and callus forms. If the auxin and cytokinin are present in the correct amounts and ratios (for shoots, high cytokinin: low auxin levels and for roots high auxin: low cytokinins level) depending on the plant species being regenerated shoots and roots develop rapidly (Chrispeels and Sadava, 2003). Hardening of plants *in vitro* is done during micropropagation by reducing the nutrient levels in the medium, supplementing with root primordial auxins and culturing in elevated levels of irradiance (Dodds and Robert, 1995).

Currently, several crops including the sweet potato have been produced using *in vitro* techniques by culturing of shoot tips, petioles, terminal buds, stem segments, callus and roots in suitable media (Sihachakr *et al*., 1997). Use of cell cultures to produce plants by use of somatic embryogenesis, callus and protoplasts has enabled application of techniques such as gene transfers and protoplast fusion (Vasil and Vasil, 1973). In sweet potato, plant regeneration via organogenesis and embryogenesis was achieved in different tissues but with limited success with protoplasts (Dhir *et al*., 1998). Protoplasts are plant cells devoid of their cell wall removed physically or enzymatically. Protoplast cultures have been used to improve plant species through protoplast fusion enabling combination of attributes from diverse species (Ducreux and Sihachakr, 1987) to produce hybrid plants (Dodds and Robert, 1995).

The success in production of transformed material ultimately depends on the ability to grow these plants from cell or tissue level to whole, mature plants by applying various protocols in tissue culture. Sweet potato improvement through genetic transformation was launched in Kenya in 1991 through collaboration between KARI and Monsanto Company (Wambugu, 1995). The initiative was to develop a genetically modified sweet potato with ability to resist viral diseases (Odame *et al*., 2002; KARI, 2000) by gene transfer and incorporation of desirable genes for specific traits into pre-adapted cultivars. Various germplasm were collected, transformed and trials done to regenerate the transgenic plants in tissue culture. Although well documented, transformation was restricted to only a few and exotic cultivars such as Jewel, Beauregard and CPT 560 (Brink *et al*., 2000).

However, sweet potato has not been easily regenerated using protoplast cultures. Most sweet potato genotypes failed to regenerate shoots (Sihachakr *et al.,* 1997; Otani *et al*., 1998) as they were fragile and difficult to manipulate. Consequently severe somaclonal variation occurred as cells divided (Zhange *et al.,* 1998). Successful protoplast culture depends on the ability to isolate viable protoplast and to provide the correct culture conditions for cell wall formation, division and plant regeneration (Otani *et al.,* 1998). This was seen in cultures of tobacco (*Nicotiana tabacum)*, potato (*Solanum tuberosum)* and cotton (*Gossypium hirsutum)* (Vasil and Vasil, 1973). It was easy to isolate protoplasts from sweet potato petioles, stems, callus and suspensions but mesophyll tissue resisted enzyme digestion (Sihachakr *et al*., 1997).

Regeneration of sweet potato in tissue culture has limitations. Adventitious roots develop easily from callus while differentiation of adventitious buds does not form as rapidly as the roots (Mukherjee, 2002). Ammirato (1984) demonstrated that plant regeneration depended on the variety and species of *Ipomoea*. The problem of inducing morphogenesis depended on manipulations of the media components and the external environment. The important group of phytohormones commonly used are cytokinins: Benzlyaminopurine (BAP) ($C_{12}H_{11}N_5$), Zeatin ($C_{10}H_{13}N_5O$), Kinetin ($C_{10}H_9N_50$) or Thiodiazuron (TDZ) ($C_9H_8N_4OS$) for shoot formation and auxins such as Napthalene Acetic Acid (NAA) ($C_{12}H_{10}O_2$), Indoleacetic Acid (IAA) ($C_{10}H_9NO_2$) and Indole 3-Butric Acid (IBA) ($C_{12}H_{13}NO_2$) for root formation (Litz and Concover, 1978).

Somaclonal variation often occurs in regeneration systems causing differentiation and alteration of genome among regenerants (Otani *et al.,* 1998). Increased variations occur in callus tissue mainly due to lack of cell differentiation and varying optimal growth conditions. This results in defects during cell division and hence an increase in *in vitro* variability especially in somatic cells (Liu and Cantilfee, 1986). Nguyen *et al*. (1997) observed that the degree of variation was dependent on the species and duration of culture. Consequently callus should not be cultured for too long as the cells lose the capacity to differentiate and develop abnormalities (Brown and Thorpe, 1991).

Several techniques have been developed for improvement of sweet potato. These include the exploitation of direct and indirect embryogenesis, somaclonal variation and gene transfers by genetic transformation (Sihachackr *et al*., 1997). The sweet potato cultivar White Star has been produced by both direct and indirect embryogenesis (Chee *et al*., 1990). Protocols for plant regeneration from cultured protoplasts have also been developed. Protoplast-derived calli from sweet potato cultivars had great phenotypic and genetic variation in growth patterns and in particular, tuber formation (Sihachackr *et al*., 1997).

2.3 Callus Induction

Callus is generally tissue arising from undifferentiated, disorganized proliferation of plant cells and was first observed in the 1920's -1930's (Sharp *et al.,* 1984). It is often naturally produced by plant tissue in response to wounding. With the stimulus of endogenous growth substances or by addition of exogenous growth regulators to the nutrient medium, cell division, cell growth and tissue differentiation are induced (Tripathi and Tripathi, 2003). The induction of callus growth and subsequent differentiation and organogenesis is accomplished by the differential application of growth regulators and the control of conditions in the culture medium. Totipotency which is the ability for undifferentiated plant cells to develop into whole plants or plant organs *in vitro* when given the correct conditions is necessary, for not all plant cells are totipotent (Herbert, 1998). The callus mass forms new cells on the periphery of the leaf edges. To avoid phenolic waste build up, activated charcoal was introduced in the medium to absorb the waste (Cassells and Plunkett, 1984).

Callus is induced for its potential in mass production of new plants when it is separated and induced to differentiate into somatic embryos (Merkle *et al.,* 1995). Callus arises as a result of plant growth regulators introduced in media and specifically auxins. The type of auxin was found to be critical for inducing embryogenic callus. The most common auxin used for sweet potato cultures is 2,4-Dichlorophenoxyacetic Acid ($C_8H_6Cl_2O_6$) (Al- Mazrooei *et al*., 1997). When leaf explants were cultured on 2,4-D containing medium small pieces of embryogenic callus and of isolated embryos reverted to whitish and/or brownish watery dispersed calli which subsequently produced several secondary embryos (Nguyen *et al*., 1997). With callus cultures it is necessary to set up a large number of replications as the growth rate varies widely between explants on the

same medium. Stable and uniform callus is produced as long as frequent transfers are done. However, prolonged stays in media lead to unstable non-uniform cell formation regimes that varied genetically (Brown and Thorpe, 1991).

Callus has been successfully induced in many plant species; for example in Pea (*Pisum sativa*) the pods have been cultured and plants regenerated from callus (Bencheikh and Gallais, 1996). In tobacco (*Nicotianum tabacum*) callus was formed on parenchyma tissues and in rice (*Oryza sativa*), it was obtained from leaf sheath nodes. In Soyabean (*Glycine max* L. Merr) callus was induced from immature seeds (Orczyk and Orczyk, 1994). Amitha and Reddy (1998) reported successful callus induction in cowpeas (*Vigna unguiculata* L.) using immature embryo axes and immature cotyledon while in sunflower (*Helianthus annum*) Saji and Sujatha (1998) used anther culture to induce callus under dark conditions.

In sweet potato, plant regeneration occurs easily from callus so long as suitable auxins are supplemented in the media because this crop is recalcitrant to regeneration and transformation attributed to the fact that each cultivar shows different response during *in vitro* studies. Newell *et al.* (1995) reported that several protocols had to be tested in order to establish an efficient methodology but failed to work for the cultivars used. Nevertheless, juvenile tissues of an explant are most suitable for callus induction (Rolando *et al*., 1999) as the cells are at a relatively early stage of development; for example non-dividing undifferentiated parenchyma meristematic and embryonic tissues that are undetermined. These were reported as capable of switching to different pathways of development depending upon the environments imposed upon them. Such undetermined cells rapidly proliferate to produce cell masses known as callus (Herbert, 1998).

The site of initiation of callus proliferation is situated at the excised surface and only a small percentage of the cells in a given explant contribute to formation of callus (Sihachakr *et al.,* 1997). Zhange *et al*. (1998) suggested that the puncturing of holes on the excised surface of leaf explants maximized exposure to culture medium which led to increased callus production. Callus in sweet potato has been induced using tubers (Rolando *et al*., 1999), leaf primordia and petioles (Torres *et al*., 2001), shoot tips (Chee *et al*., 1990; Al-Mazrooei *et al*., 1997), anthers (Sihachakr *et al*., 1997), stem and root (Liu and Cantilfee, 1984) and lateral buds (Zheng *et al*., 1996).

2.4 Somatic Embryogenesis

Embryogenesis is the process of embryo initiation and development. Somatic embryogenesis is an alternative *in vitro* technique which offers a very efficient system for rapid multiplication and mass production of plants within a short period and without somaclonal variation (Radhakrishnan *et al*., 2001). Somatic embryos are organ structures originating from somatic cells but whose morphology resembles that of a developing zygotic embryo (Tripathi and Tripathi, 2003). Somatic embryos were first observed in cell suspension cultures of carrot callus grown in agar. The embryos which developed *in vitro* from vegetative cells of mature carrot plants were further propagated into intact whole plants (Finer, 1994).

Somatic embryogenesis offers significant potential for improvement of sweet potato through generation of tissues for use in genetic transformation which is concerned with mass clonal propagation (Newell *et al*., 1995), resistance to potyvirus complex (Zheng *et al*., 1996), synthetic seed production and germplasm conservation (Chee *et al*., 1990). In addition regeneration of the crop using somatic embryogenesis has resulted in low frequency of chimeras but with a high number of regenerants (Al- Mazrooei *et al*., 1997).

The process of somatic embryogenesis has great potential for achieving rapid clonal micropropagation of genetically identical plants (Ducreux and Sihachakr, 1987). Somatic embryogenesis in *I. batatas* can occur in two ways: directly from the explant or indirectly through callus. With direct embryogenesis, pre-embryogenic cells are conditioned to produce embryos even before the explant is cultured and need only an exogenous stimulant such as medium components supplemented by either an auxin or cytokinin to be able to express them (Merkle *et al*., 1995). Finer (1994) observed that the process of somatic embryogenesis was enhanced when higher levels of sucrose and reduced time of exposure to auxins during culture were allowed. Direct embryogenesis has applicability in the improvement of *I. batatas* and other tuber crops as it omits the callus stage making it less subject to genetic variability (Chee *et al*., 1990).

Indirect somatic embryogenesis occurs through use of callus formation by manipulation of appropriate phytohormones, which programme embryogenic tissues to produce callus. Once

induction process is initiated the callus cultures are transferred to auxin-free media for further embryo development. Somatic embryos formed appear distinctly green against the yellowish background of the callus (Merkle *et al*., 1995). More significantly, some callus cultures, under certain nutritional and hormonal conditions can be induced to develop somatic embryogenesis and follow a sequence through pro-embryoid, globular and torpedo stages (Herbert, 1998).

The synthetic auxin 2,4-D is commonly used to form calli from tissue explants (Radhakrishnan *et al*., 2001). The callus is then transferred to a 2,4-D free medium supplemented with reduced forms of nitrogen in the form of NH_4^+ salts or amino acids. Such manipulations in plant tissue culture have been particularly successful in the case of tobacco and carrot tissue (Finer, 1994). However, not all crop tissues can be manipulated as readily as these two species and much intensive research has been employed in optimising plant tissue culture conditions in agricultural crops. This is particularly true of the cereal and tuber crops (monocotyledonous plants) which are particularly difficult to manipulate in culture (Herbert, 1998).

2.5 Organogenesis

Organogenesis refers to the process whereby tissue cells or callus may be induced to form shoots and complete plants (FAO/IAEA, 2003). In tissue culture it has been used to refer to the process of differentiation by which plant organs are formed *de novo* or from pre-existing structures (Torres, 1989). With the stimulus of endogenous growth substances or by addition of exogenous growth regulators to the nutrient medium, cell division, cell growth and tissue differentiation are induced (Tripathi and Tripathi, 2003). Hence, the induction of callus growth and subsequent differentiation and organogenesis is accomplished by the differential application of growth regulators and the control of conditions in the culture medium, the nature of the plant organ and the plant genotype (Torres, 1989).

Sweet potato cultivars has been propagated *in vitro* and shoots regenerated on media supplemented with MS media, the cytokinin BAP. When meristematic tips of axilliary shoots were cultured on modified MS agar medium, differences in shoot development were noted (Rolando *et al*., 1999). Rooted plants were successfully rooted and transferred to green house conditions. The auxin source, type and concentration were significant, and in sweet potato, NAA

produced a few shoots and callus, where as IAA resulted in highest shoot production (Torres, 1989). In addition, Rolando *et al*. (1999), observed that in sweet potato cultivar CEMSA-78354, shoot and root emergence were obtained at very low frequency on MS media free of a plant growth regulator was used.

Growth regulator concentration in the culture medium is critical to the control of growth and morphogenesis. Once the selection, plating out or development of callus has reached the appropriate stage, growth regulator balance is shifted in order to induce plant regeneration out of the callus or embryos so that cells start organize themselves into tissue structures hence producing organs, shoots and eventually, entire plantlets. In many cases, the axillary buds formed in the culture undergo repetitive proliferation, and produce large number of tiny plants. The plants are then separated from each other and rooted either in the next stages of micropropagation or *in vivo.* The number of shoots and roots that were regenerated and the regeneration frequency were considered for the selection of a competent condition for sweet potato regeneration. For regeneration studies it is very important to have high plant conversion in order to increase the likelihood of regenerated plants and subsequent transformed plants (Al-Mazroei *et al.,* 1997).

2.6 Response of Sweet Potato Cultivars to Embryogenesis

Several studies exist on direct and indirect embryogenesis in sweet potato cvs. but none has been reported in the locally adapted cultivars found in Kenya. The frequency of formation of embryogeneic tissues varied significantly between cultivars (Zheng *et al*., 1996; Al- Mazrooei *et al*., 1997). Different cultivars within a species when grown in culture exhibited genotype-dependent differences in the ease of plant regeneration (Ammirato, 1984).

Al- Mazrooei *et al*. (1997) was able to regenerate sweet potato plantlets while optimising different auxins suitable for somatic embryogenesis. Out of 16 cultivars, successful induction and maintenance of somatic embryogenic tissue was achieved in 14 cultivars. The most effective auxin was 2,4-D but the optimum type and concentration was specific to the individual cultivars. Although the embryogenic tissues from all cultivars increased over the subculture period, the production of secondary embryogenic tissue varied among all cultivars. In the study, the

cultivars that produced less embryogenic tissues tended to form greater amounts of non - embryogenic friable callus.

Successful somatic embryogenic tissue has also been induced from leaf and petiole explants of the exotic sweet potato genotypes White Star and P1 318846-3 (Zheng *et al*., 1996). The optimal response was obtained in the genotype P1 318846-3 which was highly regenerative with high adventitious shoot regeneration. From the study, induction of embryogenic tissues at high frequencies was restricted to one or a few cultivars and when attempts were made to extend this to a wide range of cultivars the majority were found to be recalcitrant or to respond at low frequencies (Zheng *et al*., 1996).

Sihachackr *et al*. (1997) used lateral buds from 10 cultivars of sweet potato to evaluate the potential of formation of somatic embryos and plant regeneration via embryogenesis. In this study, embryogenesis and formation of somatic embryos as well as their maturation occurred in the presence of high auxin levels notably 10 mg L^{-1} 2,4-D. Some leaf explants produced friable non-embryogenic callus or mucilagenous whitish calli from which emerged compact embrogenic callus while others gave rise to small calli depending on genotype. The evaluation of embyogenic responses from the 10 sweet potato cultivars showed a highly significant effect of genotype.

Nguyen *et al*. (1997) used 22 sweet potato cultivars to test their potential to produce plants from somatic embryos and regenerate plants. Callus induction media containing different 2,4-D levels ranging from 0 to 10 mg L^{-1} were tested. The formation of embryogenic callus depended on the genotype and media composition. The best response was attained at 4.3 mg L^{-1} 2,4-D level. Each genotype formed embryos at a specific concentration of 2,4-D and in all genotypes further embryo development was obtained after transferring embryogenic calli onto hormone-free medium. Plants were regenerated from somatic embryos in 14 of the 22 cultivars. The results confirmed genotypic dependence and differences of response in culture.

2.7 Factors Influencing Direct and Indirect Embryogenesis

Several factors have emerged that influence the ability of sweet potato embryogenic cells in culture to grow in an organised way and that modify their subsequent development in both liquid and solid media (Ammirato, 1984). These factors include explant, growth regulators, nutritients and culture conditions.

2.7.1 Explant

The embryogenic capability of a sweet potato cultivar to produce callus or somatic embryos is influenced by the type, size and the physiological condition of the explant (Merkle *et al*., 1995). Tripathi and Tripathi (2003) reported that a high frequency of shoot, root, and microtuber production from *Solanum melongena* depended on the type of explant from which the calli originated and the photoperiod. Explants that have been used for sweet potato embryogenesis include leaf explants, stem segments, lateral buds, shoot tips and anther derived callus (Sihachakr *et al.,* 1997). Each of these explants has resulted in different embryogenic patterns during culture. The origin of the explant plays a key role for successful and efficient formation of friable callus (Dovzhenko and Koop, 2003).

Raemakers *et al*. (1995) agreed that explants influenced both direct and indirect embryogenesis. Zygotic explants showed a larger percentage of direct somatic embryogenesis while vegetative explants showed a larger percentage with indirect embryogenesis. Nguyen *et al.* (1997) reported that a significant response was achieved when somatic embryos were produced from meristems than with intact buds. The size of the explant was found to be critical in affecting somatic embryogenesis. In the study explant sizes ranging between 0.5 - 1 mm were capable of producing the highest amounts of embryogenic tissues and at high frequencies. Explants greater than 1 mm in size tended to form non – embryogenic callus while those under 0.5 mm failed to grow or develop in any manner (Al- Mazrooei *et al.,* 1997).

Liu and Cantilfee, (1984) reported that the implication of explant size and type was that as long as the correct auxiliary bud is used each *in vitro* plantlet can yield numerous explants rather than just one apical meristem thereby reducing time, labour and space necessary to establish and maintain a large number of *in vitro* plantlets.

2.7.2 Plant growth regulators

The most important factors regulating organogenesis/embryogenesis *in vitro* are plant growth regulators (PGRs). Five classes of PGRs can be distinguished: auxins, cytokinins, gibberellines, abscisic acid and ethylene (Panis, 2006). Auxins, cytokinins and auxin-cytokinin interactions are considered to be the most important ones for regulating growth and organised development in plant tissue and organ cultures (Chrispeels and Sadava, 2003). Auxins and cytokinins have been shown to play an important role in the development and maturation of callus and somatic embryos. The ratio of auxins to cytokinins in the culture medium is important since their combinations determine the morphogenic response for root and shoot formation.

The type and concentration of plant growth regulators used during culture, the interaction between the regulator and time of application all affect the process of direct and indirect embryogenesis (Ammirato, 1984). Generally, 2,4-D has been the most effective auxin for plant regeneration despite continuous application resulting in inhibition of embryo development. This was attributed to gene products produced by pre-embryogenic masses which can inhibit the continuation of the embryogenesis process (Karanja, 1997). Removal of auxins inactivates the genes and activates the embryogenesis process. Lower levels of the auxins have been reported to yield better quality embryos resulting in higher embryos, with greater germination rates. Nguyen *et al*. (1997) cultured sweet potato plants on media with 2,4-D ranging from 0 – 10 mg L^{-1} and reported best stimulation of embryo formation at lower levels.

Herbert (1998) reported that tissues could be induced to form callus by application of plant growth regulators or hormones. Callus was induced to form roots in an auxin to cytokinin concentration of 0.2 to 3.0 mg L^{-1} and to induce shoot formation in 0.03 to 1.0 mg L^{-1}.Ammirato (1984) reported that a chemical imbalance results in abnormalities of embryo budding, especially when auxin levels are high in media. Cytokinin has shown to be significant in induction of somatic embryos (Silvertand *et al*., 1996). Recent studies have revealed that ABA can induce quiescent state similar to zygotic embryos and modify the formation of all aberrant forms of somatic embryos. ABA in sweet potato also stimulates callus growth and enhances shoot or bud proliferation (Torres *et al*., 2001). Plant growth regulators thus are a fundamental requirement in somatic embryo production, callus induction growth and development.

