

**GENETIC TRANSFORMATION OF SELECT AFRICAN  
SWEETPOTATO (*Ipomoea batatas L. Lam.*) CULTIVARS WITH  
WEEVIL RESISTANCE GENES AND EVALUATION OF  
PHYTOALEXIN LEVELS FROM INFECTED ROOTS**

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**WAMALWA LYDIA NANJALA (B.Sc., M.Sc.)**

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of the Degree of Doctor of Philosophy (Plant Biotechnology and  
Molecular Biology) in the School of Pure and Applied Sciences of  
Kenyatta University**

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## DECLARATION

I declare that this thesis is my original work and has not been presented in any other university/institution for consideration of any certification. This thesis has been complemented by referenced sources duly acknowledged. Where text, data (including spoken words), graphics, pictures or tables have been borrowed from other sources, including the internet, these are specifically accredited and references cited using the current APA system and in accordance with anti-plagiarism regulations.

Signature.....  ..... Date ..... 10/6/15 .....

**Lydia Nanjala Wamalwa** (I56/11942/2007)  
Department of Biochemistry and Biotechnology

## SUPERVISORS' DECLARATION

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

Signature.....  ..... Date ..... 11/6/15 .....

**Dr. Steven Runo**  
Department of Biochemistry and Biotechnology  
Kenyatta University

Signature.....  ..... Date ..... 10/6/2015 .....

**Dr. Marc Ghislain**  
Department of Genomics and Biotechnology  
International Potato Centre, Nairobi

Signature.....  ..... Date ..... 17/6/2015 .....

**Dr. Allan Mgutu Jalemba**  
Department of Biochemistry and Biotechnology  
Kenyatta University

**DEDICATION**

I would like to dedicate this thesis to my husband Dr. Stephen Indieka, my daughter Laura Indieka and Junior Indieka. I also dedicate it to my parents Mr. John Wamalwa and Mrs. Eunice Wamalwa as well as my siblings.

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### Abbreviations and Acronyms

|            |   |
|------------|---|
| BAP        | Benzylaminopurine                                 |
| <i>Bt</i>  | <i>Bacillus thuringiensis</i>                     |
| CIP        | International Potato Centre                       |
| CTAB       | Cetyl trimethylammonium bromide                   |
| DAS        | Double Antibody Sandwich                          |
| DCM        | Dichloromethane                                   |
| DNA        | Deoxyribonucleic acid                             |
| DM         | Dry matter  |
| dNTPs      | Deoxyribonucleoside triphosphates                 |
| 2, 4-D     | 2,4-Dichlorophenoxyacetic acid                    |
| ELISA      | Enzyme-linked Immunosorbent Assays                |
| 4-FA       | 4-flourophenoxyacetic acid                        |
| GC-MS      | Coupled Gas Chromatography Mass Spectrophotometry |
| GUS        | $\beta$ - glucuronidase                           |
| HPLC       | High Performance Liquid Chromatography            |
| IAA        | Indole acetic acid                                |
| <i>ipm</i> | <i>Ipomeamarone</i>                               |
| ITS        | Internal Transcribed Spacer                       |
| KEPHIS     | Kenya Plant Health Inspectorate Service           |
| LB         | Luria Broth                                       |
| MS         | Murashige and Skoog                               |
| NMR        | Nuclear Magnetic Resonance                        |
| PCR        | Polymerase chain reaction                         |
| PDA        | Potato Dextrose Agar                              |
| PGR        | Plant Growth Regulator                            |
| RE         | Regeneration efficiency                           |
| RNA        | Ribonucleic acid                                  |
| RT         | Room temperature                                  |

|       |                            |
|-------|----------------------------|
| SSA   | Sub-Saharan Africa         |
| SSR   | Simple Sequence Repeats    |
| TBE   | Tris Borate EDTA           |
| TDZ   | Thidiazuron                |
| TE    | Transformation efficiency  |
| TEMED | Tetramethylethylenediamine |
| TLC   | Thin Layer Chromatography  |

### Abstract

Sweetpotato is an important root and tuber crop in Sub-Saharan Africa (SSA) used as food and feed but weevil infestation remains a major concern. Weevils account for production losses between 28 and 100% but no resistance genes are available in the natural gene pool. When weevils attack, they create avenues for secondary infection, which in turn elicit phytoalexin production, toxic to farm animals. Since genetic transformation has been widely used to introduce genes into germplasm that may not be available in the natural gene pool, it was used in this study. Objectives of this study included (i) to develop a regeneration protocol of 32 SSA cultivars through indirect organogenesis, (ii) to develop a transformation protocol for the best cultivars using *uidA* gene and the double gene construct (*cry7Aa1* and *ET33-34*), and (iii) to isolate a fungus that infects sweetpotato after weevil infestation and subsequent analysis of the furanoterpenoid elicited from infected roots. Regeneration and transformation were conducted via indirect organogenesis. Four gene constructs were used in this study: pCIP100, pCIP87, pCIP88 and pCIP85. Phytoalexin levels were evaluated on four consumer-preferred sweetpotato cultivars using coupled gas chromatography mass spectrophotometry (GC-MS), thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and Nuclear Magnetic Resonance (NMR). Results showed 6 SSA cultivars with RE above 40% while TE of 5 SSA cultivars revealed GUS-stained calli between 6 and 98%. Six transgenic events were produced using the double gene construct as confirmed by southern blot but they had low protein levels. For phytoalexins, levels between 0.3 and 2,900 mg/kg were recorded. The results suggested that pre-screening for high RE and TE was important in identification of the best cultivars. Low protein concentration levels obtained could be due to post-transcription or/and post-translational factors. Such high variations in furanoterpenoid levels have previously been reported, and could be an indicator of a potential health concern to both animals and humans on consumption.

# CHAPTER ONE

## INTRODUCTION

### 1.1 Background

Sweetpotato is mainly grown by small-scale resource poor farmers especially women and is commonly known as a poor man's crop in sub-Saharan Africa (SSA). Apart from being utilized as food and feed, sweetpotato is also used a source of income for many rural and urban families (Omosa, 1997). Sweetpotato requires relatively less labour and fewer inputs, therefore increasingly grown by many rural families affected by the human immunodeficiency virus (HIV) (Manyumwa *et al.*, 2012). Its production is more important for many rural families especially due to the availability of early maturing varieties/cultivars (Sitango and Dopo, 2004) and piece meal harvesting practised in many parts of SSA (Ebregt *et al.*, 2007). This enables the rural families to have food all year round even during the dry seasons when the other staples are not available. In SSA, sweetpotato is therefore an important food security crop and this could be used to save lives during very dry seasons or even drought.

### 1.2 Constraints to sweetpotato production in SSA

There has been slow progress in increasing sweetpotato production in Africa owing to biotic and abiotic factors. Some of abiotic factors that affect sweetpotato production are drought, salinity, temperature and soil nutrients (Agili *et al.*, 2012). During drought, a young developing sweetpotato is unable to effectively initiate storage root development due to lack of moisture but in cases when they are already developing and then drought is experienced, if it gets water periodically, it will still develop (Ghuman and Lal, 1983).

The biotic factors include pests, diseases, inadequate clean planting material, low soil fertility, drought, limited availability of improved varieties, post-harvest storage and stress metabolites (Yasuda and Kojima, 1986). In addition, sweetpotato is known as a 'poor man's crop' and not many people in urban centres consume them (Kapinga *et al.*, 2007). Furthermore, it is mainly grown by small-scale farmers primarily practising subsistence farming and not considered by large-scale farmers as an income generating crop (Gruneberg *et al.*, 2009).

The major biotic stresses include viral infections and weevil infestation. Viral diseases are responsible for more than 50% losses (Gutierrez *et al.*, 2003) whereas weevils cause more production damage of between 28-100% (Stathers *et al.*, 2003; Kiiza *et al.*, 2009). The main weevils affecting sweetpotato in Eastern Africa are *Cylas puncticollis* and *Cylas brunneus* (Harriet *et al.*, 2012). The weevils affect the vines, roots and to a lesser extent, foliage. In the field, the cryptic feeding habits of larvae and nocturnal activity of adults make it difficult to detect infestation. Additionally, approximately 80 to 90% of weevil population occurs within vines and roots, which are below ground, hence limiting the effectiveness of chemical insecticides. Integrated pest management regimes are difficult to adopt because the crop is grown all year round. In-ground storage (piece-meal harvesting) is practiced to guarantee availability of fresh roots for consumption all year long, which means that such sweetpotato roots are exposed to weevils all year round.

### **1.3 Problem statement and justification**

Global food production has been on the upward trend since the 1800s; however in the 21<sup>st</sup> Century production has been faced with more challenges among them increased human

population due to increased life expectancy (James, 2009). Land under agricultural production, on the other hand, is not increasing to provide for the ever-growing hungry population (Chrispeels and Sadava, 2003). In this regard, increasing crop production in lands currently under cultivation is crucial and this can be achieved through development of new varieties to improve crop yield. Concerted efforts will play an important role in crop improvement, through both conventional and modern biotechnological techniques.

For sweetpotato, efforts directed towards eradication of the prevalent problem of weevil infestation have made little progress thus far, with no resistant cultivars or varieties having been developed as yet. However, there are reports on control measures such as the use of cultural methods such as mulching with elephant grass (Mansaray *et al.*, 2013); the use of biological controls such as entomopathogenic fungi (Reddy *et al.*, 2014); the use of sex pheromones (Reddy *et al.*, 2012) and conventional breeding through development of better yielding cultivars (Mwanga *et al.*, 2011). Although these methods have been yielded some gain, they will be mostly limited by the land tenure systems in SSA. This is due to the land tenure systems particularly for the rural small-scale farmers who have very small land parcels and all the other crops are grown alongside.