2.7.3 Nutrients

A plant structure needs an array of nutrients for proliferation and development of cells to maturity. During the past decades, many types of media have been developed for *in vitro* plant culture (Pierik, 1993; Torres, 1989). In sweet potato callus cultures, both organic and inorganic nutrients are essential for induction, development, maturation and germination of embryos. These nutrients have to be supplemented exogenously through the media (Raemakers *et al*., 1995).

The basic nutrient media is made up of vitamins, amino acids, sugar, a solidifying agent, macronutrients, including elements such as nitrogen, phosphorous, potassium, calcium, magnesium and sulphur. Micronutrients include manganese, zinc, boron, copper and molybdenum for plant cell and tissue growth. Vitamins such as thiamine (B_1), pyridoxine (B_6) and myo-inositol are required as catalysts in various metabolic processes. A carbon source preferably sucrose is useful to replace carbon dioxide assimilation during photosynthesis (Dodds and Robert, 1995).

Media compositions have been formulated for specific plants and tissues. Some tissues respond much better on solid media while others on liquid media. In general, the choice of medium is dictated by the purpose and the plant species or variety to be cultured. A variety of other media components have also been used for specific purposes. The osmolarity of the culture medium, agitation, and aeration of suspension cultures have an important influence on plant cell division.

The medium can be solid, semi-solid or liquid, depending on the presence or absence of gelling agents (Taji *et al.,* 1992). Gelling agents are usually added to the culture medium to increase viscosity as a result of which plant tissues and organs remain above the surface of the nutrient medium. Many gelling agents are used in plant culture media and they include agar, agarose and phytagel (Duchefa, 2005). Agar is the most commonly used gelling agent for preparation of solid and semi-solid media as it contributes to the matrix potential, humidity, availability of water and dissolved substances in the culture medium (Pierik, 1993).

2.7.4 Culture conditions

Most culture rooms need to be illuminated; however some plant cultures require complete darkness. The plant species and/or propagation scheduling determines the light intensity. The developmental stage of the plants also determines if wide spectrum or cool white-fluorescent lights are to be used. Rooting is strongly influenced positively with far-red light; therefore, wide spectrum lights should be provided especially when *in vitro* micropropagated plants are hardened (Ahloowalia *et al.,* 2004).

In sweet potato, embryogenesis has been obtained through solid, liquid, semi solid and cell suspension cultures. Embryos in liquid medium are bathed by the culture medium therefore evenly exposed to media components. For successful induction of both callus and somatic embryos and development of shoots and roots, well controlled culture conditions in terms of temperature, humidity, air circulation, light quality and duration must be provided (Ammirato, 1984). The environmental factors may influence growth and differentiation process directly during culture or indirectly affect their responses in subsequent generations.

Herbert (1998) reported that totipotent cells exhibit a high degree of plasticity in their response to the physical and environmental stimuli. Correct culture medium can induce growth of callus in plant tissue explants. Protoplast cultures as well as cell suspension cultures have been reported to be particularly sensitive to environmental conditions. In *Ipomoea batatas* absence of light leads to etiolation which results in higher frequencies of embryogenic abnormalities. The temperature varies from plant to plant but in sweet potato the ideal temperature should be maintained between 25 °C and 27 °C throughout the entire culture room. Somatic embryos formed in the dark at a temperature of 24 $\pm$ 0.5 °C (Dhir *et al*., 1998). Zheng *et al*. (1996) reported induction of embryogenic tissue when explants were incubated in a 16/8 hour photoperiod at a temperature of 25 °C.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experiment 1: Effect of 2,4-D Concentration on Callus Induction and Maturation of 5 Sweet Potato (*Ipomoea batatas* (L.) Lam.) Cultivars

3.1.1 Site Description

Laboratory experiment

The aim of this experiment was to determine the optimum 2,4-D concentration for callus induction and thereafter determine the best tissue culture regeneration protocol for locally adapted sweet potato cultivars. This experiment was carried out in the Biotechnology laboratory at the Kenya Agricultural Research Institute (K.A.R.I.) Njoro under controlled temperature (27 ºC) and light conditions (700 µ mol m^{-2} s^{-1}) unless otherwise specified for each experiment.

Field experiment

The experiment aimed at comparing growth and yield between regenerated plantlets and conventionally planted sweet potato cultivars. A one season experiment was set up at Njoro (36^{o} E 0^{o}20'S), which lies at an altitude of 2160 meters above sea level, in the agro-ecological zone LH_2 (low highlands$_2$). The soils are vintric mollic andosols, well drained, deep to dark reddish brown friable and silt clays with humic top soil. The cropping period falls between March to August (long rains) and October to February (short rains) (Jaetzold and Schmidt, 1983). The rainfall is bimodal and well distributed (Appendix 1) with which night and day temperatures range from 7 –10 ºC (Appendix 2).

3.1.2 Experimental design and data analysis

The experimental design was a factorial arrangement in a completely randomized design with five levels of the factor cultivar and six levels of 2,4-D replicated three times in an incubator. Calli strength was square-root transformed ($\sqrt{x+1}$) to standardise the variance before subjecting to ANOVA (Hills and Little, I978). The laboratory data was then subjected to Statistical Analysis Systems (SAS) (Proc:anova) and where F- test was significant at 5 % level, means were separated by Duncan's Multiple Range Test (DMRT) (Hills and Little, I978; SAS Institute, 2001).

Sweet potato cultivars

Vines for this study were sourced from Centre Internationale de Potato (C.I.P) Nairobi, Kenya. The cvs. *SPK004, Kemb 10, Mugande, Japon tresmesino* and *Zapallo* were chosen based on their popularity, preferred taste by most farmers and growth performance under local conditions. The origin and morphological description is as follows: *Mugande* 440163 originated from Nyanza province, has broad leaves, dark green petioles, with reddish purple skin, *SPK004* originated from Kakamega, has a light green petiole, white skin and flesh, *J. tresmesino* and *Zapallo* originated from South America have a purple petiole, skin and orange flesh, *Kemb 10* 440169 originated from Embu, has parted leaves, green petiole, white skin and yellow flesh. Cuttings obtained were grown in a greenhouse to provide a readily available supply of stock plants. For constant availability of *in vitro* material shoot tips (5 cm) were collected as explants and established *in vitro* for further multiplication as described in section 3.1.4.

Levels of 2,4-D

The synthetic auxin, 2,4-D was chosen based on its property as a strong auxin in including callus as compared to natural auxins such as NAA or IAA. The effect of six levels of 2,4-D on callus induction, proliferation and maturation was investigated on the five sweet potato cultivars. The levels used in the study included 0.5, 1.0, 2.0, 3.0 and 5.0 mg L^{-1} and a control with no 2,4-D was also included in the experiment.

3.1.3 Establishment of sweet potato material for *in vitro* micropropagation

Standard sterilisation procedures followed were as described by Pierik (1993). Shoot tips measuring about 1.5 cm long were obtained from each cultivar. These explants were washed under running tap water for an hour to remove any surface organisms and soil residues. Shoot tips were then dipped in 70 % ethanol for 10 seconds and rinsed thrice using sterile distilled water. A 1 % (w/v) sodium hypochlorite (NaOCl) solution was prepared, 2 drops of Tween 20 added, mixed gently and used to surface sterilise shoot tips for fifteen minutes on a rotary shaker (model GallenKamp) set at 50 rpm. The sterilizing was followed by thorough rinsing five times in double-distilled water to remove all traces of NaOCl.

Undamaged shoot tips were then inoculated onto multiplication medium composed of 4.4 g L^{-1} Murashige and Skoog Basal Medium (MSBM) (Murashige and Skoog, 1962) supplemented with 100 mg L^{-1} myo-inositol, 5 mg L^{-1} gibberellic acid and 30 g L^{-1} sucrose. The pH of the media was adjusted to 5.8 prior to adding Phytagel at a rate of 3 g L^{-1} (Zheng *et al*., 1996) and autoclaved at 121 °C and 1.035 kPa for 15 minutes. The medium was cooled to a temperature of 40 °C in a water bath and 100 mls poured into sterile 10 cm x 10 cm Kilner glass jars. In each jar 7 shoot tips were inoculated and each treatment repeated 10 times.

Several cultures were established using only *in vitro* germinated plantlets until 100 % aseptic material were obtained. Stock plantlets were maintained by continuous subculturing at 2 - 3 week intervals. The *in vitro* plants were maintained in a growth chamber set at a temperature of 27 $\pm$ 0.5 °C and a 16 hour photoperiod of 700 µ mol m^{-2} s^{-1} light intensity provided by cool fluorescent tubes (Phillips).

3.1.4 *In vitro* establishment of callus culture

The source of plantlets used to set up callus cultures was *in vitro* material established above (3.1.3). To maintain aseptic conditions the laminar flow and all incubators were surface sterilised with 95 % ethanol (C_2H_5OH) and allowed to run for 30 minutes before using to initiate callus cultures. Equal amounts of media composed of half strength MSBM, supplemented with 100 mg L^{-1} myo-inositol, 2mg L^{-1} thiamine – HCl, 40 g L^{-1} sucrose, 10ml L^{-1} vitamin stock solution (200 mg L^{-1} calcium pantothenate, 2 g L^{-1} gibberelic acid, 10 g L^{-1} ascorbic acid, and 10 g L^{-1} putrescine) and 3 g L^{-1} Phytagel was dispensed into six 500 ml volumetric conical flasks, autoclaved and cooled to 42 °C (Zheng *et al*., 1996; Nguyen *et al*., 1997). After autoclaving the 2,4-D was filter sterilised and added according to each treatment. BAP was added at a rate of 1 mg L^{-1} to inhibit development of shoot primordia during culture. The media contents were mixed gently by swirling and 30 ml poured into each sterile 9 x 1.5 cm Petri-dish.

For each cultivar, leaves from most recently expanded *in vitro* plantlets were sectioned into 50 pieces of 5mm length. In each petri - dish containing callus culture media, five leaf pieces were placed with the adaxial side in contact with the solid surface of the medium, in a circular

arrangement, labelled and sealed using parafilm. The cultures were transferred to a dark incubator set at a temperature of 29 °C.

Light was provided after six weeks in the dark and induced calli transferred whole to fresh media as above. After 3 – 4 weeks the treatments that had formed callus were noted and transferred to calli maturation media composed of half strength MSBM, supplemented with 100 mg L^{-1} myo-inositol, 2 mg L^{-1} thiamine – HCl, 40 g L^{-1} sucrose and 3 g L^{-1} Phytagel and cultured under light conditions in a growth chamber set at a temperature of 27 $\pm$ 0.5 °C and a 16 hour photoperiod of low light intensity (700 µ mol m^{-2} s^{-1}) provided by fluorescent tubes.

3.1.5 Data collected

Data collection to determine the optimum 2,4-D concentration began two weeks after culturing leaf explants on the media. The data collected included:

Days to first calli formation: The treatments were observed daily and noted for calli formation around the leaf edges. Days to calli formation were derived by the difference between date of first calli formation and date when calli was transferred to fresh media.

Colour of calli: The colour of calli formed was carefully observed through its different phases as calli colour was an important indicator of suitable time for calli isolation and separation. Observations were done on a daily basis, with transfers to fresh media done when calli was whitish in colour. Prolonged stay of calli in media resulted in the colour turning light-brown.

Calli weight: The calli weight was recorded at an interval of two weeks and data recorded at four, six and finally at eight weeks after culture when transfer to fresh media for callus maturation was done. The calli weight was derived by the difference between the initial weight obtained by measuring the weight on the day of transfer of leaf explants to callus induction media and when calli was observed to be fully formed (no more changes in calli proliferation) all measured using a sensitive weighing balance.

Calli diameter: The measurements for calli diameter were taken once using a ruler for all the treatments. The diameter was taken just before transfer to callus maturation media.

Quality of calli: The quality of calli was determined once by visual observation of the callus on the leaf surfaces. This was done just before transfer to fresh media by comparing with previous preliminary results that had been obtained by destructive sampling of calli during culture. The

intensity of calli qualtity was hence scored on a scale of 0 - 4 according to findings by Sihachakr *et al*. (1997). In this study 0 indicated no callus formed, 1 indicated ≤ 25 soft watery callus, 2 indicated 25 – 50 % jelly-like yellowish–pale brown callus, 3 indicated 50 – 75 firm callus dark-brown while 4 indicated an overly mature calli that was solid and compact.

3.2 Experiment 2: Effect of Regeneration Protocols on Sweet Potato Plant Regeneration, Survival and Growth to Maturity

The experiment was set up to determine the best tissue culture regeneration protocol for 5 sweet potato cultivars by evaluating callus culture and somatic embryogenesis regeneration protocols in the laboratory.

3.2.1 Experimental design and statistical analysis

The experimental design was a factorial arrangement in a completely randomized design with the factor cultivar at five levels, regeneration protocols at two levels and three levels of 2,4-D, replicated three times in an incubator. Treatments were randomly assigned after which data collected was subjected to Statistical Analysis Systems (SAS) (Proc:anova) and where F- test was significant at 5 % level means were separated by LSD (Hills and Little, I978; SAS Institute, 2001).

3.2.2 Regeneration protocols

The three optimum levels of 2,4-D concentration determined in experiment 1 as 0.5, 1.0 mg L^{-1} and the control with no 2,4-D were used to establish the suitable protocol of regeneration in *I. batatas* using the two regeneration methods. All the five sweet potato cultivars used in Experiment 1 were also used in Experiment 2. Leaf explants were also used for callus induction and axilliary buds for somatic embryogenesis.

3.2.2.1 Callus induction

Medium for callus culture was prepared as described previously and divided into equal amounts in three 500 mls volumetric conical flasks, autoclaved and cooled to 42 ºC. After autoclaving 2,4-D was filter sterilised and added to the basal medium according to each treatment. The control had no 2, 4-D added. BAP was filtered sterilised and added at a rate of 1 mg L^{-1}. The

media contents were mixed gently by swirling and 30 ml poured into each sterile 9 x 1.5 cm petri - dish. Leaves from most recently expanded *in vitro* plantlets were cut to a length of 5mm and 45 pieces obtained from each of the five cultivars. In each petri - dish containing callus culture media, five pieces were placed with the adaxial side in contact with the solid surface of the medium, in a circular arrangement, labelled and sealed using parafilm.

The cultures were transferred to a dark incubator set at a temperature of 29 °C. Lights were turned on and induced calli transferred whole to fresh media. After 3 to 4 weeks under light conditions treatments that had formed callus were recorded and transferred to calli maturation and embryo initiation media. The basal medium comprised of half strength MSBM (Murashige and Skoog, 1962) supplemented with 100 mg L^{-1} myo-inositol, 2 ml L^{-1} thiamine – HCl solution, 40 g L^{-1} sucrose and 3 g L^{-1} Phytagel (Chee *et al*., 1990; Zheng *et al*., 1996). After autoclaving at 121 °C and 1.035 kPa for 15 minutes, the medium was cooled to a temperature of about 40 °C in a water bath, 2 ml L^{-1} of BAP was filter sterilised and 0.5 or 1.0 mg L^{-1} 2,4-D added post autoclave in conical flask before pouring into petri dishes.

The calli formed were cultured for not longer than two weeks before transfer to plant regeneration medium which consisted of the above components but with the exception of plant growth regulators. The medium was poured into sterile 10cm x 10cm Kilner glass jars. In every jar calli already forming embryos were transferred whole onto the medium and cultured under light conditions in a growth chamber set at a temperature of 27 $\pm$ 0.5 °C and a 16 hour photoperiod of low light intensity (700 μ mol m^{-2} s^{-1}) provided by 56 cool fluorescent tubes (Phillips) to regenerate plantlets.

3.2.2.2 Somatic embryogenesis

Somatic embryos were initiated from axilliary buds grown *in vitro* on sweet potato multiplication media (Liu and Cantilfee, 1984). The somatic embryo induction media comprised of 4.4 mg L^{-1} MSBM (Murashige and Skoog, 1962), supplemented with 30 g L^{-1} sucrose, 0, 0.5 or 1.0 mg L^{-1} 2,4-D, 100 mg L^{-1} myo-inositol, 1 mg L^{-1} thiamine HCl, 0.01 mg L^{-1} nicotinic acid, 0.01 mg L^{-1} pyridoxine, 1ml L^{-1} BAP and 2.2 g L^{-1} potassium chloride. The pH of the media was adjusted to 5.8 prior to adding 3 g L^{-1} phytagel. The medium was autoclaved at 121 °C at 1.035 kPa for 15

minutes, cooled in a water bath for 20 minutes and poured into 20 (9x 1.5 cm) petri-dishes (Newell *et al*., 1995; Zhange *et al*., 1998).

Axilliary buds were excised from shoot tips under a light microscope and cut to 2 mm length from the *in vitro* plants to obtain 15 tips from each sweet potato cultivar. Three developing buds from each cultivar were placed in each petri - dish, labelled and sealed using parafilm. The cultures were incubated in the growth room at a temperature of 27 °C for a 16/8 hour day: night photoperiod for up to six weeks under low light intensity provided by white fluorescent lamps. After six weeks the initiated embryos were transferred using a scalpel to fresh media containing half strength MSBM (1962) medium, 30 g L^{-1} sucrose, 0.03 g L^{-1} abscisic acid and 0.1 ml L^{-1} BAP, for two weeks in the dark to promote embryo development (Zheng *et al*., 1996). Embryos were observed under a microscope after which those that had developed to the heart-shaped stage were transferred to a light chamber set at 25 °C for two weeks, after which the developing embryos were transferred to hormone (2,4-D) free media to initiate shoots.

3.2.3 Data collection

Data collection began two weeks after culturing leaf explants and axilliary buds on the media. The data collected included:

Days taken to form shoots: The number of days taken to form shoots were counted and recorded for each treatment.

Days taken to form roots: The number of days taken to form roots were counted and recorded for each treatment.

Growth rates: The number of leaves and roots that formed was observed on a daily basis.

Number of plants regenerated: The total number of plants regenerated and the percentage (%) survival of regenerated plantlets *in vitro* were determined for each cultivar.

3.3 Experiment 3: Effect of Plant Regeneration Methods on Yield of Sweet Potato Cultivars under Field Conditions

Each regenerated plant was soaked in distilled water for one hour to rinse off *in vitro* culture media from roots. Plants were acclimatized first *ex vitro* in soil mixture prepared by mixing forest loam soil, 2mm size sand particles and farmyard manure in the ratio 3:1:1, sieved and autoclaved to destroy soilborne pathogens. Plantlets were grown in 10 cm x 15 cm plastic polythene bags, covered with transparent bags to prevent moisture loss. Hardening was done for 7 days in the growth room followed by two weeks in the greenhouse after which the plants were transplanted to the field.

Conventional cuttings were planted in ridges during the long rains (March to July 2006) in a caged field to protect from pests. The main plot size measured 16m x 4m and had 15 plots each measuring 4m x 3m. The inter-row and intra-row spacing was 0.6m x 0.5m, respectively. One seedling cutting was planted per ridge (30cm high and wide). A 0.75 cm path was left between the replicates as suggested by Munga *et al*. (2000). Each plot had five rows spaced at 60 cm apart and each row had eight vine cuttings at 0.5 cm apart. Vine cuttings planted were 20 cm long.

3.3.1 Experimental design and statistical analysis

The experimental design was a RCBD replicated three times. A control experiment of propagated by conventional means was included in the study and compared with the tissue culture regeneration methods: callus induction and somatic embryogenesis. The data was subjected to ANOVA using Statistical Analysis Systems (SAS) (Proc: anova) and mean separation done using DMRT (SAS Institute, 2001).

3.3.2 Data collected

Plant stand count: The number of plants that survived after acclimatization of *in vitro* plants and those that survived after transplanting to the field was counted three weeks after transplanting and at harvesting. Plants were monitored to identify healthy, damaged, diseased and off types in the field.

Growth: The growth variables determined included leaf area, plant height, number of leaves, number of buds and number of branches that formed, on a two week interval after transplanting

followed by a fortnightly determination until time of maturity. The leaf area (LA) of the most fully expanded leaf was also measured using a leaf photometer (LI- COR- 3000) and leaf area index calculated as shown below.

$$\text{Leaf Area Index (LAI)} = \frac{\sum \text{Leaf area } (m^2) \text{ x number of leaves}}{\text{Ground area occupied by plants } (m^2)}$$

The plant height from each treatment was measured from the base of each stem to the tip of the main vine using a tape measure. All growth measurements were taken on 5 randomly selected plants from each row. A useful indicator of time of maturity was taken to be the period when maximum flowering occurred.

Yield: Agronomic characteristics including tuber size, length, weight and yield per m^2 and marketable yield were recorded during harvesting. Marketable tubers were separated from non-marketable tubers by visual assessment. Tiny malformed, pest-attacked or damaged tubers were considered non-marketable.