Conventional breeding of sweetpotato cultivars to build resistance to weevils has not yielded much success. This is mainly due to the complexity of ploidy level and heritability issues including difficulty to break gene linkages between desirable and undesirable traits, male sterility and self-incompatibility (Anderson and deVicente, 2010). Due to these limitations, genetic transformation is an attractive alternative to overcome the problems associated with conventional breeding of sweetpotato. Prior to developing a

transformation protocol, an amenable regeneration protocol should be in place. Two main methods used for regeneration include somatic embryogenesis and organogenesis (George *et al.*, 2008). Somatic embryogenesis takes a relatively long time to produce regenerants (up to one year) (Manrique *et al.*, 2013) when compared to organogenesis, which takes less than 8 weeks (Gosukonda *et al.*, 1995) to produce regenerants.

When initiating sweetpotato regeneration studies, it is known that different cultivars vary in the ability to produce regenerants based on tissue culture conditions they are subjected to. When the regeneration protocol has been optimised for a specific cultivar, the same protocol could be used to produce regenerants after genetic transformation. Genetic transformation has successfully been used to address pest related problems in crop species including maize (Mugo *et al.*, 2011) and potato (Ghislain *et al.*, 2003). Therefore genetic transformation by introducing weevil resistance genes into the genome holds promise to protect sweetpotato against weevils. In addition, weevil resistance technology has been reported to create welfare gains of up to US\$ 9.9 million and an approximate internal rate of return on biotechnology research investment of 33-77% in Kenya (Qaim, 2001). These gains notwithstanding, sweetpotato weevils research has received relatively little attention from the private sector, which has been the key player in development of *Bacillus thuringiensis* (*Bt*) technology to control crop pests.

*Bacillus thuringiensis* (*Bt*) toxins, which affect the feeding habits of insect pests in crops, currently represent the most feasible means for achieving sustainable control of weevils under African small scale farming conditions. In a previous study, a number of *Bt* toxins namely Cry1Ab, ET33-34, ET70, Cry3Aa3, Cry3Aa3-K, CryBb2, CryBb3, Cry3Ca1 and

Cry7Aa1 with activity against *Cylas puncticollis* and *Cylas brunneus* were tested under *in-vitro* conditions using artificial diet and 3 proteins were confirmed to be effective (Ekobu *et al.*, 2010). These proteins were Cry3Ca1, ET33-34 and Cry7Aa1 toxins, which were repeatedly most toxic to *C. puncticollis* and *C. brunneus* larvae (Ekobu *et al.*, 2010). The corresponding genes were then identified, synthesized and used in sweetpotato transformation as reported herein this thesis.

In the best case scenario, if transgenic events were produced with resistance to weevils, it would mean that there will be reduced damage to sweetpotato roots hence less secondary infection by microbes and elicitation of phytoalexins. Therefore, another objective was added to this research to establish the quantities of phytoalexins produced once microbes attack the roots. Once infection by microbes occurs, storage roots produce phytoalexins to fight it off (Kuc, 2001). Phytoalexins are known to cause hepatotoxicity, lung oedema and even death in animals (Clark *et al.*, 1981; Shen, 1997). Doses that cause hepatotoxicity are between 250-500 mg/kg in experimental rats (Pandey, 2008). If the avenues through which weevils affect storage roots are sealed by developing weevil resistant sweetpotato, there will be a subsequent reduction in microbial infection on the storage roots and consequently a reduction of phytoalexin production.

#### **1.4 Null hypothesis**

Selected Sub-Saharan Africa sweetpotato cultivars can be regenerated *in-vitro* through organogenesis and can be genetically transformed using *Bt* genes to confer resistance against *C. puncticollis*.

## 1.5 Objectives

To investigate the potential of regenerating selected Sub-Saharan African sweetpotato cultivars *in vitro* through organogenesis and genetically transform them using *Bt* genes to confer resistance towards *C. puncticollis*.

The specific objectives of this study are to: -

- a) To determine the potential of developing regeneration protocols of selected Sub-Saharan Africa sweetpotato cultivars *in vitro* through organogenesis.
- b) To establish a GUS transformation protocol and screen for effect of promoter-used on staining. Use the established transformation protocol to transform selected SSA cultivars using *Bt* genes *ET33-34* and *cry7Aa1* conferring weevil resistance towards *C. puncticollis*.
- c) To isolate a fungus that infects sweetpotato after infestation by *Cylas* spp. and subsequent furanoterpenoids elicited in the infected roots.

## 1.6 Outputs

- (a) A regeneration protocol for several SSA sweetpotato cultivars developed.
- (b) A transformation protocol for SSA sweetpotato cultivars through organogenesis developed.
- (c) Furanoterpenoid concentration levels on fungal infected sweetpotato storage roots developed.

## CHAPTER TWO

### LITERATURE REVIEW

#### 2.1 Sweetpotato

Sweetpotato is classified as a root and tuber crop; it has the shortest growing cycle of all the root crops grown (FAO, 1998). The crop belongs to the family Convolvulaceae, class Ipomoeae, sub-genus Quamoclit, section Batatas, genus *Ipomoea* and species *Ipomoea batatas* (L.) Lam (Huaman, 1999). It is a perennial dicot herb mainly grown for its storage roots, which are consumed as food and feed as well as the leaves and vines (Low *et al.*, 2009b). The sweetpotato cultivars produce storage roots which differ in size and sweetness that is dependent on the variety or cultivar type. The family Convolvulaceae has many other species but *I. batatas* is the only one that is cultivated.