Virus indexing: A Double Antibody Sandwich Enzyme Linked Immuno Sorbent Assay (DAS ELISA) procedure was used for diagnosis and detection of SPFMV in the regenerated plant samples after transplanting to the field. The antibodies used were obtained from Agdia ELISA kit (Jericho and Thompson, 2000) and the three day procedure followed is described below.

DAY 1: Extraction of the sap

Leaves from sweet potato plants showing symptoms of SPFMV were collected from the laboratory and the field. One gram of the fresh plant leaves was weighed using a sensitive weighing balance, transferred into a sterilised mortar and pestle and crushed using extraction buffer (Appendix 3). The ratio of the extraction buffer to the volume of leaves used was 1g: 2ml. The extract was stored in sterile Eppendorf tubes (2 mls) at 4° C.

Coating of plates: Polystyrene micro-titre well plates (Tetra Inc) were coated with 100 µl of SPFMV polyclonal antibody solution per well at a dilution of 1µg antibody per ml in coating buffer. Plates were sealed in a humid box to prevent desiccation and incubated overnight at 4 °C.

DAY 2: Loading of samples

The plates were washed four times by flooding individual wells with generous amounts of washing buffer (PBS –Tween) (Appendix 3). Each ELISA plate was left to stand for one minute before the wash buffer was dumped out and dried by tapping on a paper towel after the final wash.

Antigen: One hundred microlitres of extracted sap from each sample was added to horizontally duplicated wells, and the plates incubated in a humid box overnight at 4 °C.

DAY 3: Preparation of enzyme conjugate buffer

The plates were washed four times by flooding individual wells with generous amounts of washing buffer. A commercial antibody enzyme conjugate was used (Sigma Chemical Co.) (Appendix 3) to prepare the enzyme conjugate buffer. One hundred µl of the conjugate solution were added to each well at 1 µg per ml of conjugate buffer concentration and diluted with conjugate buffer. The covered plates were incubated for 2 hrs at 37 °C. The wells were washed as described above.

Blocking: The background of the plates was blocked using powdered milk which was diluted at the ratio of 1:20 (non-fat powdered milk/distilled water). One hundred µl of the milk solution was then added to each well and incubated at room temperature for 30 minutes followed by washing.

Preparation of substrate buffer: Freshly prepared di-sodium p-nitrophenyl phosphate (PNP) (Sigma Chemical Co.) diluted at 1mg/ml in substrate buffer was added to the wells. Plates were incubated at room temperature (22 - 25 °C) for 30 minutes after which readings were taken.

Reading of results: An ELISA micro-reader photometer (Dynatech MR 5000 reader) was used to obtain the results. Absorbance values were taken at wavelength of 405 *nm*. Readings that were twice that of the healthy controls were considered positive. Visual assessment of colour development in the wells was confirmed with the ELISA readings. The readings were taken at an interval of 15 minutes.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Effects of 2,4-D Concentration on Callus Induction and Maturation of 5 Local Sweet Potato (*Ipomoea batatas* (L.) Lam.) Cultivars

4.1.1 Optimal 2,4-D

The concentrations 0, 0.5 and 1.0 mg L^{-1} 2,4-D that produced calli early and in high frequencies were adopted and used with the two protocols (callus induction and somatic embryogenesis culture) to determine the efficient tissue culture regeneration protocol(s) suitable for each of the five local sweet potato cultivars.

4.1.2 Calli incidences

4.1.2.1 Days to calli formation

The analysis of variance (ANOVA) indicated no significant ($P \leq 0.05$) interaction between the sweet potato cultivars and 2,4-D levels for the mean number of days taken to form calli (Appendix 4), however, significant ($P \leq 0.05$) differences among the cultivars and the 2,4-D levels for the mean number of days taken to form calli were established The results revealed that overall mean time taken to form calli ranged from 39 to 56 days (Table 1). The shortest mean number of days taken to form calli was obtained when the callus induction medium was devoid of 2,4-D (40 days) followed closely at 0.5 mg L^{-1} 2,4-D (41 days) observed with cultivar *Kemb10* and *Mugande*. The longest time taken to form callus was observed when callus induction medium was supplemented with 1.0 mg L^{-1} and 3.0 mg L^{-1} (48 days) and lastly at 5.0 mg L^{-1} 2,4-D levels (43 days) (Table 1) with the cultivar *Zapallo* and *J. tresmesino*.

From these results, increasing 2,4-D levels in media led to an increase in the mean days taken to form calli and varied significantly with the test cultivar. The mechanism by which inhibition by 2,4-D occurred in cells and increased the number of days taken to form calli was uncertain as also reported by Lyndsey and Alderson (1986) who related it to genetic aberrations. The increase in the 2,4-D levels meant high levels in growth media and hence could have caused the uptake of 2,4-D by the cells to occur at a slower rate leading to excess auxin accumulation in the cells. As observed in this study and also as reported by Radhakrishnan *et al*. (2001) the cells used up 2,4-D as required and any excess began to actively show the herbicidal effects that

Table 1: Interaction effect of 2,4-D concentration and cultivar on the mean number of days taken to form calli and calli weight in sweet potato callus cultures grown *in vitro* at KARI-Njoro in 2006.

Source	2,4-D conc. (mg L^{-1})	Cultivar					Mean
		Mugande	J. tres	SPK004	Zapallo	Kemb 10	
Number of days taken to form calli	0.0	32.7 ± 1.8 c	45.3 ± 0.8 bc	36.0 ± 2.1 c	52.3 ± 1.3 b	33.3 ± 0.7 c	39.9 c
	0.5	34.3 ± 2.3 bc	42.7 ± 0.8 c	42.3 ± 2.5 ab	51.7 ± 1.1 b	33.3 ± 1.6 c	40.8 c
	1.0	37.7 ± 2.2 abc	41.7 ± 2.4 c	43.0 ± 1.7 ab	53.3 ± 2.7 b	38.7 ± 1.7 b	48.1 a
	2.0	40.0 ± 1.9 abc	47.3 ± 0.4 b	46.0 ± 1.2 a	60.0 ± 1.5 a	39.0 ± 1.2 b	46.5 ab
	3.0	45.7 ± 3.3 a	50.0 ± 1.2 a	40.7 ± 2.7 b	62.7 ± 1.7 a	42.7 ± 1.4 a	48.3 a
	5.0	44.0 ± 2.5 ab	49.3 ± 1.4 a	42.1 ± 2.0 b	61.7 ± 2.2 a	43.7 ± 1.7 a	42.9 bc
Means		39.1 c	46.1 b	41.7 c	56.9 a	38.4 c	
LSD (5 %)		9.05					
CV (%)		12.59					
Calli weight (g)	0.0	132.7 ± 5.8 a	121.1 ± 0.3 bc	135.1 ± 4.9 a	130.5 ± 4.9 ab	97.50 ± 16.4 d	123.4 bc
	0.5	130.5 ± 4.2 ab	125 ± 2.1 abc	135.2 ± 3.9 a	136.2 ± 2.3 ab	132.60 ± 1.9 a	131.90 a
	1.0	117.9 ± 6.8 b	115.2 ± 2.6 c	130.2 ± 5.4 ab	135.2 ± 3.2 ab	102.7 ± 9.4 cd	120.20 c
	2.0	133.7 ± 6.0 a	132 ± 3.8 ab	126.1 ± 1.3 ab	123.9 ± 9.5 b	104.9 ± 7.4 bcd	124.2 bc
	3.0	130.1 ± 4.0 ab	135.7 ± 1.8 a	125.7 ± 0.7 ab	137.5 ± 3.1 a	113.4 ± 14.5 bc	128.5 ab
	5.0	121.2 ± 1.7 ab	128.3 ± 1.4 ab	118.1 ± 6.0 b	134.3 ± 1.6 ab	116.8 ± 3.0 b	123.6 bc
Means		127.7 b	126.3 b	132.9 a	128.4 ab	111.3 c	
LSD (5%)		5.7					
CV (%)		15.5					

Data is presented as mean number of explants producing embryogenic callus ± Standard error (S.E).

Means followed by the same letter within a column are not significantly ($P \leq 0.05$) different, based on Duncan's Multiple Range Test (DMRT).

therefore slowed down the callus induction process. The 0 and 0.5 mg L^{-1} 2,4-D gave suitable calli within a shorter time period which was transferred to fresh medium to induce further calli maturation. Suitable calli for the study was identified as white and/or brownish to light-yellow. These calli were allowed to proliferate (Plate 1), as they had been reported as having higher ability to form embrogenic cells (Nguyen *et al*., 1997). However, in leaf explants treated with 2.0, 3.0 or 5.0 mg L^{-1} 2,4-D although explants lost their bright green colour within a week of culture no substantial calli formed until after 7 weeks. In addition the calli that formed at these levels were dull/dark-brown, dispersed on the surface (Plate 2) and not suitable for plant regeneration. This indicated that the 2,4-D levels 2.0, 3.0 or 5.0 mg L^{-1} could have been too high for suitable induction of calli for the local sweet potato cultivars. Amitha and Reddy (1998) observed dull-brown, dispersed and watery callus at high levels of 2,4-D in cowpea (*Vigna unguiculata L*.) and discarded the calli as it was subject to variation due to the unstable non-uniform cell formation regimes that were unsuitable for plant regeneration.

Significant ($P \leq 0.05$) differences in response to 2,4-D levels on mean number of days taken to form calli were detected among the sweet potato cultivars. In this study, 2,4-D was found effective in inducing calli to form but the optimum concentration depended on the individual cultivar. The shortest duration taken to form calli was obtained in the cultivar *Mugande* which gave a mean of 32 days with 0.5 mg L^{-1} 2,4-D followed by the cultivar *Kemb10* with 33 days, *SPK 004* with 36 days when no 2,4-D was supplemented in the medium. *J. tresmesino* took 41 days with 1.0 mg L^{-1} 2,4-D and *Zapallo* took 52 days with 0.5 mg L^{-1} 2,4-D (Table 1).

In cultivar *Mugande, Kemb10* and *SPK004* treatments devoid of 2,4-D took a significantly ($P \leq 0.05$) shorter time to form calli, indicating that addition of 2,4-D during calli formation was inhibitory for certain cultivars, a finding in agreement with Nguyen *et al*. (1997) with the sweet potato genotypes Q23728 and 1560E. It took the cultivars longer to form callus when medium was supplemented with high 2,4-D levels and less calli was produced as also reported by Radhakrishnan *et al.* (2001) with peanuts. High 2,4-D levels probably inhibited formation of embryogenic callus, an observation similar to that of Silvertand *et al*. (1996), who reported that at high 2,4-D levels friable calli that was soft, remained stunted in growth and had lost the ability to form embryogenic calli.

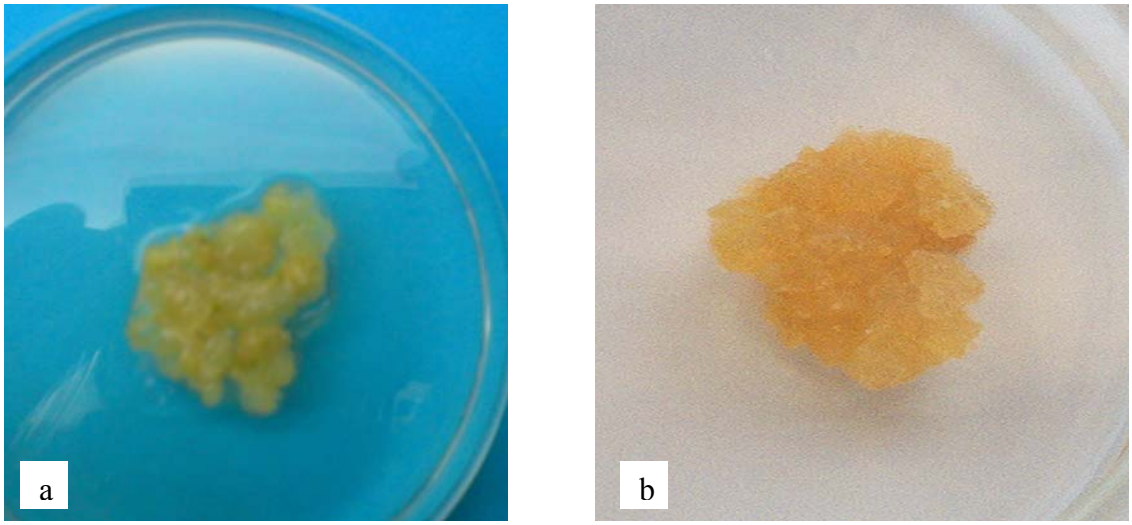


Plate 1: Leaf explants from the cultivar *Kemb10* at 4 (a) and 6 (b) weeks culture on callus initiation medium supplemented with 0.5 mg L^{-1} 2,4-D.

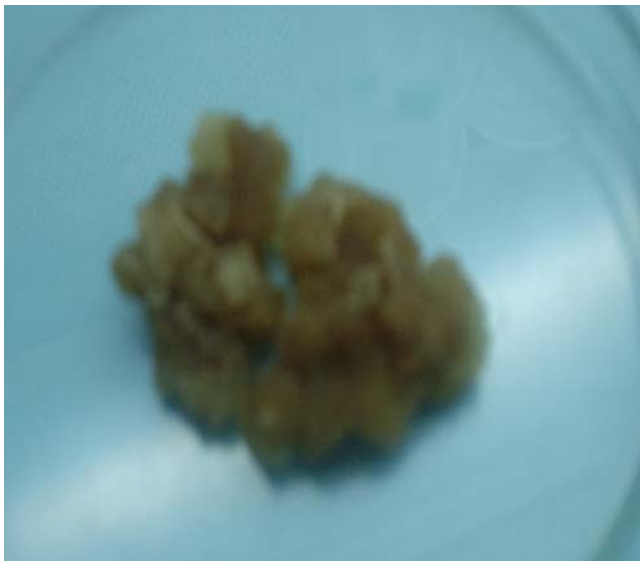


Plate 2: Leaf explants from the cultivar *Kemb10* at 10 weeks culture after initiating callus cultures on 3.0 mg L^{-1} 2,4-D.

Differences in callus formation patterns were also observed among the cultivars. In *Kemb10, SPK004* and *Mugande* embryogenic calli formed in a circular pattern and spread out on the leaf surface while in the cultivars *J. tresmesino* and *Zapallo* calli formed mostly around the leaf edges. For all cultivars no calli developed below the leaf explant surfaces. These differences in patterns were attributed to the different morphological features of the leaf associated with each cultivar. It was observed that the epidermis of leaves from the cultivars *Mugande, SPK004* and *Kemb10* appeared to be visually slightly thicker than for the cultivars *J. tresmesino* and *Zapallo.* Since 2,4-D was naturally more concentrated outside than inside the cell, uptake from the culture medium to the inside of the cell occurred rapidly by diffusion and active uptake depending on the dissociation of the 2,4-D molecules via the cut leaf explants surfaces. A leaf from a cultivar with an epidermal tissue that was less thick rapid depletion of auxin from the medium to cells occurred soon after subculturing onto fresh medium (Duchefa, 2005) thereby exposing the epidermal tissues to reduced sensitivity to 2,4-D hence reducing time taken to initiate calli.

4.1.2.2 Calli weight

The incidence of calli formation and subsequent calli weight depended on the 2,4-D concentration supplemented in the callus induction medium and test cultivars used. Significant ($P \leq 0.05$) interaction between test cultivar and 2,4-D treatments for calli weight were established in this study (Appendix 4). It was observed that using 0.5 mg L^{-1} 2,4-D led to a significant increase in calli weight (131.9 g), while the lowest mean was obtained at 1.0 mg L^{-1} (120.2 g) (Table 1) followed by 3.0 and lastly 5.0 mg L^{-1} 2,4-D. The highest decline in calli weight occurred when high levels of 3.0 and 5.0 mg L^{-1} 2,4-D were used. This results confirmed that despite 2,4-D being an effective auxin in producing callus in sweet potato, it was active for callus induction when used in small amounts (Sihachakr *et al.*,1997). As earlier reported by Radhakrishnan *et al*. (2001), high 2,4-D concentration has been shown to have herbicidal effects on plants. Comparison of calli weights indicated genotype dependent responses to the 2,4-D added in the culture medium. The highest calli weights were attained with the cultivar *Zapallo* (132.9 g) followed by *SPK004* (128.4 g) while the lowest overall mean calli weight were observed in *Kemb10* (111.3 g) (Figure 1).

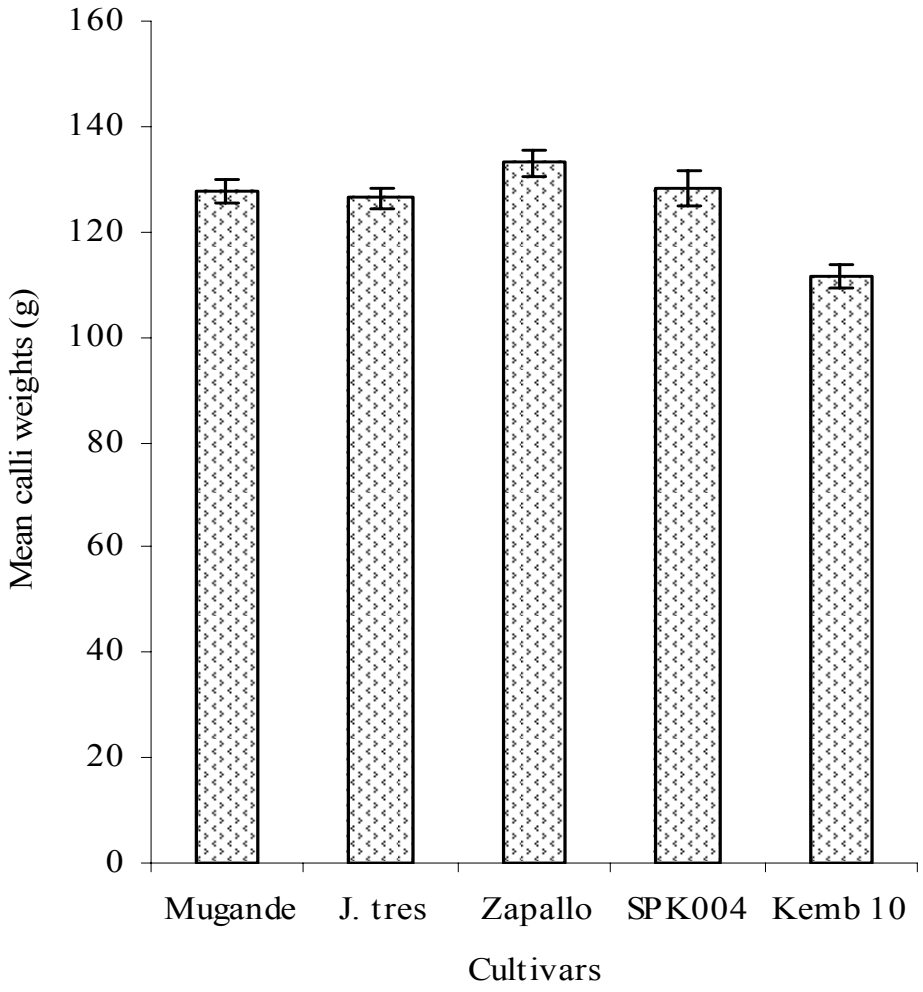


Figure 1: Mean calli weights of sweet potato cultivars cultured on callus induction media composed of different 2,4-D levels and grown *in vitro* at KARI-Njoro in 2006.

An increase in 2,4-D level from 1.0 to 2.0 or 3.0 mg L^{-1} resulted in a significant ($P \leq 0.05$) increase in mean calli weights in the cultivars *Mugande, J.tresmesino* and *Kemb10* which emphasized the cultivar differences in response to different 2,4-D levels for calli weight (Table 1). Studies by Nguyen *et al*. (1997) showed that cultivar differences played a significant role in determining the amount of calli that formed. *Kemb10* was more sensitive to an increase in the amount of 2,4-D compared to other cultivars.

Calli weights recorded at 4, 6 and 8 weeks in culture when morphological changes were expected to occur showed that despite differences in calli weights being observed at these times the results indicated no significant ($P \leq 0.05$) differences. Prolonging the period of callus culture on callus initiation medium resulted in decreased mean calli weights. The reduction in weight was attributed to the exhaustion of nutrients in the medium by leaf explants during calli formation, therefore destabilizing callus culture initiation. It could also have been due to moisture and aeration conditions during culture that influenced hormones to cause rapid cell re-adjustment during cell division as reported by Vasil and Vasil (1983). The first four weeks in culture corresponded to the time when leaf explants were first cultured on the medium to the time when callus began to grow and hence increased calli weights. During this period it took the longest time for an increase in calli weights to be recorded an observation also reported by Dovzhenko and Koop (2003), with sugar beet (*Beta vulgaris* L.) callus. For successful induction of callus to occur, chemical signals are sent to the leaf explant cells that are to form calli to counteract and adjust to the 2,4-D levels within the medium (Sachs, 1993).