#### 2.2 Sweetpotato production

Root and tuber crops collectively occupy about 50 million hectares of land worldwide, with about two thirds being in the developing world; they provide energy and nutrition requirements for about 2 billion people (Scott *et al.*, 2000). Sweetpotato alone is grown on about 9 million hectares worldwide mainly in the developing countries (Thottapilly, 2009). Sweetpotato ranks among the top seven staple food crops worldwide after wheat, rice, corn, potato, barley and cassava. It is produced in all the continents with Asia ranking as the highest producer with more than 80% of total worldwide production followed by Africa (Loebenstein, 2009).

The top ten sweetpotato producers include 5 countries from Africa and 5 from Asia (Table 2.1). United States of America made an entry in the year 2010 and is among the top ten highest producing countries in the world (FAOSTAT, 2012). China is the largest producer of sweetpotato in the world (Table 2.1) with an average yield of 20 tonnes per hectare; 70% is used as animal feed especially pigs (Gruneberg *et al.*, 2009; Loebenstein, 2009). Total production of sweetpotato in China has been reducing over time (Table 2.1) due to growth and manufacture of soybean for pellets as feed. Apart from Angola in the top ten sweetpotato producing countries, the rest have consistently increased production over a period of 5 years (FAOSTAT, 2008-2013) (Table 2.1).

Sweetpotato production contributes towards addressing food security issues in many developing countries. Although sweetpotato has been important for years, emphasis towards food self-sufficiency had been placed mainly on cereals, unlike the orphan crops, which include sweetpotato (Naylor *et al.*, 2004). The slow progress in increasing sweetpotato production in many developing countries has somehow stagnated over time (Table 2.1), which could partly be attributed to fewer sweetpotato research efforts or funds directed towards sweetpotato improvement in many countries. Nevertheless, China has been the only country known to heavily invest in sweetpotato research and the world's top producer for many years (Table 2.1).

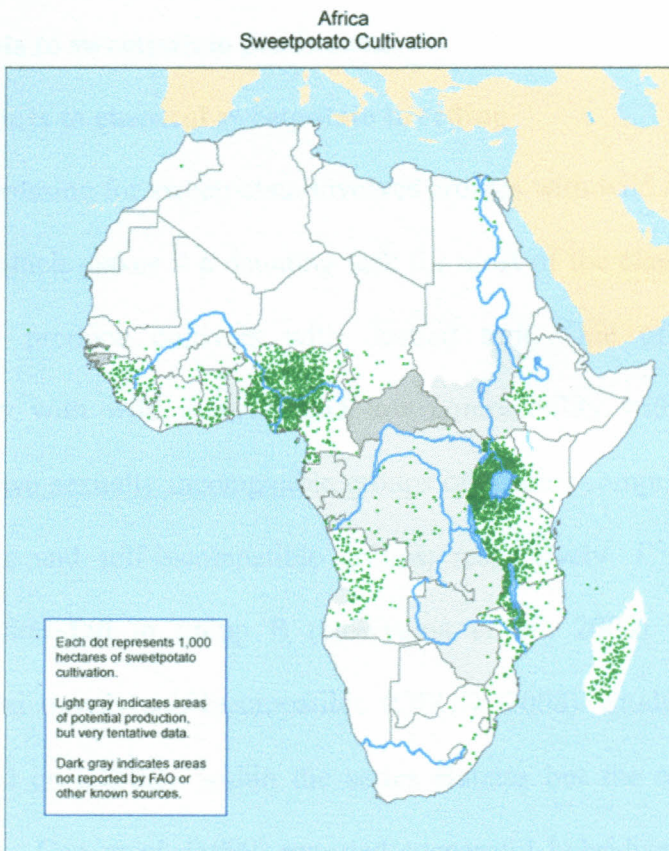
**Table 2.1:** Worldwide sweetpotato production for the period between 2008 and 2013 as reported by FAO

| Sweetpotato production in metric tonnes over time (years) |            |            |            |            |            |            |
|---|------------|------------|------------|------------|------------|------------|
| Country   | 2008       | 2009       | 2010       | 2011       | 2012       | 2013       |
| China   | 78,442,881 | 76,772,593 | 81,175,660 | 75,362,000 | 77,375,000 | 70,526,000 |
| Uganda  | 2,707,000  | 2,766,000  | 2,838,000  | 2,554,000  | 2,645,700  | 2,587,000  |
| Nigeria   | 3,318,000  | 2,746,820  | 2,703,500  | 3,300,000  | 3,400,000  | 3,450,000  |
| Indonesia   | 1,876,940  | 2,057,910  | 2,051,050  | 2,192,242  | 2,483,467  | 2,386,729  |
| Tanzania  | 1,379,000  | 1,381,120  | 1,400,000  | 3,573,302  | 3,018,175  | 3,470,304  |
| India   | 1,094,000  | 1,119,700  | 1,094,700  | 1,046,600  | 1,072,800  | 1,132,400  |
| Vietnam   | 1,325,600  | 1,207,600  | 1,317,060  | 1,362,194  | 1,422,501  | 1,358,175  |
| Japan   | 1,011,000  | 1,026,000  | 863,600    | 885,900    | 875,900    | 942,300    |
| Angola  | 819,772    | 982,588    | 986,563    | 1,045,104  | 644,854    | 1,199,749  |
| Kenya   | 894,781    | 930,784    | 820,971    | 759,471    | 859,549    | 1,150,359  |

In sub-saharan Africa (SSA), the major sweetpotato producing countries are Uganda, Nigeria, Tanzania, Angola, Kenya, Mozambique, Rwanda, Burundi and Ethiopia (FAOSTAT, 2013). Sweetpotato is mostly produced in areas surrounding lakes and water masses in these countries (Low *et al.*, 2009). There has been an increase in the land under sweetpotato cultivation in these SSA countries probably due to spread of cassava and banana diseases around the lake regions, especially L. Victoria. This could be because many farmers cultivate both cassava and sweetpotato, when the former is infected by disease; many farmers opt for sweetpotato (anonymous: <http://cipotato.org/research/sweetpotato-in-africa>). Although sweetpotato production is increasing in SSA, the yields are still low at about 4 tones per hectare (Bashaasha *et al.*, 1995). This could significantly be increased if certain measures like improvement of post-harvest handling, planting of clean material and availability of markets are put in place for the rural small scale farmers.

Nigeria and Kenya are the only countries that have reduced sweetpotato production in the 2008 to 2013 period, while the other 3 SSA countries have consistently increased its production (Table 1). The reduction in sweetpotato production in Kenya between 2009 and 2010 could have been due to the drought that struck the country over that period. Although there is a slight increase in sweetpotato production in SSA, more needs to be done to increase its yields from 4 to 6 tonnes per hectare to above 20 tonnes per hectare like in China (Gruneberg *et al.*, 2009; Low *et al.*, 2009; Zhang *et al.*, 2009).

Sweetpotato also grow over a wide range of climatic zones from 0 to 2000 m above sea level depending on cultivar or variety (Gbigbi, 2011) (Fig. 2.1).



Source: <https://research.cip.cgiar.org/confluence/display/WSA/Sweetpotato+and+Malnutrition+in+Africa>

**Figure 2.1:** Sweetpotato growing regions in Africa

### **2.2.1 Cultural practices in sweetpotato production in SSA**

Sweetpotato is mainly grown by rural families as a subsistence crop (Gruneberg *et al.*, 2009). Although there is a relative increase in land under sweetpotato cultivation, access to clean planting material has been an issue for farmers producing the crop partly because of the cost implications (Yanggen and Nagujja, 2006). Farmers also share planting material amongst them thus providing avenues for infestation and facilitate the spread of diseases or pests. The farmers have frequently used various methods to control pests such as use of mounds, crop rotation and mulching over the growing storage roots (Talekar, 1991), but these are only temporary measures.

### **2.3 Constraints to sweetpotato production**

#### **2.3.1 Constraints to classical sweetpotato breeding**

Interspecific crossing for sweetpotato involves crosses with wild relatives. Sweetpotato is a hexaploid, which makes it a daunting task for most of the classical crop improvement techniques to produce cultivars with desired trait. One of the main reasons is incompatibility with wild relatives and low compatibility within species. The genus *Ipomoea* has two sexually incompatible groups of species comprising of group A and B, self-compatible and self-incompatible groups, respectively. *I. batatas* and its closest relative *I. trifida* fall in group B (Kowyama *et al.*, 2000) with the former being allogamous and mostly self-incompatible (OECD, 2008). Studies done by Diaz *et al.* (1996) showed crossability within the series Batatas but the crosses did not produce viable progeny. Cao *et al.* (1999) reported successful hybridization between cultivated sweetpotato and diploid wild relatives (*I. grandifolia* and *I. purpurea*) using plant growth hormones, however only two hybrids were produced. The difficulty in hybridization of

sweetpotato and its wild relatives includes differences in ploidy levels, compatibility and sterility related issues (Anderson and de Vicente, 2010). However, human interventions such as artificial ploidy level manipulations, artificial hybridization, embryo rescue and ovule culture are some techniques that have been used to address the incompatibility barriers (Mont *et al.*, 1993).

Intraspecific crosses involve crosses between sweetpotato cultivars. Crosses between sweetpotato cultivars are possible and have produced landraces that have been used in breeding programs (Mwanga *et al.*, 2007) but the rate of producing viable offspring varies with cultivar. Sweetpotato plants produce many flowers, however, it is followed by low seed set due to self-incompatibility (Murata and Matsuda, 2003); the few seeds produced normally have a hard testa making germination difficult unless scarification is done (Anderson and de Vicente, 2010). Nonetheless, there have been successful sweetpotato crossing programmes in East Africa as reported Mwanga *et al.* (2007) where 2 cultivars were developed from 25 clones. Among the evaluations done before releasing these cultivars include dry matter content of storage roots, root yield, taste alongside agronomic attributes such as earliness, root size and shape.