Maximum calli growth occurred at six weeks duration of leaf explants in culture. At this stage embryogenic calli began to proliferate and spread around the edges of leaf explants, resulting in increased calli size but not calli weight. At eight weeks in culture there was a stationary stage when no significant ($P \leq 0.05$) changes in calli growth occurred, resulting in very low mean calli weights. The ability of cells to divide was very slow and limited by factors such as aeration rate, gas composition and agitation speed during cell regeneration process as suggested by Prasertsongskun (2003). The occurrence of fluctuations in calli weights were high after 8 weeks and especially at 3.0 or 5.0 mg L^{-1} 2,4-D. Frequent transfers (on weekly basis) had to be done severally to enable cell division to take place.

4.1.2.3 Calli quality

The analysis of variance confirmed significant ($P \leq 0.05$) interaction among the sweet potato cultivars and 2,4-D levels for the mean calli quality (Appendix 4). Calli formed by the local sweet potato cultivars were of different quality with quality of values above 2.3 being deemed suitable for embryogenesis. These calli were light-yellow and transferable to callus maturation medium for further development. The most suitable calli quality was scored with the treatment

devoid of 2,4-D followed by 0.5 and 1.0 mg L^{-1} 2,4-D both with a mean of 2.3 with cultivar *Kemb 10*. The lowest means were attained at 2.0 mg L^{-1} 2,4-D which gave a mean of 1.7 with *Mugande* and *SPK004*. This was probably because calli formed at high 2,4-D levels was small, undeveloped and took long to form therefore influencing the strength of the calli in media.

Cultivar influences on calli quality were also observed. The cultivar *Mugande* formed calli of favourable quality at both high and low 2,4-D levels contrary to the response in cultivars *Zapallo, J. tresmesino* and *Kemb10* which gave favourable calli quality at only low levels of 2,4-D (Table 2). From previous results, the calli weight in *Mugande* had increased with an increase in 2,4-D levels. Likewise the 2,4-D levels may have increased amount of calli thereby increasing the number of embryogenic calli formation. At 2.0 mg L^{-1} 2,4-D levels the cultivars *Kemb10, SPK004* and *J. tremesino* were unable to develop calli of suitable quality. This was attributed to the differential responses of the cultivar to the 2,4-D levels in the medium which affected calli formation patterns, as reported by Rolando *et al*. (1999).

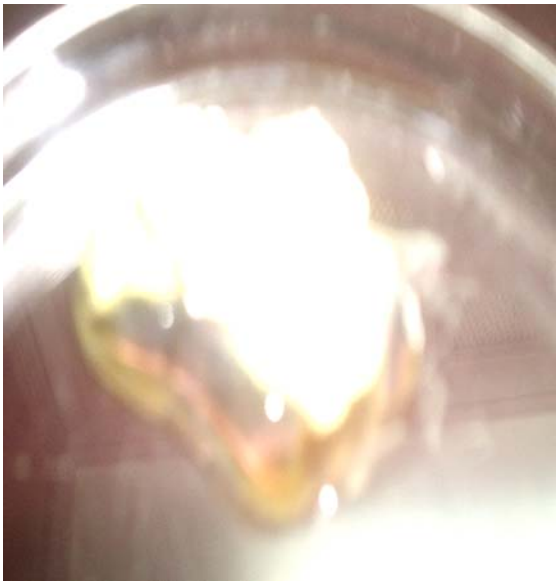
A soft watery callus was induced from the leaf explants within 4 weeks of culture in callus initiation media (Plate 3a). These calli were undeveloped (Plate 3b) but with further culture the calli differentiated and developed into a smooth circular mass of calli after six weeks culture (Plate 3c). By the 9^{th} week the calli gradually changed colour from yellow to pale brown (Plate 3d). During the 12^{th} week of culture, it was observed that the calli turned completely white became compact and embryos could be seen beginning to develop along the edges of calli just as observed by Amitha and Reddy (1998). The light yellow to white callus was the most suitable calli type for this study because these calli were easier to isolate from media and transfer whole without contamination or callus breaking (Plate 3d). Small, undeveloped, soft dispersed callus, having a yellowish-brown, loose callus developed on the medium surface, a finding conforming to observations by Nguyen *et al*. (1997), who reported that these calli hardly developed and were discarded. Early and timely transfers were done because prolonged cultures have been shown to negatively affect callus proliferation and also lead to increased genetic variations (Brown and Thorpe, 1991). In the current study, it was evident that depending on the 2,4-D level supplemented in the culture medium and the test cultivar, different types of calli and strength developed.

Table 2: Interaction effect of 2,4-D concentration and cultivars on the mean calli quality and diameter of sweet potato callus cultures grown *in vitro* at KARI-Njoro in 2006.

Variable	2,4-D Conc.	Cultivar					
		Mugande	J. tres	SPK004	Zapallo	Kemb 10	Mean
Calli quality (%)	0.0	3.0 ± 1.1 a	2.7 ± 0.5 a	2.7 ± 0.2 a	3.0 ± 0.0 a	3.7 ± 0.4 a	3.0 a
	0.5	2.7 ± 0.2 ab	2.7 ± 0.2 a	2.0 ± 0.3 a	2.7 ± 0.2 ab	3.3 ± 0.5 ab	2.7 ab
	1.0	1.3 ± 0.2 ab	2.7 ± 0.5 a	2.0 ± 0.5 a	3.0 ± 0.6 a	1.7 ± 0.4 ab	2.3 abc
	2.0	1.0 ± 0.1 b	2.0 ± 0.2 a	1.0 ± 0.0 b	2.7 ± 0.4 ab	1.7 ± 0.4 ab	1.7 c
	3.0	3.0 ± 0.6 a	2.0 ± 0.6 a	2.3 ± 0.5 a	2.3 ± 0.5 ab	1.7 ± 0.4 ab	2.0 bc
	5.0	3.0 ± 1.7 a	2.0 ± 0.6 a	2.0 ± 0.7 a	2.0 ± 0.6 b	1.0 ± 0.0 b	2.1 bc
Means		3.0 a	2.7 ab	2.3 abc	1.7 c	2.0 bc	
LSD (5%)		0.09					
CV (%)		18.5					
Calli diameter (cm)	0.0	1.10 ± 0.06 a	1.02 ± 0.06 a	1.00 ± 0.03 a	1.10 ± 0.12 b	1.13 ± 0.11 a	1.07 a
	0.5	0.97 ± 0.05 ab	0.93 ± 0.04 a	0.93 ± 0.02 a	0.96 ± 0.02 bc	0.97 ± 0.02 ab	0.95 bc
	1.0	0.87 ± 0.04 b	0.93 ± 0.05 a	0.87 ± 0.02 a	0.87 ± 0.02 c	0.87 ± 0.02 b	0.96 bc
	2.0	0.83 ± 0.02 c	0.87 ± 0.04 a	0.82 ± 0.01 a	0.80 ± 0.01 c	0.87 ± 0.02 b	0.84 d
	3.0	0.80 ± 0.01 c	0.85 ± 0.02 a	0.88 ± 0.03 a	1.47 ± 0.34 a	0.80 ± 0.01 b	1.00 ab
	5.0	0.83 ± 0.82 b	0.90 ± 0.00 a	0.93 ± 0.05 a	1.43 ± 0.40 a	0.90 ± 0.03 b	0.88cd
Means		0.9 b	0.92 b	0.91 b	1.11 a	0.92 b	
LSD (5%)		0.50					
CV (%)		32.3					

Data is presented as mean number of explants producing embryogenic callus ± Standard error (S.E).

Means that are significantly ($P \leq 0.05$) different within columns are indicated with different letters based on Duncan's Multiple Range Test (DMRT).



a) Soft non-embryogenic calli, with a translucent white appearance at 4 weeks in culture medium

b) Soft watery undeveloped callus at the callus initiation stage

c) Dull brown non-embrogenic calli showing compact appearance with calli growing closely attached around the leaf explant

d) Firm, yellow and fully developed embryogenic callus, growing away from the explant

Plate 3: Distinction of calli types of sweet potato formed *in vitro* on callus induction medium at KARI-Njoro.

4.1.2.4 Calli diameter

The highest mean calli diameter was recorded with cultivar *Kemb 10* (1.13 cm) at 0 mg L^{-1} 2,4-D while the lowest calli weight was 0.8 cm with cultivars *Mugande* and *Kemb 10* at 3.0 mg L^{-1} 2,4-D and *Zapallo* at 2.0 mg L^{-1} 2, 4-D. These results indicated no cultivar differences in response to 2,4-D levels (Figure 2) and no significant interaction for calli diameter. Treatments involving *Zapallo* only gave the highest calli diameter at high 2,4-D level. Similarly with *Mugande*, calli diameter was highest when the 2,4-D level was increased to 5.0 mg L^{-1} (Table 2). For cultivars *SPK004, J. tresmesino* and *Kemb10* calli diameter declined at 3.0 or 5.0 mg L^{-1} 2,4-D (Table 2). The results indicated that the genotypic differences which existed among the sweet potato cultivars seemed to be related to different sensitivities to 2,4-D.

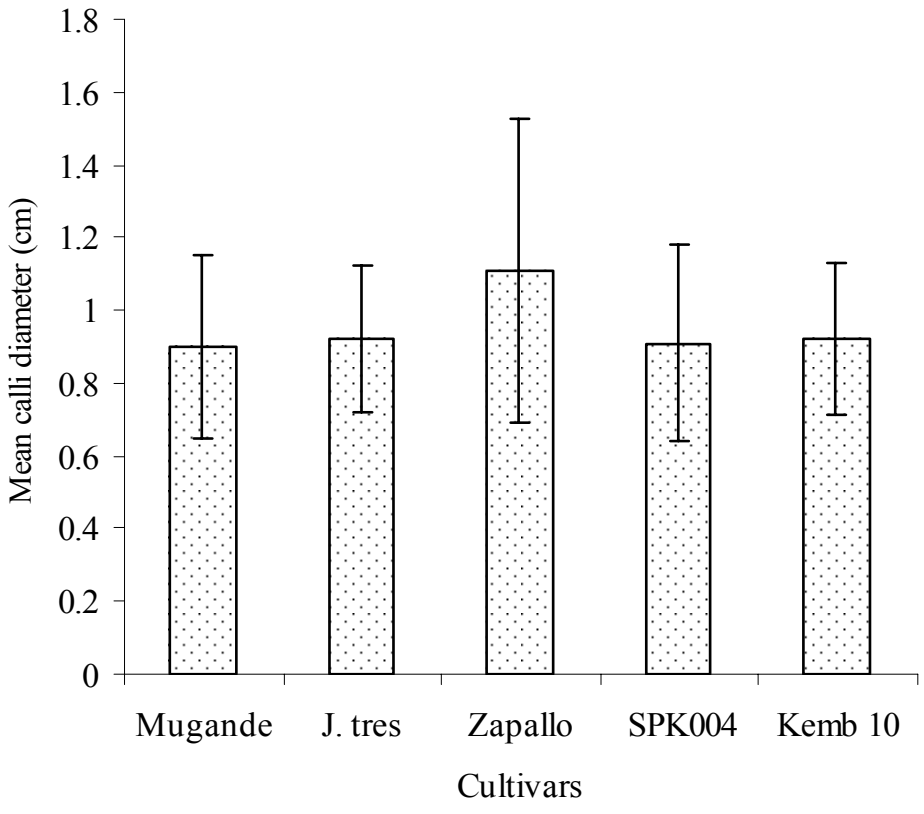


Figure 2: **Mean calli diameter of sweet potato cultivars grown *in vitro* at KARI-Njoro in 2006.**

Amitha and Reddy (1998) observed that high 2,4-D levels hindered calli formation and in some cases led to failure to produce embryogenic callus. Calli diameter was related to the ability of the leaf explant in culture medium to proliferate and spread out. A wide calli spread resulted in a higher calli diameter and vice-versa, depending on the sweet potato cultivar. Increasing 2,4-D levels from 0.5 to 2.0 mg L^{-1} 2,4-D significantly ($P \leq 0.05$) reduced the calli diameter before an increase was observed to occur at 3.0 and 5.0 mg L^{-1} 2,4-D (Table 2). This was probably because amorphous amounts of tissue formed as cells multiplied to form calli. 2,4-D played an important role in influencing calli diameter and there was a definite threshold level at 1.0 mg L^{-1} where cell expansion occurred leading to increased calli growth, Nguyen *et al*. (1997) had made similar observations.

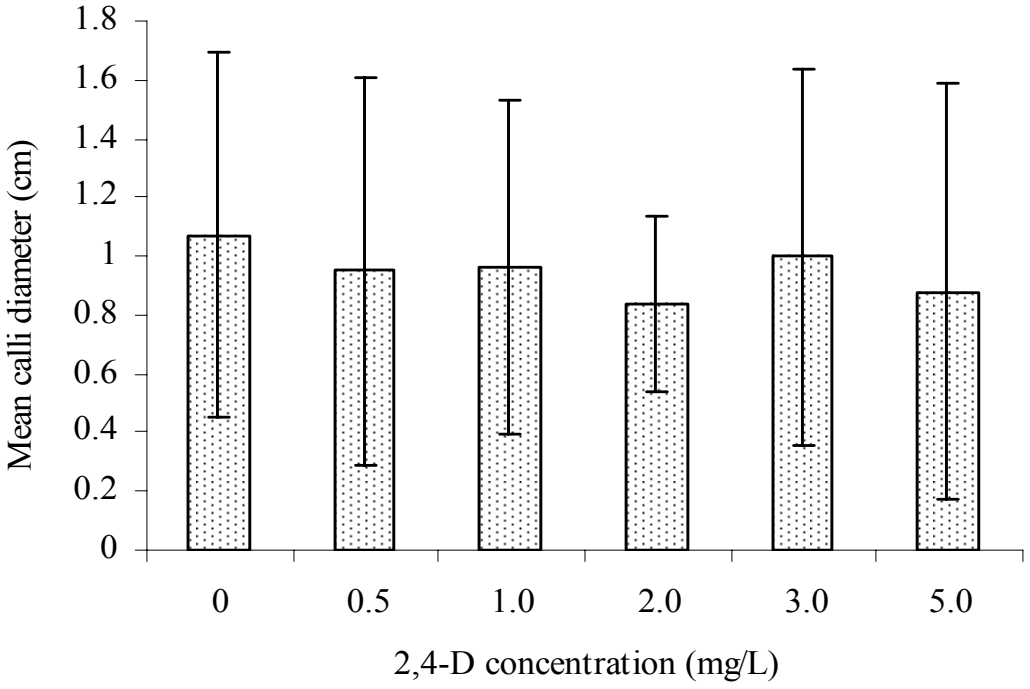


Figure 3: Mean calli diameter of sweet potato cultivars cultured *in vitro* on different 2,4-D concentrations at KARI-Njoro.

4.1.2.5 Correlation

There was a negative correlation between 2,4-D levels applied in the culture medium and all the calli incidences. This implied that increasing 2,4-D levels significantly influenced the calli incidences such that there was a decrease in calli weight, calli quality and calli diameter and an increase in number of days taken to form calli (Table 3). The disadvantage of increasing the 2,4-D levels was evident by a strong negative correlation being established between the 2,4-D concentration for days taken to form calli (r^2 = -0.36, P = 0.0005). These results conformed with findings of Amitha and Reddy (1998) who reported that at high 2,4-D levels, non-embryogenic calli was formed and development of embryogenic calli was suppressed leading to a delay in days taken to form calli and decreased calli weights, calli quality and calli diameter.

Table 3: Correlation between 2,4-D levels and calli incidences in sweet potato grown *in vitro* at the biotechnology laboratory of KARI-Njoro.

	2,4-D levels (mg L^{-1})	Calli weights (g)	Days calli formation (DCF)	Calli diameter (cm)	Calli quality (%)
2,4-D levels	1.0				
Calli weights	-0.01*****	1.0			
DCF	-0.36	0.32	1.0		
Calli diameter	-0.06	0.02******	0.15	1.0	
Calli quality	-0.27	-0.05******	-0.14	0.03******	1.0

* Correlation significant at P $\leq$ 0.05.

Highest decline in calli weight occurred at 3.0 and 5.0 mg L^{-1} 2,4-D, as shown in Table 1 and in most cultivars at this level the calli diameter and calli quality was observed to also reduce. In agreement with studies by Nguyen *et al.* (1997) and Al- Mazroooei *et al*. (2001), the results in this study confirmed that 2,4-D was effective in inducing embryogenic calli in the local sweet potato cultivars and was required in small amounts. Prolonging callus cultures in callus initiation media for a period longer than six weeks led to declining calli weights, quality and diameters.

Nguyen *et al*. (1997) reported that the medium composition had a significant effect ($P \leq 0.001$) on the overall production of nodular embryogenic calli and subsequently growth. The negative correlation observed between the 2,4-D levels, calli diameter and calli quality was due to the declining potential of explants to initiate and induce calli at high 2,4-D levels. It was important to determine the optimum 2,4-D concentration that was capable of inducing formation of embryogenic calli early and in high frequencies.

The study showed that the level of 2,4-D supplemented in the culture medium significantly affected the incidence, type and quality of calli that was formed. The optimum concentration of 2,4-D was identified to be effective when it was supplemented at low levels of 0, 0.5 or 1.0 mg L^{-1}. These concentrations formed calli in high frequencies for all the cultivars contrary to when high 2,4-D levels of 2.0, 3.0 and 5.0 mg L^{-1} were used. Using the low levels of 2,4-D resulted in a decrease in the mean number of days taken to form calli and lower calli weights. There was a significant ($P \leq 0.05$) increase in the mean calli diameter at low 2,4-D levels which led to the calli being formed having suitable calli quality for further plant regeneration. Significant differences in cultivar responses to the 2,4-D treatments supplemented in culture medium on the variables mentioned above were established. The cultivar *Zapallo* was most responsive in all the variables mentioned as it gave higher ratings when compared to other cultivars. Therefore, the order of ease of regeneration was *Zapallo* followed closely by *SPK004, Mugande, Japon tresmesino* and finally *Kemb10*.

4.2 Effect of Regeneration Protocol on Sweet Potato Plant Regeneration, Survival and Growth to Maturity

With plant regeneration incidences by the callus induction process, organized morphological tissues arose on the adaxial surface of the leaf explants within 49 days after culturing on callus maturation and embryo initiation medium. For somatic embryogenesis, somatic embryos developed directly from the axilliary buds within 34 - 45 days when placed on embryo initiation medium.

4.2.1 Days taken for shoots to form

Significant ($P \leq 0.001$) interaction between cultivar and regeneration method for mean number of days taken to form shoots were observed but there was no significant ($P \leq 0.001$) three-way interaction between cultivar, 2,4-D concentration and regeneration method (Appendix 5). From this study, using callus induction method generally increased the mean number of days taken for shoots to develop compared to regeneration by somatic embryogenesis (Table 4). This was because regeneration by somatic embryogenesis involved embryogenic cells already conditioned to produce embryos therefore enhancing organogenesis (Merkle *et al*., 1995). These cells were subject to less genetic variability, while regeneration through callus, the embryogenic cell mass had to be initiated and only a small percentage contributed to formation of calli, as reported by Chee *et al*. (1990), hence increasing the mean number of days taken to form shoots.

Both regeneration methods took a significantly ($P \leq 0.001$) shorter time to form shoots at 0.5 mg L^{-1} 2,4-D levels (Table 4) an observation similar to that of Dovzhenko and Koop (2003) and Rolando *et al*. (1999) with sugar beet and sweet potato shoot regeneration, respectively. The shortest time taken to form shoots was obtained when the 2,4-D level was 0.5 mg L^{-1} (37 days), followed by the treatment devoid of 2,4-D (39 days). Increasing 2,4-D level to 1.0 mg L^{-1} increased the time taken for shoots to form (41 days) (Table 4). The influence of 2,4-D on the mean number of days taken to form shoots was a function of amounts supplemented in the culture medium. Low 2,4-D levels probably supported production of secondary embryogenic calli that led to organogenesis as compared to high levels which in this study caused cultivars to form non-embryogenic calli, therefore hindering shoot formation (Al-Mazrooei *et al*., 1997).

Table 4: Interaction effect of cultivar, 2,4-D concentration and regeneration methods on the mean days taken to form shoots and days taken to form roots in sweet potato grown in tissue culture at KARI-Njoro in 2006.

2,4-D Levels	Cultivars	Days to form shoots			Days to form roots		
		CI	SE	Means	CI	SE	Means
0 (mgL^{-1})	Mugande	53.3± 1.3	34.6± 1.3	44.2±1.1	58.3± 1.2	42.3± 1.4	50.3± 0.9
	J. tres	41.3± 1.5	26.0± 0.7	33.7±0.9	53 ± 1.2	35.3± 0.4	44.2±0.9
	Zapallo	52.3± 0.5	26.3± 0.2	39.3±1.4	58.3± 0.4	31 ± 0.0	44.6±1.4
	SPK004	46.0 ± 0.6	25.3± 0.8	35.6±1.1	51 ± 0.3	33 ± 1.0	42.0±1.0
	Kemb 10	51.0 ± 0.7	33.7± 0.2	42.3±1.0	58.7± 2.0	38.3± 1.0	48.5±1.1
0.5 (mgL^{-1})	Mugande	54.3± 1.0	31.7± 0.2	43.0±1.2	60 ± 0.9	37.3± 0.2	48.6±1.2
	J. tres	40.0 ± 1.3	24 ± 2.7	32.0±0.9	51.7± 1.7	33 ± 0.9	42.3±1.0
	Zapallo	46.3± 0.8	25.3± 0.8	35.8±1.1	51.3± 0.7	27 ± 0.9	39.2±1.3
	SPK004	45.7± 0.2	23.3± 0.4	34.51.2	52 ± 0.7	30.3± 0.5	41.1±1.2
	Kemb 10	50.7± 0.2	33 ± 1.5	41.8±1.0	58 ± 1.5	37.7± 1.5	47.8±1.2
1.0 (mgL^{-1})	Mugande	58.3± 0.4	37.6± 0.2	48.0±1.1	63.7± 0.2	46.3± 1.5	55. ±0.9
	J. tres	47.0 ± 0.6	26.6± 0.8	36.8±1.1	61.7± 0.2	39 ± 0.1	50.3±1.2
	Zapallo	50.7± 0.7	26.6± 0.4	38.6±1.3	55.3± 0.7	31.3± 0.6	43.3±1.3
	SPK004	46.7± 1.4	27.7± 1.7	37.1±1.1	53.0± 1.7	36 ± 0.0	44.5±0.9
	Kemb 10	58.3± 1.2	38.3± 0.2	48.3±1.1	67.7± 3.3	45 ±2.0	56.3±1.4
Means		49.5 a	29.4 b		56.9 a	36.2 b	
LSD (5%)		1.79			0.57		
CV (%)		6.7			22.8		

CI and SE denote callus induction and somatic embryogenesis.

Data shown represents mean ± standard error (S.E). Means within a column are not significantly ($P \leq 0.05$) different based on Least Significant Differences (LSD)

With the transfer of embryogenic callus to culture media free of 2,4-D but supplemented with 2ml L^{-1} ABA embryos began to form clearly on the surfaces of calli. These embryos turned green against the surface of calli (Plate 4), confirming the role of ABA in enhancing maturation of the somatic embryos before shoots began to appear, an observation similar with findings of Bencheikh and Gallais (1996) studying variation in embryogenesis of Pea lines. For this study, periodic observations established that embryo appearance was directly on the surface or centre of leaf primordial where calli had been formed (Plate 5) indicating differences in the site of initiation of callus.

Significant differences in cultivar response to regeneration method on mean number of days taken to form shoots were observed (Table 4). In cv. *Mugande* the number of days taken to form shoots increased from 35 with somatic embryogenesis method to 56 days when the callus induction method was used. *Kemb10* followed with 50 days with callus induction and 26 days with somatic embryogenesis. With callus induction method the cv. *J. tresmesino* took the overall least time to produce shoots (43 days) while with somatic embryogenesis *SPK004* took the shortest time of 25 days. The mean number of days taken to form shoots were longest with *Kemb10* (44) and shortest with *Zapallo* (34) followed by *SPK004* (38) (Table 4). The results indicated no significant ($P \leq 0.01$) difference in the number of days taken to form shoots through callus induction method among the cv. *Zapallo*, *SPK004*, *Kemb10* and *J. tresmesino* but were significant with *Mugande* which took the longest mean time to form shoots at all 2,4-D levels.

The regenerated shoots from the test cultivars had similar phenotypic characteristics as mature plants. For example, the regenerated shoots of *Zapallo* was observed as having a purple stem (Plate 6), while the regenerated *Mugande* had broad leaves (Plate 7) an observation in agreement with findings of Taylor *et al.* (2001), who reported similarities in the nature of regenerated plants to their parent. Low 2,4-D levels supported production of secondary embryogenic calli, which promoted organogenesis therefore enhancing shoot growth. This finding was reinforced by Liu and Cantilfee, (1986) who reported that low 2,4-D levels (< 5 mg L^{-1}) promoted shoot formation within a short period of time. As the shoots developed a thickening wall began to gradually form around the shoot (Plate 8), which was followed by progressive elongation of the shoot. Further shoot development resulted in the gradual regeneration of leaves (Plate 9).

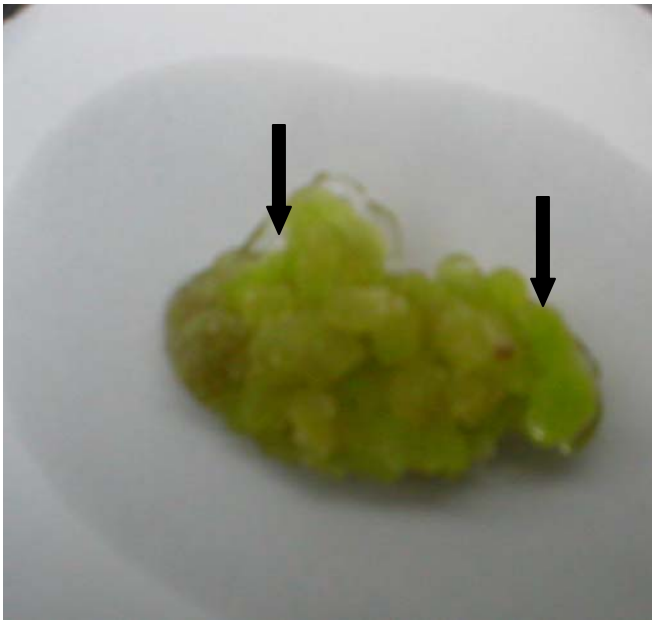


Plate 4: Embryos begin to form on the surfaces of leaf explants from sweet potato cultivar *SPK004* cultured on media supplemented with 2ml L^{-1} ABA and no 2,4-D.

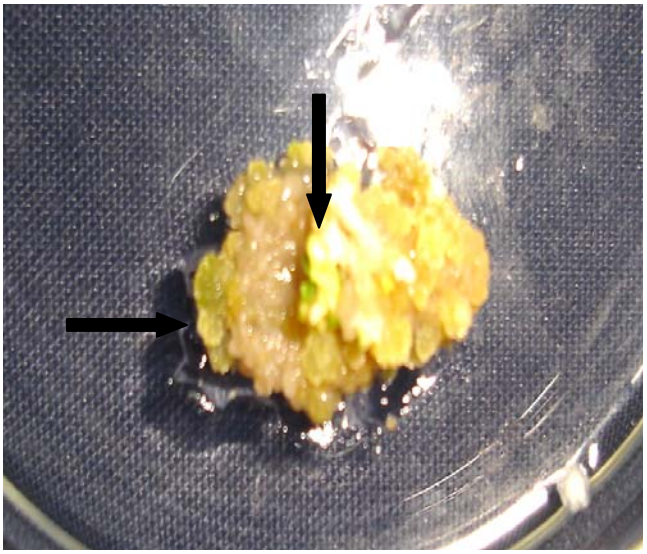


Plate 5: Embryos begin to form at the centre of embryogenic calli of leaf explants from sweet potato cultivar *Zapallo* cultured on media supplemented with 2ml L^{-1} ABA and no 2,4-D.

Plate 6: Shoots regenerated from the cultivar *Zapallo* showing purple stem colouration and narrow leaves characteristic of a mature plantlet.

Plate 7: Shoots regenerated from the cultivar *Mugande* showing green stem colouration and broad leaves characteristic of a mature plantlet.

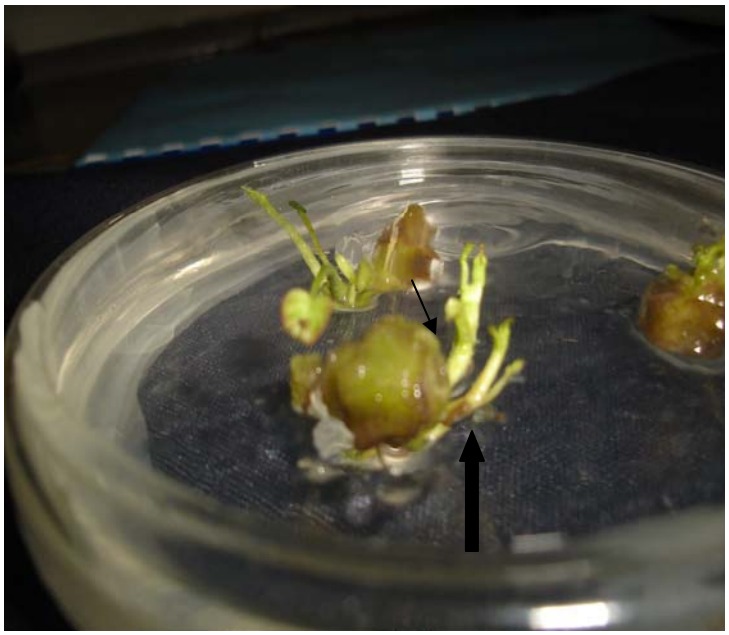


Plate 8: Somatic embryos from the sweet potato cultivar *Kemb 10*, five weeks after culture in ½ strength MS medium and 1ml L^{-1} BAP, begin to develop shoots.

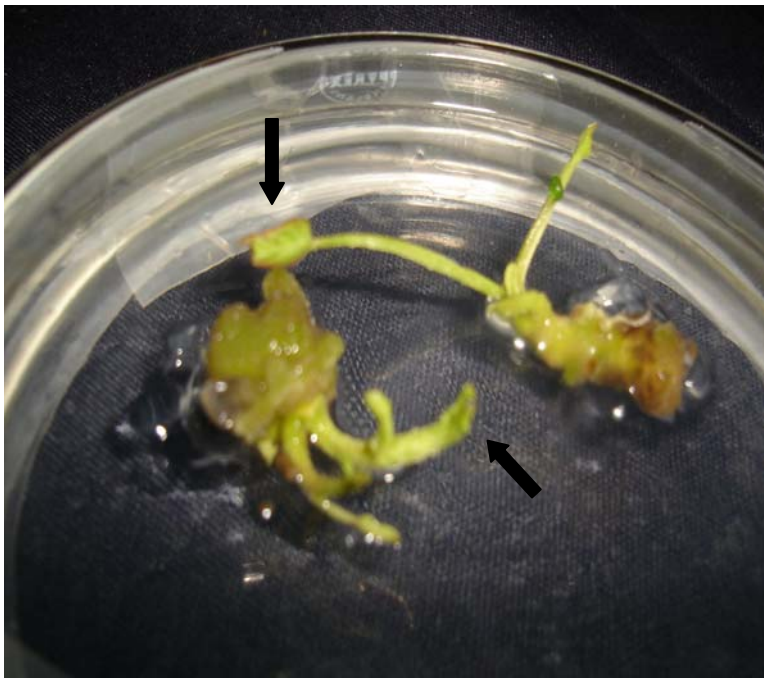


Plate 9: Shoots from the sweet potato cultivar *Kemb 10* increase in length and leaves begin to develop seven weeks after culture in ½ strength MS medium and 1ml L^{-1} BAP.

4.2.2 Number of leaves formed per plant

The growth of leaves from somatic embryos of sweet potato explants cultured *in vitro* was highly dependant on the three way interaction between the test cultivar, 2,4-D level and regeneration method (Appendix 5). Generally, regeneration by callus induction method resulted in higher mean number of leaves (3.9) than somatic embryogenesis (3.5) (Table 5). Using callus induction method at 0 mg L^{-1} 2,4-D resulted in the highest number of leaves with the cultivar *Zapallo* (6.0) followed by *SPK004* (5.3).

However, at 0.5 mg L^{-1} 2,4-D cultivar *SPK004* recorded the highest number of leaves (5.6) with callus induction method while with somatic embryogenesis *Mugande* developed the highest number of leaves (5) at 0.5 mg L^{-1} 2,4-D (Table 5). These results contradicted the hypothesis that *in vitro* regeneration method was independent of genotype as the mean number of leaves that were regenerated per plant varied significantly with the cultivar. Using 1.0 mg L^{-1} 2,4-D reduced the number of leaves across all treatments but reducing these level to 0.5 mg L^{-1} 2,4-D developed the highest number of leaves per plant (5.7) (Table 5). It could be that increasing the 2,4-D was inhibitory to leaf formation a result similarly observed in cowpea by Amitha and Reddy (1998).

The lowest mean number of leaves (2) was recorded with cultivar *J. tresmesino* at 1.0 mg L^{-1} 2,4-D using the callus-induction regeneration method at 1.0 mg L^{-1} 2,4-D with somatic embryogenesis method. This was followed by *Kemb10* with a mean of 2.3 and 3 leaves per plant with the callus and somatic embryogenesis method respectively at 1.0 mg L^{-1} 2,4-D. The high production of leaves with callus induction method was probably because somatic embryos arose from a large mass of disorganized and undifferentiated cells, unlike with somatic embryogenesis where the cells were already organized to develop into leaves or shoots, as suggested by Merkle *et al*. (1995). When the disorganized cells differentiated into organized cells they may have differentiated into more shoots, which subsequently increased the mean number of leaves that formed per plant.

Table 5: Interaction effect between the sweet potato cultivars, regeneration method and 2,4-D concentration on the mean number of leaves formed per plant, grown *in vitro* at KARI-Njoro in 2006.

	Cultivars										
2,4-D	Mugande		Kemb10		Zapallo		SPK004		J. tresmesino		
mg L^{-1}	CI	SE	CI	SE	CI	SE	C1	SE	CI	SE	Mean
0.0	3.0 ab	4.0 ab	2.0 b	3.0 ab	6.0 a	4.0 a	5.0 ab	3.0 b	4.0 a	3.0 b	3.83 b
0.5	4.0 a	5.0 a	3.0 a	4.0 a	5.0 ab	4.0a	6.0 a	4.0 a	4.0 a	4.0 a	4.3 a
1.0	4.0 a	2.0 c	3.0 a	2.0 c	4.0 c	4.0 a	3.0 c	3.0 b	2.0 b	3.0 b	3.03 c
LSD (%)	0.54										
CV (%)	21.7										

CI and SE denote callus induction and somatic embryogenesis respectively.

Data shown represents mean $\pm$ standard error (S.E). Means within a column followed by the same letter are not significantly ($P \leq 0.05$) different based on Least Significant Differences (LSD)

4.2.3 Days taken for roots to form

Significant ($P \leq 0.01$) interaction between the cultivar and 2,4-D concentration and cultivar and regeneration method were established for mean number of days taken to form roots (Appendix 5). From the results the number of days taken to form roots ranged from 42 – 51 days with cultivar *Mugande* and the longest time taken to form roots was 50.3, 48.7 and 55 days at 0, 0.5 and 1.0 mg L^{-1} 2,4-D, respectively (Table 4). For most treatments the shortest time taken to form roots was attained with 0.5 mg L^{-1} 2,4-D. The mean number of days taken to form roots increased by almost six days when the medium was without 2,4-D (Table 4) and the type of roots produced were adventitious and appeared at the base of the developing shoots (Plate 7). The results also showed significant ($P \leq 0.05$) interaction between test cultivars and regeneration methods for the number of days taken to form roots (Figure 4).

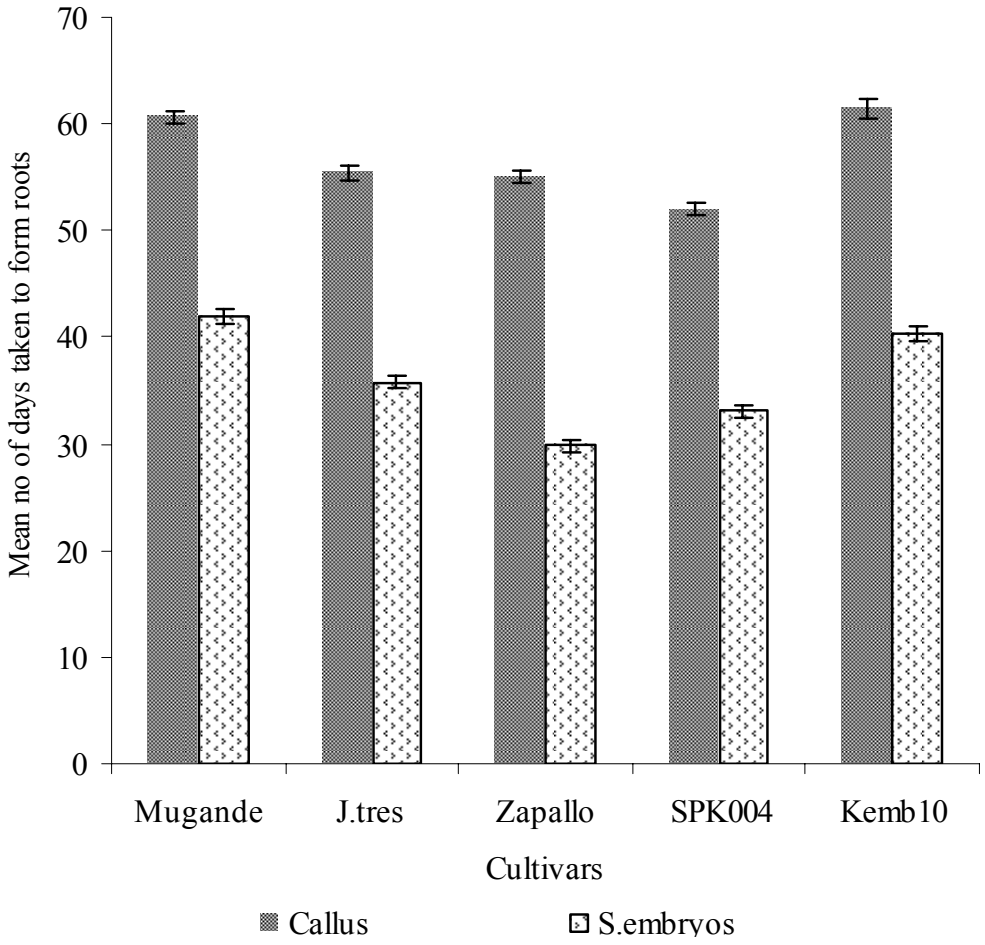


Figure 4: Interaction effect of cultivar and regeneration method on mean number of days taken to form roots in sweet potato grown *in vitro* at KARI-Njoro.

Plant regeneration through somatic embryogenesis method took fewer days to form roots. The cultivar *Zapallo* took the shortest time (27 days) while *Mugande* took the longest time to form roots with somatic embryogenesis method (42 days) (Figure 4). In initiation of plants from callus, *Kemb10* took the longest period of time (67 days) while *SPK004* took the shortest period of time (51 days) (Figure 4). These confirmed genotypic influences on regeneration method for mean number of days taken to form roots. Rolando *et al*. (1999) reported that root emergence was obtained after a long frequency on plant growth regulators. This was owed to the endogenous growth regulator's effect on root emergence from the local sweet potato cultivars and the genotypic differences related to the different sensitivities to 2,4-D by the tested cultivars, as indicated by Nguyen *et al.* (1997).

4.2.4 Number of roots formed per plant

A significant ($P \leq 0.001$) three way interaction between the sweet potato cultivars, 2,4-D concentration and regeneration method was revealed for the mean number of roots formed per plant (Appendix 5). The highest number of roots formed with *SPK004* at 0.5 and 1.0 mg L^{-1} 2,4-D (4.7) using callus induction method, followed by *Zapallo* at 0.5 mg L^{-1} 2,4-D (4.7) (Figure 5). With somatic embryogenesis the highest number of roots was with *Kemb 10* which developed a mean of 8 and 6 roots at 0.5 and 0 mg L^{-1} 2,4-D level, followed by *SPK004* with 4 roots per plant. Likewise, using the concentration 1.0 mg L^{-1} 2,4-D reduced the number of roots per plant across all treatments with the exception of *Mugande* (Figure 5), thereby confirming cultivar differences in response to 2,4-D levels.