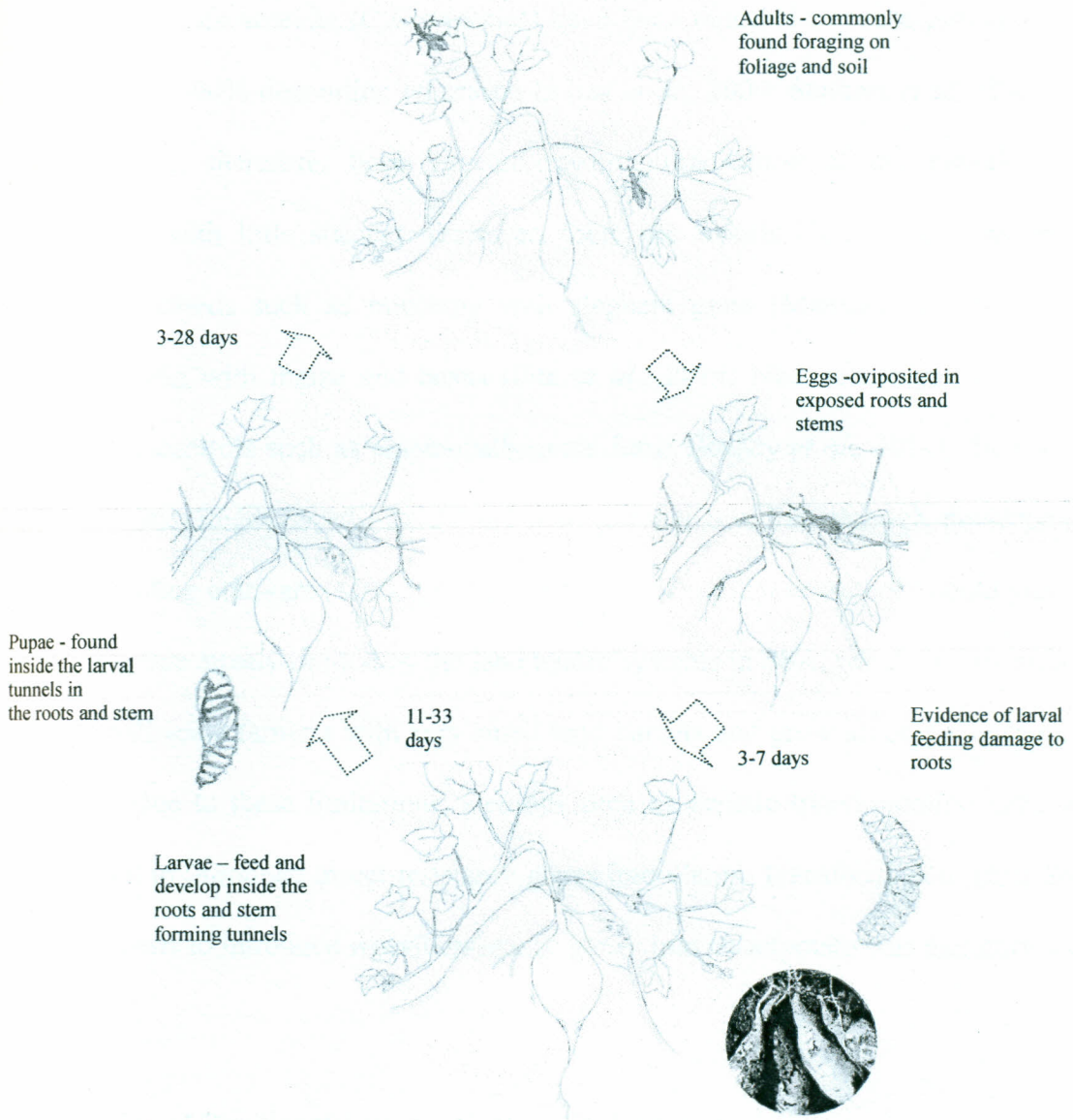
### **2.3.2 Sweetpotato insect pests**

#### **2.3.2.1 Sweetpotato weevils (*Cylas* species)**

Many weevil species affect sweetpotato production in SSA (Ames *et al.*, 1996); however *Cylas puncticollis* and *Cylas brunneus* are the species of great economic importance (Muyinza *et al.*, 2012). The weevils damage storage roots, vines and sometimes foliage (Fig. 2.2). They persist in areas where drought is prevalent. Kiiza *et al.* (2009) reported

an average of 28% crop loss caused by the *Cylas* spp. Although survival of *Cylas* spp. reduces with time, they can live up to an average of 120 days with *C. puncticollis* surviving longer than *C. brunneus* (Smit *et al.*, 1994). Mating of *Cylas* species is nocturnal and oviposition follows between 15 and 85 days after mating but the rate is reduced with time. Typically, *C. brunneus* lay their eggs during the day (Smit *et al.*, 1994) just below the epiderm of the storage root, while *C. puncticollis* lay them slightly deeper (Smit and Huis, 1999). The female weevils delay laying eggs until the environment is favourable and adult weevils emerge between 24 and 34 days after oviposition (Smit and Huis, 1999).

When weevils attack foliage, the storage roots produced are reduced both in size and numbers due to damaged vascular system. To infest sweetpotato storage roots, weevils pass through cracks in the soil to get to them where they lay eggs and the larvae cause massive damage as it burrows through the root. This encourages secondary infection by microbes such as *Ceratocystis fimbriata* and *Rhizopus stolonifer* fungi.