Cultivars *SPK004* and *J. tresmesino* revealed no significant ($P \leq 0.05$) differences in the regeneration methods for number of roots formed (Figure 5). Plant regeneration was reported to be genotype specific by Nguyen *et al*. (1997), hence there was a threshold level of 2,4-D at which plants were able to initiate shoots and roots. Similarly, depending on the species being regenerated, if the level of auxin and cytokinins were in correct amounts, shoots and roots developed rapidly, as reported by Chrispeels and Sadava (2003).

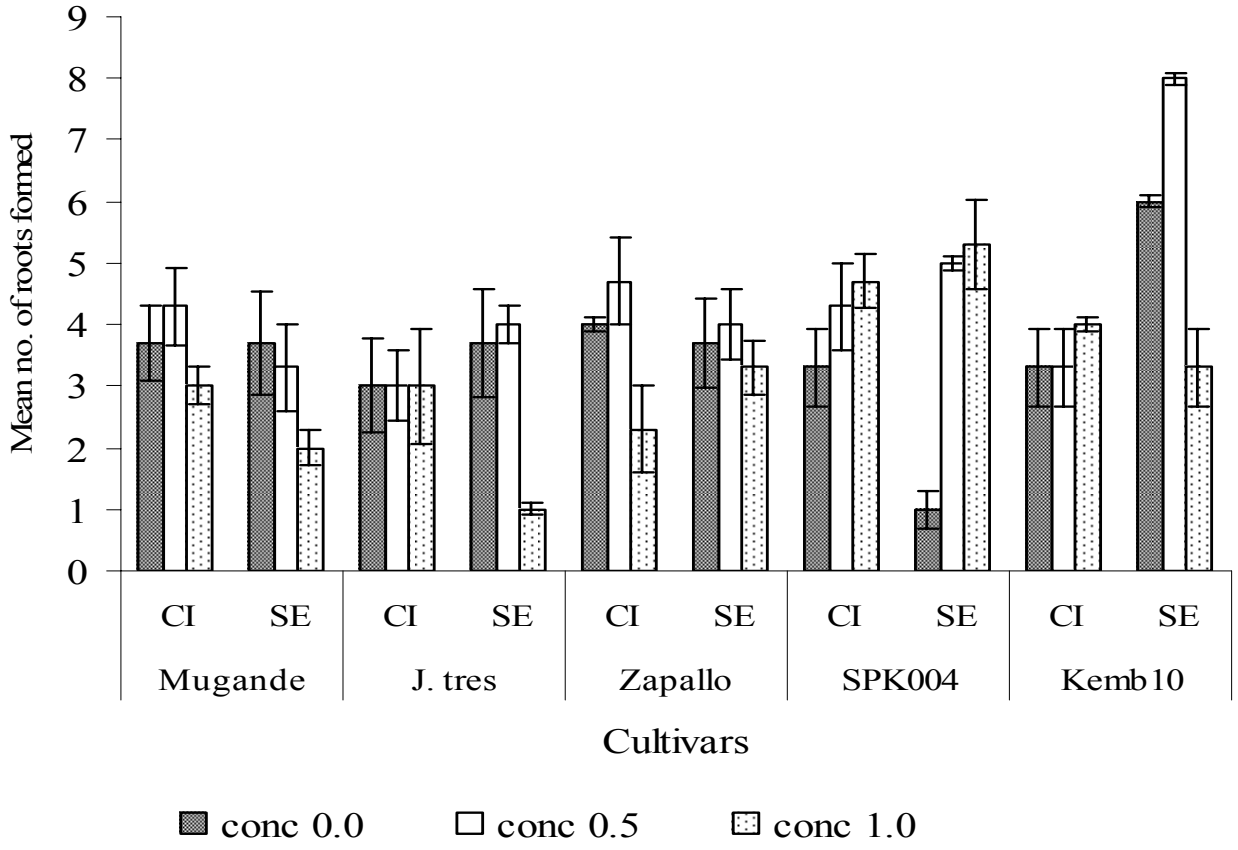


Figure 5: Interaction effect between the sweet potato cultivars, regeneration method and 2,4-D concentration on the mean number of roots formed per plant, grown *in vitro* at KARI-Njoro

CI and SE denote callus induction and somatic embryogenesis respectively.

4.2.5 Number of plants regenerated

In this study, the 2,4-D levels were important in influencing overall plant regeneration. Significant ($P \leq 0.001$) three way interaction between 2,4-D concentration, cultivar and regeneration method was established (Appendix 5). The number of plants regenerated was highest with the callus induction method when no 2,4-D was added to the medium which resulted in 12 plants with cultivar *SPK004*, followed by when 0.5 mg L^{-1} 2,4-D was added resulting in 9 plants of the same cultivar. *Zapallo* followed, regenerating 6.3, 7.3 and 7 plants at 0, 0.5 and 1.0 mg L^{-1} 2,4-D with the callus induction method. The number of plants regenerated per cultivar decreased at 1.0 mg L^{-1} 2,4-D (Figure 6).

Previous results, during 2,4-D optimization for embryogenic calli induction, established that the quality, quantity and frequency of cell embryogenic induction affected the ability to regenerate plants. This was because decreasing levels of 2,4-D in culture medium led to increased plant regeneration as plant cell division and development of embryogenic tissue being generated also increased (Prakash and Varadajaran, 1992). The process of somatic embryogenesis was enhanced when reduced time of exposure to auxins was allowed during culture (Finer, 1994). In addition, high 2,4-D levels have been reported to affect many biochemical processes in plants though it is still not clear which of the biochemical alterations 2,4-D as a herbicide causes that were ultimately responsible for weakening plants during *in vitro* culture (Tu, 2001).

Cultivars *Mugande* and *SPK004* responded best when the regeneration method was by callus induction, while *J. tresmesino*, *Kemb10* and *Zapallo* produced more plants with somatic embryogenesis. Plant regeneration varied among the local cultivars and the regeneration method (Carswells and Locy, 1984). With somatic embryogenesis method highest plant regeneration was recorded at 0.5 followed by 0.0 and lastly 1.0 mg L^{-1} 2,4-D (10, 9 and 8) of *Zapallo*. The lowest number of plant regenerated was of cultivar *Mugande* (3, 1 and 2). This could be because with callus induction method plant regeneration increased due to remaining meristematic and differentiating tissues from cultured leaf explants that continued developing into normal plants under the influence of growth regulators and culture conditions (Rolando *et al*., 1999). Newly formed plants, showing normal and similar phenotypic characteristics were separated from the medium, grown on sterile soil before transferring to the greenhouse.

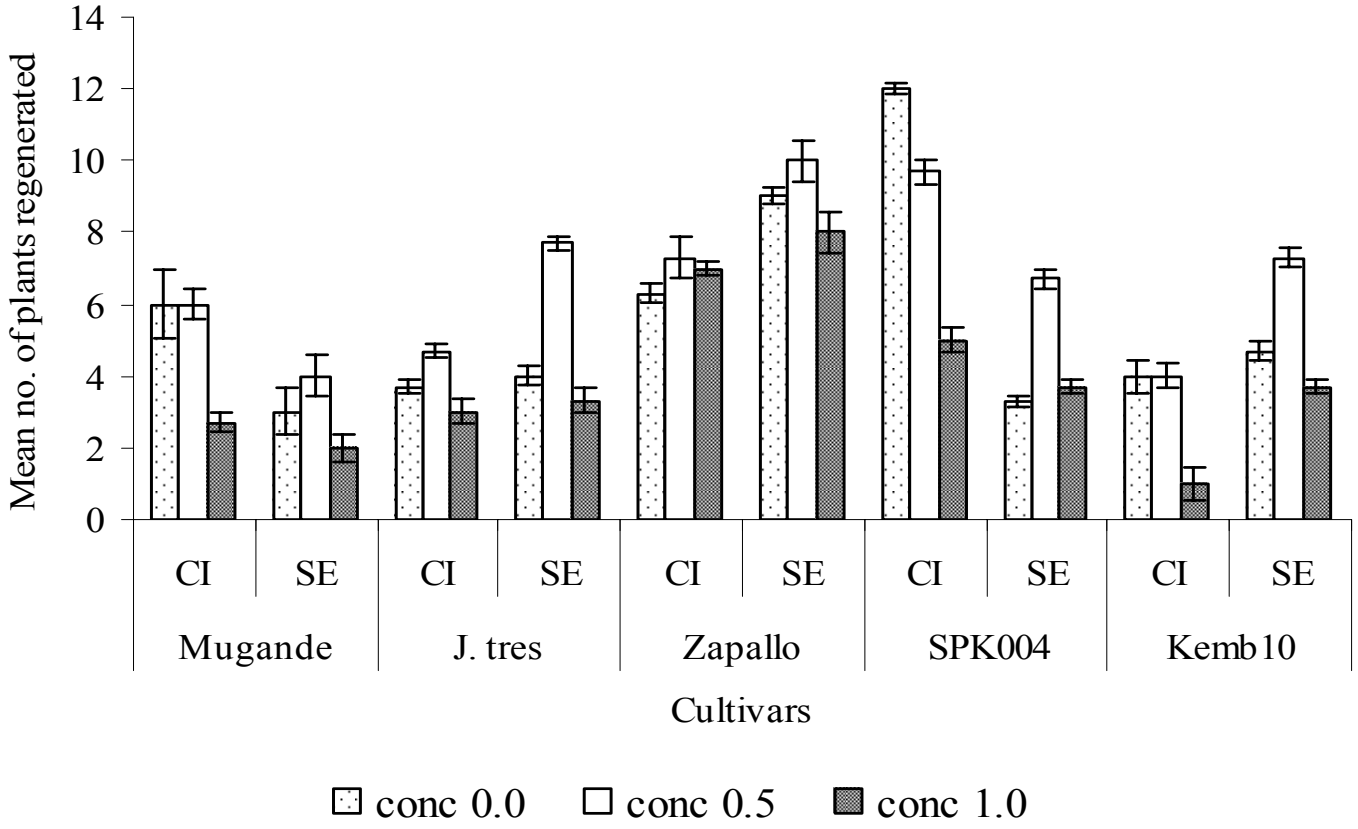


Figure 6: Interaction effect between the sweet potato cultivar, regeneration method and 2,4-D concentration on the mean number of plants regenerated *in vitro* at KARI-Njoro.

CI and SE denote callus induction and somatic embryogenesis respectively.

4.2.6 Correlation

The results indicated that there was a strong negative correlation between 2,4-D levels and the number of leaves that were formed or the number of plants regenerated. The correlation was not significant with the number of roots that were formed (Table 6). This implied that increasing the 2,4-D levels in the culture medium led to a reduced number of leaves that were produced as well as the number of plants that ended up being regenerated. Prasetsongskun (2003) reported that plant regeneration in tissue culture depended on the embryogenic callus type. The plant regeneration tendencies varied significantly with the regeneration method used and sweet potato cultivar agreeing with findings of Chee *et al*. (1990). Each cultivar varied widely in response to 2,4-D levels supplemented in the tissue culture *in vitro* plant regeneration medium.

Table 6: Correlation among 2,4-D level on days taken for shoots to form, leaves formed, days taken to form roots, number of roots formed and plants regenerated in sweet potato plants regenerated *in vitro* at KARI-Njoro.

	2,4-D levels (mg L^{-1})	Days to form shoots (DSF)	Leaves formed	Days taken to form roots (DRF)	Number of roots formed	Plants regenerated (PR)
2,4-D level	1.0					
DSF	0.098	1.0				
Leaves formed	-0.27 *****	0.08	1.0			
DRF	0.14	0.95	-0.007*****	1.0		
Number of roots formed	-0.08	-0.03*****	0.23	-0.06	1.0	
PR	-0.22 *****	-0.182	0.5	0.3	0.229	1.0

***** correlation significant at $P \leq 0.05$

There was however a strong positive correlation between 2,4-D levels and the mean number of days taken to form shoots and days taken to form roots. This implied that increasing the 2,4-D levels led to a significant increase in the number of days taken to form shoots. Nguyen *et al*. (1997) reported that great variability in genotype (cultivars) depended on the different sensitivities to the endogenous levels of 2,4-D. Rolando *et al*. (1999) observed that shoot and root regeneration took a longer time at higher 2,4-D levels. In this study it was observed that root emission and sporadic shoot formation were obtained, but at low frequency of the plant growth regulators.

The results of this study showed that the impact of the regeneration method and 2,4-D concentration in the medium largely influenced the responses of the genotype in regenerating plants. The results indicated that the levels of 2,4-D concentration affected the number of plants that were regenerated and hence sufficient 2,4-D levels had to be provided when using the two regeneration methods to promote somatic embryo formation and subsequent plant regeneration. Higher concentrations of 2,4-D resulted in low plant shooting, rooting, leafing and mean number of plants regenerated per cultivar. Sweet potato embryos began to differentiate into shoots and when hormone levels were reduced with time plants were regenerated. Hormonal concentrations could alter embryo formation and subsequently reduce overall plants regenerated per cultivar.

In the current study plant regeneration in sweet potato was highest in the cultivars *Zapallo, SPK004* and *Kemb10* respectively, at 0.5 mg L^{-1} 2,4-D level with callus induction regeneration method. These results showed that using 0.5 mg L^{-1} 2,4-D led to a decrease in the mean number of days taken to form shoots and roots and hence an increase in leaf and root formation. It was noted that 2,4-D had a great role in media for sweet potato plant regeneration. It influenced the rate of cell division and hence affected the success of morphogenesis of tissues and subsequent increased development of regenerated plants. It was important to select a protocol that had a higher plant conversion in order to increase the likelihood of getting higher transformed plants.

4.3 Effect of Cultivar and Regeneration Methods on Growth and Yield of 5 Local Sweet Potato Cultivars Grown Under Field Conditions

4.3.1 Hardening of regenerated plants and their survival *ex vitro*

The hardening process was first done *in vitro* by reducing by half the MSBM, sucrose and myo-inositol amounts as described by Taji *et al. (*1992). This was followed by gradually lowering the relative humidity by increasing the duration under light conditions in the growth chamber for two weeks after which plants were transferred to the greenhouse. *In vitro* regenerated plants were similar to the mature parent plants in the current study. Only a few abnormalities were identified by an altered mesophyll structure and mostly caused aborted and expanded embryos, as reported by Chee and Cantilfee (1988). The abnormal plants however did not survive to whole plants.

The highest plant survival of sweet potato planting material under the greenhouse study was recorded with *Zapallo*, by plants from conventionally propagated material (88%) followed by materials regenerated by somatic embryogenesis (76%). The lowest plant survival was with cv. *J. tresmesino* using regenerated planting material (Figure 7). Across all cultivars the highest plant survival was recorded with conventional propagation method. The reduced plant survival obtained using regenerated planting material meant that regenerated sweet potato plants were extremely sensitive to acclimatization *ex vitro,* especially temperature, soil and light conditions.

From earlier reports this performance was expected as regenerated plants had a very delicate shoot and root system, as reported by Lyndsey and Alderson (1986) hence resulted in greater *ex vitro* plant mortality. In addition, tissue culture regenerated plants were very small at the time of transfer to the soil, unlike conventional stem cuttings, which were larger in size and obtained from already growing sweet potato plants. Stem cuttings established faster as they already contained energy reserve, which enable rapid and sustained growth. Within the first week of transfer to the greenhouse all plants from each treatment were covered with transparent polythene bags (Plate 10a) to reduce substantial moisture loss and prevent desiccation (Dodds and Robert, 1995). The transparent bags were removed (Plate 10b to f) and plants that survived were transferred for further growth and tuber initiation to the field after 28 days (Plate 11) stay in the greenhouse.

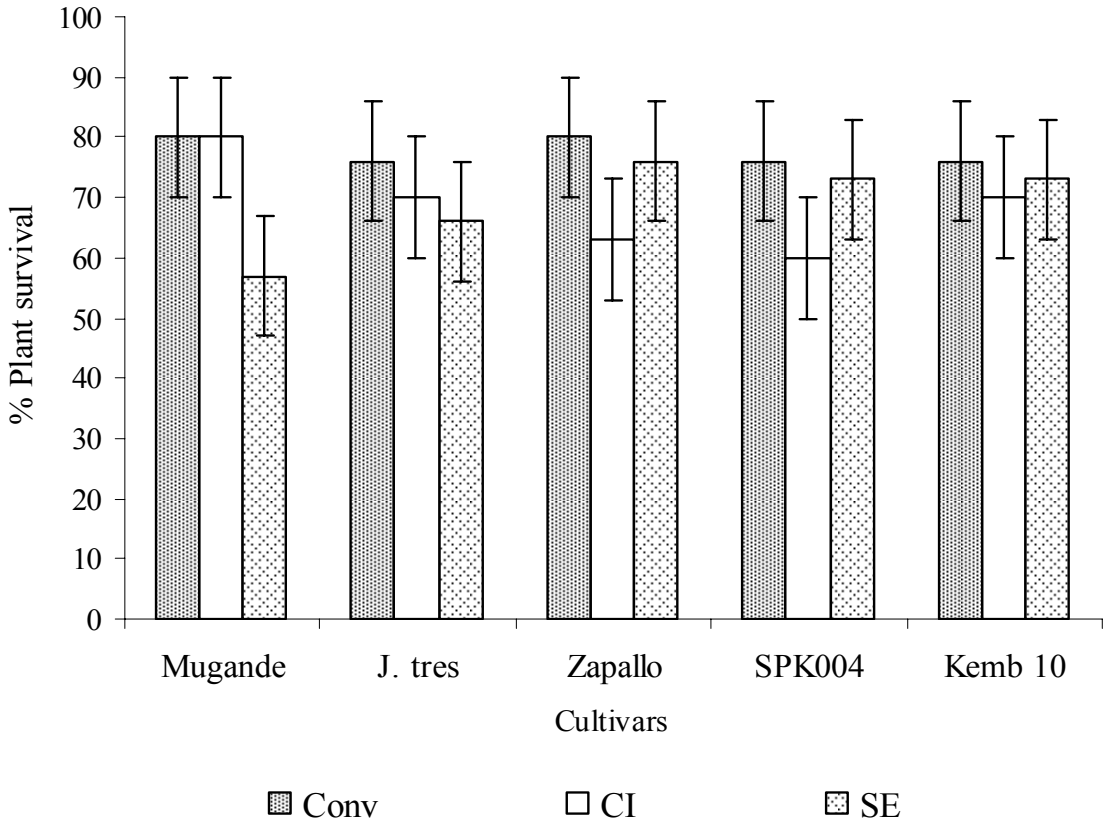


Figure 7: Plant survival (%) comparison between tissue culture regenerated plants and conventional propagated material grown under greenhouse conditions at KARI-Njoro.

Conv, CI, SE denotes conventionally propagated, callus induction and somatic embryogenesis sweet potato planting materials.

a) Regenerated and conventional sweet potato plantlets covered in transparent bags 7 days after hardening *ex vitro*

b) ***Mugande***. Broad leafed, dark green stem/petiole

c) ***Zapallo.*** Parted leaves, purple stem/petioles

d) ***SPK004.*** Shouldered leaf shape, green stem/petiole

e) ***Kemb 10.*** Parted leaves, green stem/petiole

f) ***J. tresmesino.*** Narrow leaves purple stem/petioles

Plate 10: Sweet potato cultivars begin to sprout 28 days after acclimatization *ex vitro* in the greenhouse at KARI-Njoro in 2006

Plate 11: Sweet potato cultivars at 68 days after transplanting to the field at KARI-Njoro in 2006.

4.3.2 Growth parameters

4.3.2.1 Plant stand count

The analysis of variance established significant ($P \leq 0.01$) three way interactions between the cultivar, method of propagation used and plant growth after transplanting for plant stand count (Appendix 6). The conventional propagation method had the highest plant stand count under field conditions (99.4%) followed by propagules obtained from callus induction (96.5%) and lastly propagules from somatic embryogenesis method (96.6%) (Figure 8). High stand count with conventional method could be due to the fact that propagation was by stem cuttings, which were already larger in size and had energy reserve that made them have a rapid sustained growth (CVPG, 2003).

There was a general stable stand count after 28 days of transplanting plantlets to the field (Plate 11). This was because the transplanted plants had by then acclimatized to the field soil and moisture conditions, as well as aerial conditions, therefore allowing rapid growth. Comparison among the three propagation methods revealed that lowest plant mortality was attained with conventional propagation method, followed by callus induction method and lastly by somatic embryogenesis method. However, no significant difference between the two tissue culture regeneration methods. With conventional propagation method higher stand counts were attained by *Mugande, Zapallo* and *SPK004* in that order, while the lowest stand count was attained with cultivar *J. tresmesino* followed by *Kemb10*. With callus induction plantlets, stand count was highest with *Mugande* followed by *Kemb10* and the lowest stand count was with *SPK004*. Plants regenerated from somatic embryogenesis gave lowest stand count with the cultivars *Mugande* and *J. tresmesino* (Figure 8).

The results confirmed differences in genotypic responses to the propagation method used for stand count. The decreased plant survival recorded with tissue culture regeneration methods was established by Lineberger (2006) that tissue culture plants were sensitive to environmental conditions which made young plant establishment slower in the face of intense competition for water, minerals, light and physical conditions of the soil.

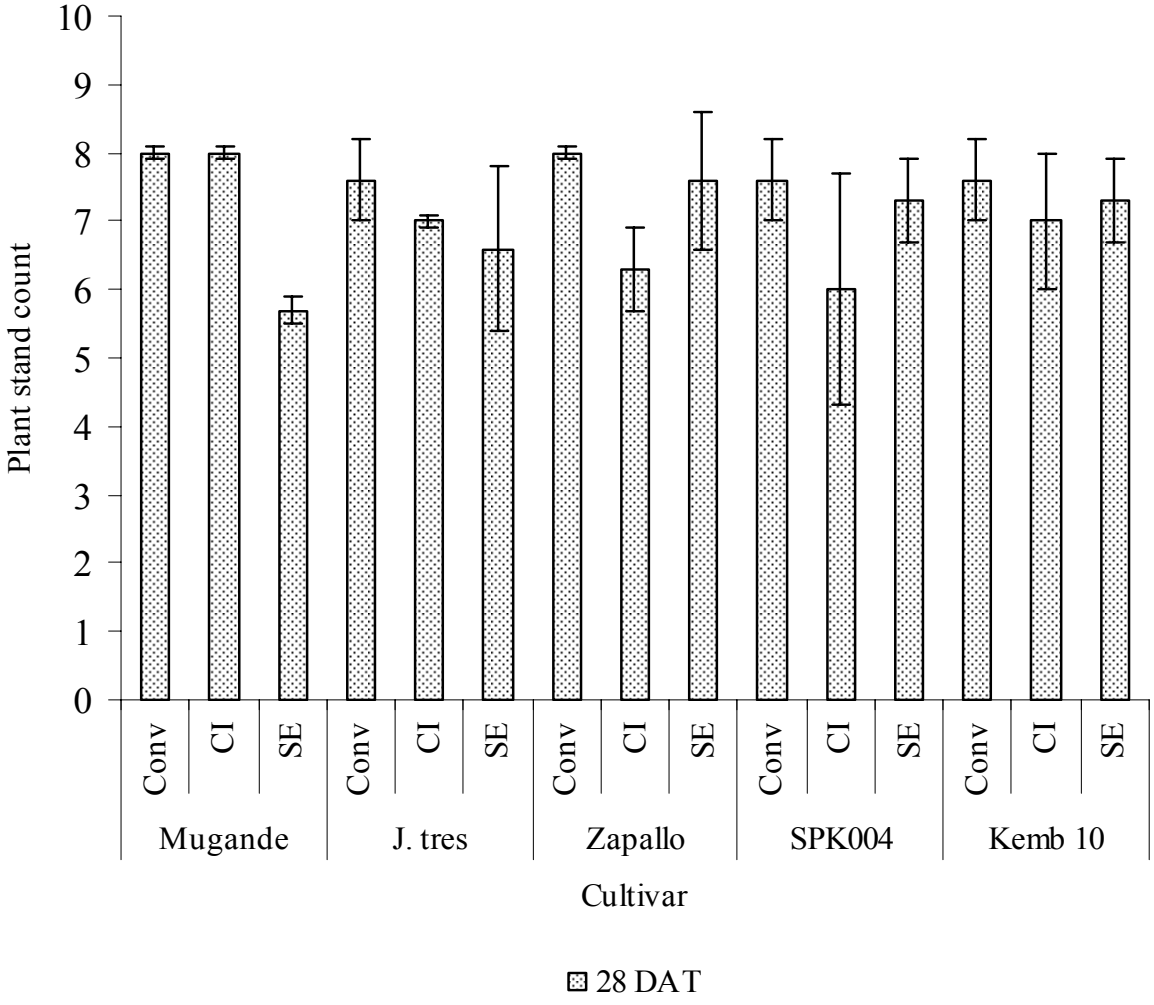


Figure 8: Effect of cultivar and regeneration method at 28 days after transplanting on plant stand count of sweet potato grown under field conditions at KARI-Njoro.

Conv, CI, SE denotes conventionally propagated, callus induction and somatic embryogenesis sweet potato planting materials.

4.3.2.2 Plant height

Significant ($P \leq 0.01$) three way interactions between test cultivars, regeneration method and plant height after transplanting was established (Appendix 6). Cultivar *SPK004* had the tallest plants (70.6 cm), followed by *J. tresmesino* (44.2 cm), *Zapallo* (38.4 cm), *Kemb10* (37.9 cm) and finally *Mugande* (28.9 cm) (Figure 9). Plant height increased among the cultivars with increase in number of days after transplanting and shortest plants were recorded at 28, 42, 56, and 150 respectively. High plant heights recorded at harvest time were expected because these measurements were taken after a longer period of time as compared to that at the other three stages of development, as reported by Ali *et al.* (2003). The low plant height in *Mugande* was due to morphological differences because this cultivar, unlike all the other cultivars, had no creeping stems but rather grew upright, as genetically determined.

Highest plant heights were attained at harvest time with conventional propagated sweet potatoes which tended to have taller plant heights except for *SPK004* where the greatest plant height was recorded with somatic embryogenesis regeneration method. Conventional plants rooted earlier, acclimatized faster to the external environment and hence had faster growth as compared to propagules from tissue culture regenerated plants (Lineberger, 2006). Using callus induction propagules resulted in shorter plants, with the exception of *Mugande*. However of the two methods, propagules from somatic embryogenesis regeneration method gave the tallest plants compared to the callus induction method. This could be that plants regenerated by callus induction method were more sensitive to the physical environment and therefore the propagules adapted poorly to field conditions. Increased plant height meant higher growth vigour, resulting in earlier maturity as suggested by similar Munga *et al*. (2000).

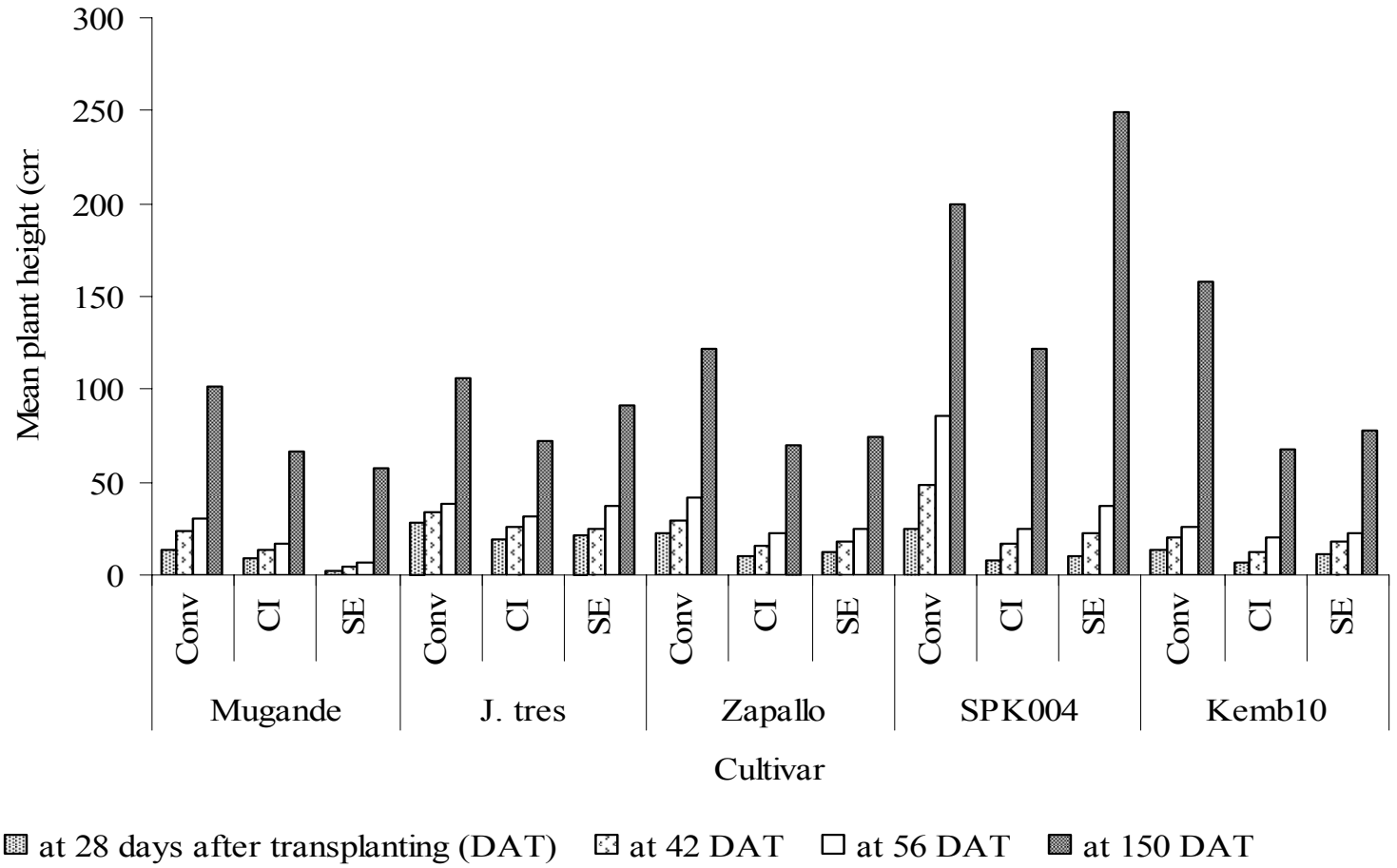


Figure 9: Effect of cultivar, regeneration method and growth days after transplanting on plant height of sweet potato grown at KARI-Njoro in 2006.

Conv, CI and SE denote conventionally propagated, callus induction ad somatic embryogenesis planting materials.

4.3.2.3 Number of branches

Significant ($P \leq 0.05$) interaction was revealed between cultivar and method as well as cultivar and growth after transplanting for number of branches (Appendix 6). Overall, the highest branching was recorded with the cultivar *Mugande* at 28, 42, 56 and 150 days after transplanting followed by *SPK004* and *Kemb10* (Table 7). The high branching recorded at harvest time was also expected as the measurements were taken when the plants had been acclimatized to environmental conditions after a longer period of time (150 days) and after completion of all developmental stages unlike at 28 days when the plants were still sensitive and fragile.

Table 7: Effect of cultivar and plant growth in days after transplanting on mean number of branches formed in sweet potato grown under field conditions at KARI-Njoro.

		Cultivars					
Source	Variables	Mugande	J. tres	Zapallo	SPK004	Kemb 10	Mean
Plant growth days after transplanting (DAT)	28 DAT	2.8 c	1.1 c	2.0 b	1.8 b	1.7 c	1.9 d
	42 DAT	4.6 b	1.9 bc	2.4 b	2.6 b	3.3 c	2.9 c
	56 DAT	7.6 a	3.4 b	3.2 b	8.1 a	5.8 b	5.6 b
	150 DAT	9.2 a	8.3 a	9.1 a	8.0 a	9.0 a	8.7 a
	Mean	6.0 a	3.7 c	4.2 bc	5.1 b	4.9 b	
	CV (%)	39.4					
	LSD (5%)	0.68					

Means followed by the same letter within a column are not significantly ($P \leq 0.05$) different based on Least Significant Differences (LSD).

The lowest overall number of branches was recorded with the cultivar *J. tresmesino* followed by *Zapallo* (Table 8) but using different propagation methods. The highest number of branches was attained with *Mugande* and *SPK004* at harvest time followed by at 56, 42 and least at 28 days after transplanting due to the gradual increase in number of branches as the sweet potato plants developed.

Table 8: **Effect of cultivar and propagation method on mean number of branches formed in sweet potato grown under field conditions at KARI-Njoro.**

Source	Variables	Cultivars					Mean
		Mugande	J. tres	Zapallo	SPK004	Kemb 10	
Propagation method	Conv	7.0 a	4.0 a	5.0 a	6.8 a	4.9 a	5.6 a
	Callus	7.1 a	2.8 b	4.4 ab	4.5 b	4.6 a	4.7 b
	SE	4.0 b	4.3 a	3.2 b	4.0 b	5.3 a	4.2 b
	Mean	6.0 a	3.7 c	4.2 bc	5.1 b	4.9 b	
	CV (%)	39.4					
	LSD (5%)	0.68					

Means followed by the same letter within a column are not significantly ($P \leq 0.05$) different based on Least Significant Differences (LSD). CI and SE denote callus induction and somatic embryogenesis respectively.

In general, the conventional planting materials gave the highest mean number of branches followed by the callus induction and somatic embryogenesis methods which were not significantly different from each other. Tissue culture plants have been reported to have a higher branching habit in studies by Lineberger (2006); however, the results of the current study contrasted the finding as tissue culture sweet potato plants formed fewer branches than those propagated by conventional stem cuttings.

4.3.2.4 Leaf area

Conventional grown sweet potato plants attained a mean leaf area of 72.9 cm^2 over the four growth durations after transplanting, in contrast to propagules generated through somatic embryogenesis (68.5 cm^2) and callus induction method (68.8 cm^2), although the differences in leaf area among the tissue culture regenerated propagules were not significant (Table 9). The significant interaction between test cultivars and regeneration method was due to the response of *Kemb 10* in which callus induction method produced significantly lower leaf area. Leaf area increased slightly with conventional methods as opposed to tissue culture-regenerated plants, as the later plants may have been sensitive to changes in the environmental conditions at early stages of growth but after 28 days they responded just as the conventionally propagated

propagules did. With conventional propagation, *Mugande* had the largest leaf area (92.2 cm^2), followed by *Kemb 10* (76.7 cm^2) while the lowest leaf area was with *Zapallo* (64.1cm^2).

Table 9: Effect of cultivar, propagation method on mean leaf area (cm^2) formed in sweet potatoes grown under field conditions at KARI-Njoro in 2006.

Source	Variables	Cultivars					Mean
		Mugande	J. tres	Zapallo	SPK004	Kemb 10	
Propagation method	Conv	92.2 a	65.7 a	64.1 a	65.1 a	76.7 a	72.9 a
	Callus	90.5 a	64.8 a	69.3 a	57.2 a	62.1 b	68.8 a
	SE	75.8 b	64.2 a	57.9 a	65.1 a	79.7 a	68.5 a
	CV (%)	5.34					
	LSD (5%)	21.1					

Means followed by the same letter within a column are not significantly ($P \leq 0.05$) different based on Least Significant Differences (LSD).
CI and SE denote callus induction and somatic embryogenesis respectively.

In propagation using callus induction, leaf area was highest with *Mugande* (90.5 cm^2), followed by *Zapallo* (69.3 cm^2). Propagation using somatic embryogenesis favoured *Kemb 10* (79.7 cm^2), followed by *Mugande* (75.8 cm^2), while the lowest leaf area was with *Zapallo* (57.9 cm^2) (Table 10). The differences in leaf area were attributed to the initial differences in planting material in that with the use of stem cuttings root and shoot development were accelerated, resulting in mature plants being produced more rapidly under field conditions than with regenerated propagules as reported by Cameron *et al*. (2001).

From previous results in this study, plant stand count was highest with conventional propagation, which could have affected the leaf area display during the initial plant stand life especially since at this time the canopy closure had not yet been achieved (Chen and Black, 1992). Faster development of leaves meant a faster subsequent leaf area development. In addition, the leaf area was genotype dependent, which for this study a cultivar with broad leaves as *Mugande* meant leaf surface area was enhanced, thereby a larger leaf area was exposed to the sun hence light interception was higher than in narrow leafed genotypes (Annes, 2005).

Table 10: Effect of cultivar and growth in days after transplanting on mean leaf area (cm^2) formed in sweet potatoes grown under field conditions at KARI-Njoro in 2006.

Source	Variables	Cultivars					Mean
		Mugande	J. tres	Zapallo	SPK004	Kemb 10	
Growth days after transplanting	28 DAT	38.4 c	24.2 d	26.1 c	24.4 c	28.3 c	28.3 c
	42 DAT	80.3 b	73.8 b	66.1 b	68.0 b	79.6 b	73.6 b
	56 DAT	119.4 a	102.5 a	90.6 a	90.6 a	108.2 a	103.3 a
	150 DAT	106.6 a	59.2 c	72.2 b	72.2 b	75.2 b	75.1 b
	Mean	86.2 a	64.9 c	63.8 c	62.6 c	72.8 b	
	CV (%)	21.9					
	LSD (5%)	6.17					

Means followed by the same letter within a column are not significantly ($P \leq 0.05$) different based on Least Significant Differences (LSD).

Canola (2005) reported that the growth rate of a crop is closely related to the amount of solar radiation captured by the leaves and hence rapid leaf development also encouraged root growth, reduced soil moisture evaporation and the more dry matter that was eventually produced the higher the potential yield was. Maximum leaf canopy was achieved at 56 DAT (103 cm^2), followed by 150 DAT (75.1 cm^2) while lowest leaf area was obtained at 28 DAT (28.3 cm^2) (Table 10). This was probably because at 28 DAT, the propagules were young and still in the early stages of active growth and hence could have been exposed to competition for light, moisture and nutrients among each other which hindered leaf expansion.

The decline in leaf area at 150 DAT occurred across all treatments probably because the reproductive structure which in sweet potato is the tuber had been initiated. Ali *et al*. (2003) reported that despite vegetative growth being an important factor determining plant production in annual crops growth had to cease or slow down at the onset of reproductive activity such as flowering or tuberization. Similarly, mature leaves senesced to allow new leaves to be formed and the new leaves became stronger assimilate sinks, during tuber onset and filling. Annes (2005) reported that mature leaves cannot act like assimilate sinks, but must be self-sufficient and hence all leaves which are net consumers of assimilate eventually died off. Leaf area, leaf

number and leaf area index were expected to decrease as maximum leaf size had already been formed and this period was critical for the genesis of alternative growth of tuberous root types (Siegfried *et al*., 2004).

4.3.3 Yield parameters

4.3.3.1 Total tuber number

No significant ($P \leq 0.05$) interaction was established between test cultivars and the propagation method for unmarketable tuber (Appendix 7). The conventional propagation method gave higher number of tubers with *SPK004* and *Kemb10* respectively while higher tubers with tissue culture regenerated propagules were with cultivar *Mugande, Zapallo* with callus induction propagules, *Kemb10* and *Zapallo* with somatic embryogenesis propagules (Table 12). Munga *et al*. (2000) reported that tubers and subsequent yield varied among cultivars, therefore suggesting genotypic and environmental responses hence tubers depended on whether the cultivar in its environment was late maturing or early maturing. The results indicated no overall significant difference in marketable tubers between conventional and tissue culture propagation methods (Table 12).

Table 11: Effect of propagation method and cultivar on the mean number of tubers in sweet potato grown under field conditions at KARI-Njoro.

Propagation Method	Cultivars					Mean
	Mugande	J. tres	Zapallo	SPK004	Kemb10	
Conventional	10.3 ± 0.1a	5.7 ± 0.6 a	7.3 ± 0.6 b	14.7 ± 0.8 a	12.7 ± 0.6 a	10.1 a
CI	12.3 ± 0.7a	7.3 ± 0.5 a	12.7 ± 0.7 a	3.7 ± 0.2 c	6.3 ± 0.5 b	8.5 a
SE	7.3 ± 0.5 b	6.7 ± 0.6 a	11.7 ± 0.9 a	7.0 ± 0.6 b	9.7 ± 0.6 a	8.5 a
Mean	10.0 a	6.6 a	10.6 a	8.4 a	9.5 a	
CV (%)	51.3					
LSD (5%)	3.47					

Means followed by the same letter within a column are not significantly ($P \leq 0.05$) different based on Least Significant Differences (LSD).

CI and SE denote callus induction and somatic embryogenesis respectively.

4.4.3.2 Total marketable tuber weight

The test cultivars did not differ significantly from each other in terms of tuber weights (Appendix 7); however, significant interaction was established between test cultivar and propagation method. Conventionally propagated plants gave highest mean total tuber weight (9.6 tons/ha) for *Zapallo*, followed by *Mugande* (5.1 tons/ha), while lowest tuber weights were with *Kemb 10* (2.7 tons/ha) (Table 12). Of the two tissue culture regeneration methods propagules from callus-induced plants outperformed somatic embryogenesis method by having higher overall tuber weights with the cultivar *J. tresmesino* (6 tons/ha) followed by *Mugande* (4.9 tons/ha), while the lowest was with *SPK004* (1.6 tons/ha).