Source: [http://cipotato.org/vitaa/manual/09\\_TM%20Chapt%204b.pdf](http://cipotato.org/vitaa/manual/09_TM%20Chapt%204b.pdf)

**Figure 2.2: Schematic representation of the life cycle of a sweetpotato weevil.** The adult weevils feed on vines and storage roots, followed by females laying eggs at the base of storage roots. The larvae then emerge after 3 to 7 days and they feed through the roots forming tunnels. After 11 to 33 days, pupation then occurs within the roots. The adults then emerge after a period between 3 to 28 days from pupation.

### 2.3.2.2 Sweetpotato weevil (*Cylas* spp.) management

The sweetpotato weevils (*Cylas* species) have been reported to cause production losses between 28-100% depending on season (Kiiza *et al.*, 2009; Stathers *et al.*, 2003). Many efforts have, therefore, been directed towards eradication of the prevalent weevil infestation with little success. However, there are reports on control measures using cultural methods such as mulching with elephant grass (Mansaray *et al.*, 2013) and intercropping with maize and beans (Fite *et al.*, 2014; Nedunchezhiyan *et al.*, 2010); biological controls such as entomopathogenic fungi (Reddy *et al.*, 2014); the use of sex pheromones (Reddy *et al.*, 2012) and conventional breeding through development of better yielding cultivars (Mwanga *et al.*, 2011). Although these methods could yield some gain, they are mostly limited by the land tenure systems in SSA, which mainly affects the rural small-scale farmers with very small land parcels and grow all crops on same piece of land. Due to these limitations, methods such as genetic transformation have widely been used to introduce insect resistance genes into plants. Transformation using *Bacillus thuringiensis* to introduce weevil resistance genes into sweetpotato was therefore a viable option.

### 2.3.2.3 Use of *Bacillus thuringiensis* to control weevils

*Bacillus thuringiensis* (*Bt*) is a gram-positive soil bacterium that produces Insecticidal Crystal Proteins (ICPs) (Macaluso and Nettus, 1991). *Bacillus thuringiensis* forms spores and a parasporal crystal during the stationary phase of its growth cycle (Schnepf *et al.*, 1998) therefore producing Cry proteins (Bravo *et al.*, 2007). Cry genes are a source of proteins with insecticidal activity important in engineering plants that have in-built insect resistance (Schnepf *et al.*, 1998). There are many strains of *Bt* with endotoxins of known

insecticidal properties (Crickmore *et al.*, 1990) such as those effective against lepidopterans, dipterans and coleopterans (Sharma *et al.*, 2000; Suzuki *et al.*, 2004).

The ICPs comprise of protoxins, which are activated into toxins by proteases once insects ingest them and this is followed by solubilisation (Hofte and Whiteley, 1989). The Cry toxins usually bind to mid-gut receptors creating pores and when this occurs the gut are made porous and makes the insect larvae unable to feed and digest the food hence starves to death (Sharma *et al.*, 2000). Solubility differences in the mid-gut particularly due to pH explain differences in the degree of toxicity among Cry proteins (Du *et al.*, 1994).

The mid-gut pH of coleopterans is neutral while lepidopterans and dipterans are slightly alkaline (Bravo and Soberon, 2010). Therefore, the Cry proteins are harmless against mammals, birds, amphibians, or reptiles, but specific to insect families (Schnepf *et al.*, 1998; Betz *et al.*, 2000).

There are many Cry proteins with insecticidal activity against different insect classes and nematodes; Cry1Ab and Cry1Ac work against yellow stem borer in rice at mortality rate up to 93% reported (Nayak *et al.*, 1997), Cry2Aa2 has activity against cotton boll worm with mortality of 100% (Kota *et al.*, 1999), Cry3Aa against Colorado potato beetle with 50% mortality (Naimov *et al.*, 2003), Cry5, Cry12, Cry13 and Cry21 work against nematodes and hymenopterans, while Cry3, Cry7, Cry8, Cry14, Cry26 and Cry28 have toxicity against coleopterans (Naimov *et al.*, 2003). In addition, CryET70 caused mortality of 85%; ET33-34 caused mortality of 95%; Cry7Aa1 caused mortality of 96%; Cry3Ca1 caused mortality of 87% in weevil bioassays conducted using artificial diet against *Cylas* species (Ekobu *et al.*, 2010).

#### **2.3.2.4 Rough sweetpotato weevil**

These are *Blosyrus* species, which are rough, ridged in appearance and texture. The adults lay eggs under fallen foliage whereas the larvae and pupae form in the soil. The larvae get to the storage roots and make shallow holes thus making the roots of less market value. This is a common pest in East Africa and cause serious problems in some regions and the control measures for *Cylas* species may also apply for this species (Ames *et al.*, 1996).