With somatic embryogenesis highest tuber weight for *Zapallo* (5.1 tons/ha) followed by *J. tresmesino* (4.2 tons/ha) and lowest weight with cultivars *Mugande* (2.2 tons/ha) and *SPK004* (2.4 tons/ha) (Table 12). Conventional stem cuttings had highest marketable tubers as these propagules established faster and were less sensitive to the physical environment compared to tissue culture planting material and hence paved way for earlier assimilate partitioning for tuber growth.

Table 12: Effect of propagation method and cultivar on the marketable tuber weight (tonnes/ha) in sweet potato grown under field conditions at KARI-Njoro.

Propagation	Cultivars					Mean
Method	Mugande	J. tres	Zapallo	SPK004	Kemb10	
Conventional	5.1 a	3.8 b	9.6 a	2.9 a	2.7 b	4.8 a
CI	4.9 a	6.0 a	3.6 b	1.6 b	2.2 a	3.7 a
SE	2.1 b	4.2 b	5.1 a	2.4 a	2.9 a	3.3 a
Mean	4.0 ab	4.7 ab	6.1 a	2.3 b	2.6 b	
CV (%)	21.1					
LSD (5%)	1.22					

Means followed by the same letter within a column are not significantly ($P \leq 0.05$) different based on Least Significantly Difference (LSD).

CI and SE denote callus induction and somatic embryogenesis respectively.

Generally, in this study propagation using tissue culture propagules and conventional stem cuttings were effective in producing tubers of morphological characteristics attributed to each cultivar as shown in Plate 12. The cultivars *Mugande* and *Kemb10* responded better to callus induction since there were no significant differences between regenerated propagules and stem cuttings for marketable tubers produced. Across all treatments *Kemb10* also gave highest marketable weights with somatic embryogenesis planting materials. From the field study, marketable tuber weights varied among cultivars and method used for propagation, suggesting that cultivar performance varied within the environmental conditions.

4.3.4 Disease detection using ELISA

Leaf samples obtained from symptomatic conventional sweet potato plants had higher photometer readings than plants obtained from tissue culture regenerated propagules (Table 13). In the laboratory study, the results showed that the mean absorbance values from leaf samples of cultivars *J. tresmesino, Zapallo, Kemb10* and *Mugande* were lower than those of the sweet potato healthy control (Table 13) indicating that these cultivars were free from the SPFMV. These results emphasized that tissue culture regenerated plants were from clean planting material as they gave lower values than the control therefore giving a negative test for the SPFMV.

However, contrary to these results *Kemb10* which had initially displayed no virus-like symptoms during *in vitro* culture was confirmed to have the virus after overnight incubation. This reaction was also described by Kartha (1986) with tobacco plants which showed different responses to Tobacco Mosaic Virus (TMV) and attributed it to the uneven distribution of the TMV during *in vitro* meristem culture. Moury *et al*. (2003) also reported a positive reaction to a virus during *in vitro* culture in sour cherry, red raspberry and peach. The ELISA results also revealed that all composite leaf samples collected under field conditions were positive to the SPFMV, despite the early prediction of being virus-free during the *in vitro* studies.

There was a high virus titre (concentration) in the field plants compared to the tissue culture regenerated plants (Table 14) probably due to the fact that during this study SPFMV-like symptoms such as mosaic, leaf purpling, mottling curling or chlorosis were more pronounced in the field plants. ELISA tests were influenced by factors such as the ability of the plant to release

Table 13: Detection of sweet potato viruses by DAS-ELISA from *in vitro* regenerated and field plants in locally adaptable sweet potato cultivars at KARI-Njoro.

	Regenerated plants			Field plants		
Readings at	30/60 mins	120 mins	24hrs	At 30/60 mins	120 mins	24hrs
Mugande	- (0.10)	- (0.10)	+ (0.14)	+ (0.15)	+ (0.16)	+ (0.20)
J. tres	- (0.13)	- (0.13)	- (0.13)	- (0.14)	- (0.14)	+ (0.23)
Zapallo	- (0.10)	- (0.13)	- (0.10)	+ (0.15)	+ (0.16)	+ (0.19)
SPK004	- (0.13)	- (0.13)	- (0.13)	+ (0.15)	+ (0.16)	+ (0.20)
Kemb 10	- (0.14)	+ (0.16)	+ (0.16)	+ (0.16)	+ (0.16)	+ (0.20)
Healthy control	- (0.14)					

-, +, represents negative, positive reaction for SPFMV, respectively.

Values in parenthesis represent mean absorbance indicating disease incidence

the virus during crushing and the thickness of the sap produced (Wangai *et al*., 2001). The field material had further been exposed to environmental conditions prone to SPFMV vector namely, aphids. Conventionally propagated planting material had higher readings than the healthy control (Table 14) probably due to the rapid virus multiplication resulting from rapidly growth of plants. Kartha (1986) reported that since conventionally propagated plants were derived from donor plants, they harboured pathogens, therefore resulting in faster transmission to areas of renewed growth. Munga *et al*. (2000) reported that transmission of viruses to healthy plants was enhanced and much faster through insects or other vectors.

Generally, the callus induction and somatic embryogenesis propagules had lower photometer readings indicating that the disease level was lower with these methods. It has been reported that tissue culture plants have little virus because they lack a vascular system therefore movement of the virus through plasmodematal connections occurs at a slower pace during the invasion of the rapidly dividing meristematic cells (Kartha, 1986).

Table 14: Detection of sweet potato viruses by DAS-ELISA using different propagation materials at KARI-Njoro.

Propagation method	Overall Disease score			
	Ist reading	2nd reading	3rd reading	Mean
Conv method	+ (0.16 a)	+ (0.17 a)	+ (0.21a)	+ (0.18 a)
Callus Induction	- (0.13 b)	-/+ (0.14 b)	+ (0.2 a)	+ (0.16 b)
S. embryogenesis	-/+ (0.14 ab)	+ (0.15 b)	+ (0.2 a)	+ (0.16)

-, +, -/+ represents negative, positive and limited reaction for SPFMV respectively.

The low virus titre with tissue culture regenerated plants could also be that these plants were propagated from small explants unlike the conventional plants where large stem cuttings were used. In this study all field-harvested plants reported as displaying clear virus, symptoms were confirmed to have the SPFMV virus unlike with the tissue cultured plants where all cultivars with the exception of *Kemb10* tested negative for the SPFMV.

a) ***J. tresmesino:*** tubers from C.I method weight / tuber 0.8 tons/ha

b) ***SPK004:*** tubers from C.I method (0.8 tons/ha)

c) ***Zapallo***: tubers from S.E method (1.7 tons/ha)

d) ***Kemb 10***: Tubers from S.E method (0.6tons/ha)

e) ***Mugande:*** tubers from C.I method (0.8 tons/ha)

Plate 12: Selected tubers and their weights obtained using regenerated planting material (CI callus induction and SE somatic embryogenesis) in local sweet potato cultivars grown under field conditions at KARI-Njoro.

a) ***SPK004*** tubers- (0.8 tons/ha)

b) ***J. tremesino*** tubers (0.8 tons/ha)

c) ***Zapallo*** tubers (0.8 tons/ha)

d) ***Mugande*** tubers (0.3 tons/ha)

e) ***Kemb10*** tubers (0.6 tons/ha)

Plate 13: Selected tubers and tuber weights obtained using conventional propagated stem cuttings from local sweet potato cultivars grown under field conditions at KARI-Njoro.

CHAPTER FIVE

CONCLUSIONS AND RECOMENDATIONS

This study was directed towards developing a suitable and efficient protocol for the production of embryogenic callus that would have long-term competence for plant regeneration in the locally adaptable sweet potato cultivars (*Ipomoea batatas* (L.) Lam). The results from the study showed that:

- Optimum calli induction varied with the cultivars and across most cultivars it was achieved by culturing leaf explants from young juvenile plant tissues on MS media supplemented with low levels of 0.5 mg L^{-1} 2,4-D. This induced highest embryogenic response in calli formed and subsequent plant regeneration in the local sweet potato cultivars.
- High 2,4-D levels led to decreased calli incidences and reduced the shooting and rooting incidences as well as plant regeneration capacity which varied significantly with each local sweet potato cultivars.
- Plant regeneration using callus induction and somatic embryogenesis regeneration methods was not achieved easily with the local cultivars. However regeneration by somatic embryogenesis took a short time to form plant shoots and roots but the number of plants regenerated was reduced as compared to callus induction method.
- Significant cultivar differences were observed for both calli and plant regeneration incidences which accounted for the observed differences in response to all the parameters measured. There were also significant cultivar differences with respect to ease of calli formation patterns and organogenesis. Cultivar *Zapallo* followed by *SPK004* at 0.5 mg L^{-1} 2,4-D performed best under both tissue culture regeneration methods.
- The optimum 2,4-D concentration in the medium was critical and had to be right in order to cause cell differentiation and totipotency among the cultivars. The local sweet potato cultivars were extremely sensitive to the 2,4-D levels that were supplemented in the media. The results revealed prolonged culture on the same medium had a negative effect on calli formation, proliferation, shooting, rooting, leafing and plant regeneration among the cultivars.
- No significant differences between the conventional stem cuttings and tissue culture-regenerated sweet potatoes were established under field conditions. Tissue culture

regenerated plants for the cultivars *Zapallo, J. tresmesino* and *Mugande* established at a slower rate but picked up and grew just as stem cuttings.

- The ELISA test established that SPFMV incidence among the cultivars was different and *in vitro* regenerated plants tested negative to SPFMV. However when transferred to the field they tested positive for the SPFMV though at very low concentration.

It is hence recommended that:

- Embryogenic calli induction for the locally adaptable sweet potato cultivars be done at 0.5 mg L^{-1} 2,4-D levels because at this level calli induction, maturation and proliferation occurred at a high and faster rate and hence promoting faster organogenesis.
- Lower levels of 2,4-D should be used as they increase the formation of embryogenic calli and hence reducing the number of days taken for the formation of calli incidences.
- Further evaluation studies should however be done to determine the mechanism by which the 2,4-D influences calli induction and similarly evaluate calli induction and plant regeneration in other local sweet potato cultivars.
- The use of callus induction regeneration method was beneficial as more calli formed from a large mass of disorganized cells thereby ensuring higher plant regeneration and increased mass propagation of *in vitro* plants.
- Further studies should also be done to understand the genetic control that favoured calli embryogenesis during plant regeneration in some local cultivars such as *Zapallo* and not in others such as *Kemb10* and *J. tresmesino*.
- At Njoro cultivar *Zapallo, J. tresmesino* and *Mugande* using *in vitro* regenerated plant-lets were most suitable as they gave the highest tuber and yield. More work should also be done to improve the hardening or acclimatisation regimes of regenerated plants *ex vitro* among the local cultivars.
- Further studies using the local cultivars should be done to check how the SPFMV incidence and severity vary at different stages of development and determine the susceptibility of the cultivars to the SPFMV over a longer period of time and under a wider agro ecological zone before any conclusive recommendation on the diseases can be given.

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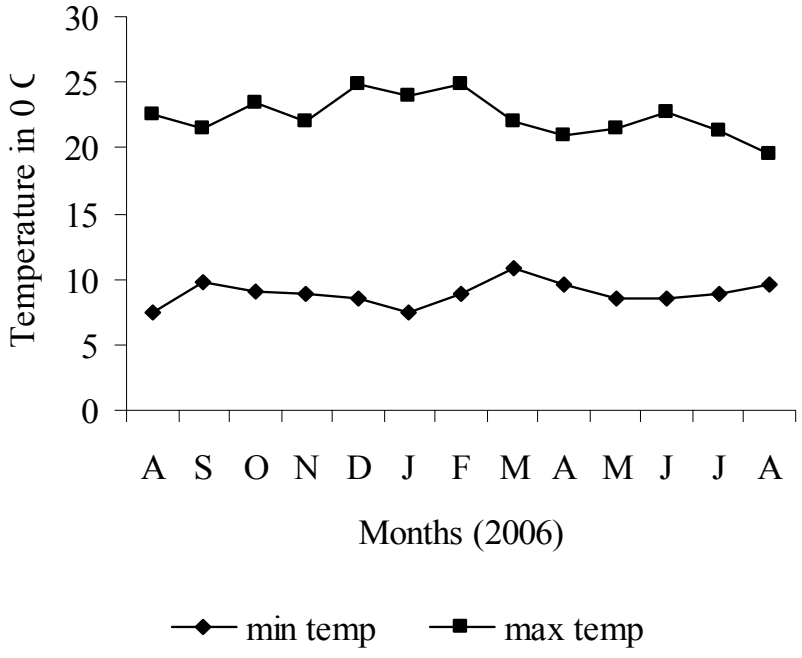
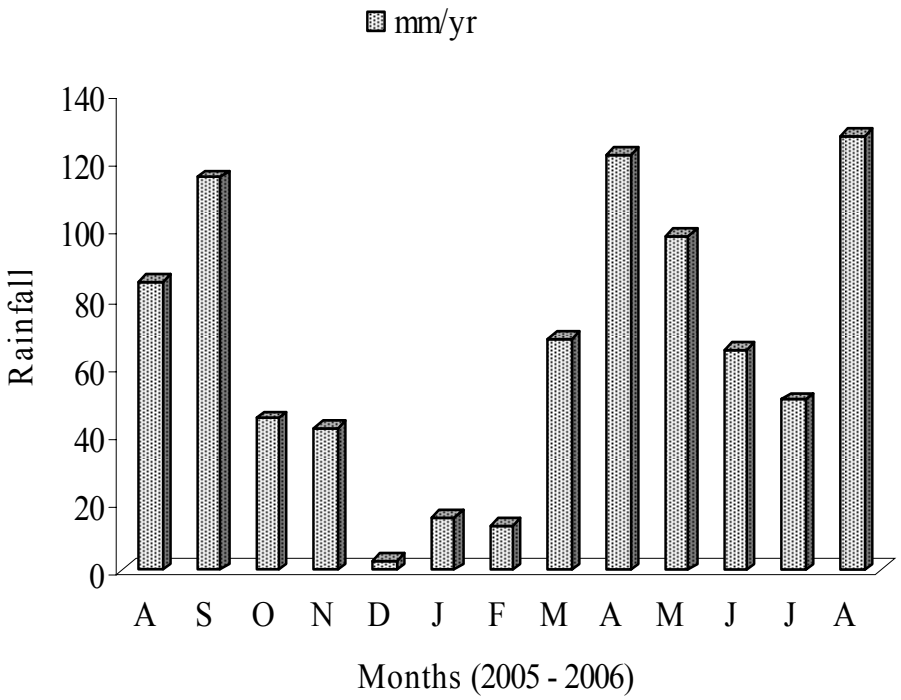
APPENDICES

Appendix 1: Temperature recorded during the field study at KARI-Njoro



(Source K.A.R.I. metrological station, 2006)

Appendix 2: Rainfall recorded during the field study at KARI-Njoro



(Source K.A.R.I. metrological station, 2006)

Appendix 3: Buffer formulations of ELISA

General extraction buffer: dissolve in 1000ml 1X PBST buffer (pH 7.35)

Sodium sulphate (anhydrous)	1.3g	
Polyvinyl pyrrolidone (PVP) MW 24-40,000	20g	
Sodium azide-	0.2g	
Powdered egg chicken, Grade	112.0g	
Tween-20	20g	(pH 7.4)

Coating buffer: Dissolve in distilled water to 1000ml

Sodium Carbonate (anhydrous)	1.59g,	
Sodium bicarbonate	2.93g,	
Sodium azide	0.2g	(pH 9.6)

PBST buffer (wash buffer): Dissolve in distilled water 1000 ml

Sodium chloride	8g	
Sodium phosphate dibasic (anhydrous)	1.15g	
Potassium Phosphate, monobasic (anhydrous)	0.2g	
Potassium chloride	0.2g	
Tween-20	0.5g	(pH 7.35)

ECL buffer: add to 1000ml 1X PBST (PH 7.35)

Bovine serum albumin (BSA)	2.0g
Polyvinylpyrrolidone (PVP) MW24-40,000	20g

Appendix 4: Analysis of variance on the effect of cultivars and 2,4-D concentration on mean calli weights, days taken to form calli, calli diameter and calli quality in sweet potato grown in tissue culture at KARI-Njoro in 2006.

		Calli weight (g)		Days taken to form calli		Calli diameter (cm)		Calli quality (%)	
Source	df	MSE	Pr< F	MSE	Pr< F	MSE	Pr< F	MSE	Pr< F
Cultivar	4	6060	0.0001**	1042.2	0.0001**	0.85	0.0001**	0.467	0.002*
Conc.	5	1320.4	0.006*	205.4	0.0001**	0.10	0.0001**	1.75	0.0001**
Cultivar x Conc.	20	892.9	0.002*	19.4	0.87	0.06	0.0001**	0.48	0.0001**

*, **, shows significant at the 0.05 and 0.001 probability levels respectively

Appendix 5: Analysis of variance on the effect of cultivar, 2,4-D level and regeneration method on mean number of days taken to form shoots and roots, number of leaves, roots and plants regenerated in sweet potato cultivars grown at KARI-Njoro in 2006.

Source of variation		Days taken to form Shoots		Days taken to form roots		No. of leaves plant $^{-1}$		No. of roots plant $^{-1}$		Number of plants regenerated	
	df	MSE	Pr < F	MSE	Pr < F	MSE	Pr < F	MSE	Pr < F	MSE	Pr < F
Cultivar	4	437.9	0.0001**	341.4	0.0001**	6.06	0.0001**	7.65	0.001*	63.63	0.0001**
Conc.	2	146.4	0.0001**	284.7	0.0001**	12.9	0.0001**	11.5	0.001*	58.9	0.0001**
Cultivar x Conc.	8	10.2	0.195	21.7	0.07	0.48	0.72	4.90	0.003*	37.6	0.0021*
Method	1	912.0	0.0001**	965.1	0.0001**	3.6	0.03	1.11	0.39	0.011	0.919
Cultivar x Method	4	28.0	0.006*	32.74	0.03*	2.8	0.007*	5.92	0.006*	42.3	0.0001**
Conc. x Method	2	2.63	0.69	5.2	0.62	0.43	0.55	3.38	0.11	9.01	0.0006**
Cultivar x Conc. x Method	8	7.58	0.39	10.2	0.51	2.57	0.002*	4.43	0.007*	5.65	0.0001**

*, **, shows significant at the 0.05 and 0.001 probability levels respectively

Appendix 6: Analysis of variance on the effect of cultivar, propagation method and growth in days after transplanting (stage) on the plant stand count, plant height, number of branches and leaf area (LA) in sweet potato grown at KARI-Njoro in 2006.

Source of variation		Plant stand count		Plant height (cm)		Number of branches		LA (cm)	
	df	MSE	Pr>F	MSE	Pr>F	MSE	Pr>F	MSE	Pr>F
Block	2	0.15	0.37	158.7	0.71	8.34	0.1	1.94	0.15
Cultivar	4	0.05	0.87	9090.7	0.001	28.93	0.001	16.01	0.0001
Method	2	1.02	0.002	10355.2	0.001	29.96	0.0004	1.63	0.19
Cult x Meth	8	0.45	0.005**	1037.7	0.03	13.4	0.0006***	2.68	0.01
Stage	3	7.2	0.0001	86632.3	0.001	422.3	0.0001	198.68	0.001
Cult x Stage	12	0.05	0.99	4066.4	0.001	10.9	0.0009***	2.32	0.01
Meth x Stage	6	7.02	0.0001	1894.9	0.001	4.52	0.28	0.68	0.67
Cult x Meth x Stage	24	0.48	0.0001***	870.7	0.002**	2.59	0.82	0.43	0.99

, * represent significant interaction at 0.01 and 0.001 probability levels

Appendix 7: Analysis of variance on the effect of cultivar and propagation method on the number and weight of unmarketable tubers, the number and weight of marketable tubers in sweet potato grown under field conditions at KARI-Njoro in 2006.

Source of variation		Number of unmarketable tubers			Weight unmarketable tubers			Number of marketable tubers			Weight marketable tubers		
	df	MSE	F value	Pr>F	MSE	F value	Pr>F	MSE	F value	Pr>F	MSE	F value	Pr>F
Block	2	88.7	88.7	0.008	0.01	0.25	0.78	0.06	0.03	0.97	0.29	0.13	0.88
Cultivar	4	16.4	16.4	0.38	0.06	1.65	0.19	5.19	3.27	0.03*	2.09	0.94	0.45
Method	2	5.35	5.4	0.71	0.01	0.15	0.86	2.38	1.50	0.24	2.02	0.91	0.41
Cultivar x method	8	23.8	23.8	0.17	0.08	2.44	0.04*	2.20	1.39	0.24	4.36	1.96	0.01*

* shows significance at the 0.05 probability level.