#### **2.3.2.5 Sweetpotato stemborer (*Omphisia anastomasalis*)**

The stem-borer lays eggs under the leaf margins and stems, and the infested plants have a characteristic brown frass at the base. The larvae make an exit hole through the stem before pupating where the adult moth will emerge. This species can be of economic importance if it attacks young plants because their infestation reduces storage root development (Coelacp, 2011). This pest is prevalent in Asia and can result to losses of up to 30%. It can be controlled by treating infested planting material with pesticides, crop rotation and hilling-up (Ames *et al.*, 1996).

#### **2.3.2.6 Sweetpotato butterfly (*Acraea acerata*)**

The adults lay many eggs on both surfaces of the leaves. The larvae are greenish-brown and hide from sunlight during the day while the adults have orange wings with black margins. The larvae feeds on the leaves and in severe infestation, they can cause complete defoliation (Coleacp, 2011). This is a pest in East and Central Africa and can be controlled by biological means such as the use of *Beauveria bassiana* or by early planting and harvesting. Pyrethrin is also effective in controlling them (Ames *et al.*, 1996).

### 2.3.3 Disease constraints in sweetpotato production

#### 2.3.3.1 Sweetpotato viral diseases

Sweetpotato feathery mottle virus (SPFMV), sweetpotato chlorotic stunt closterovirus (SPCSV) and sweetpotato virus disease (SPVD) are commonly occurring viruses. The disease is caused by a combination of virus diseases, SPFMV and SPCSV. SPFMV are aphid-transmitted potyviruses and symptoms are largely lacking but they can occur on susceptible cultivars, particularly under increase in stress level or stage of growth and virulence of the strain (Coleacp, 2011). The common strain causes no symptom, but a strain known as the "russet crack" causes external necrotic lesions or internal corking on certain varieties, but SPFMV can be latent in vines. It occurs worldwide and can be controlled by planting disease-free material (Ames *et al.*, 1996). When SPCSV occur the plants become weak, stunted and the leaves appear wavy with a chlorotic mottle (Gibson *et al.*, 1998). In SPVD, diseased plants are generally severely stunted with narrow leaves. It occurs in many parts of the world, and it can be controlled by planting disease-free material (Ames *et al.*, 1996).

#### 2.3.3.2 Alternariosis, anthracnose, blight (*Alternaria bataticola*)

This is a fungal disease and symptoms normally occur on older leaves causing brown lesions and appearance of concentric rings, while black lesions appear on petioles and stems (Coleacp, 2011). The ground under affected vines is often covered with black leaf debris and vine deaths then occur. The severity of the disease increases with increase in altitude, which is essential for infection while sporulation at such altitudes is due to the high relative humidity. The fungus spreads through infected planting material, wind and

rains. The disease occurs mostly in East Africa and Brazil; control is achieved by planting clean material (Ames *et al.*, 1996).

#### **2.3.3.3 Black rot (*Ceratocystis fimbriata*)**

This is a fungal disease that usually develops dark sunken cankers at the lower part of sweetpotato stem followed by yellowing of plant and finally to death. The affected storage roots develop black sunken areas with fungal structures protruding from the root surface producing a foul smell. The storage roots may not show the symptoms when harvesting but emerge later during post-harvest storage (Coleacp, 2011). Transmission is through wounds caused by insects and planting infected material. It is prevalent mostly in Asia and Oceania; can be controlled mainly by planting disease-free material and crop rotation (Ames *et al.*, 1996).

#### **2.3.3.4 Soft rot (*Rhizopus stolonifer*)**

This is a fungal disease and it is associated with post-harvest storage of sweetpotato roots. The storage roots become soft, wet, and stringy, from one end and have a strong alcohol-like odour (Coleacp, 2011). The fungus sporulates on sweetpotato storage roots and is spread through wounds and infested soil but resistance may vary with variety even though there is no resistant variety identified yet. This disease is found worldwide over a wide range of hosts including sweetpotato. It attacks the fleshy organs of plants that are rich in sugar or starch and can be controlled by washing storage roots and proper curing (Ames *et al.*, 1996).

## **2.4 Sweetpotato regeneration**

### **2.4.1 Plant tissue culture**

Tissue culture is regeneration of whole plants using tissues and organs as propagules or explants in nutrient-rich media under sterile conditions. Plants cells, tissues or organs under appropriate conditions are able to initiate cell division and grow to mature plants. The ability of plants to regenerate through tissue culture and express the total genetic potential of the parent plant is known as totipotency (Ribnicky *et al.*, 2002). To achieve this however, optimal culture conditions should be in place, and for sweetpotato, like many other plant species, this varies with genotype. The tissue culture conditions to be considered include growth medium and external environment such as light and temperature. The growth medium normally contains macro- and micro-nutrients essential for growth and development, plus vitamins, amino acids and a fixed carbon source, although this differs with the experiment. Murashige and Skoog (1962) worked out the composition of the nutrient elements required for plants to regenerate and are now widely used.

### **2.4.2 Factors affecting *in-vitro* plant regeneration**

#### **2.4.2.1 Plant growth regulators**

Plant growth regulators (PGR) are critical media components, which are essential in defining the pathway through which growth will take place in plant cells or explants. Naturally, plant hormones are produced in small quantities and they are responsible for specific physiological effects in plants. PGRs are classified into several groups namely gibberellins, abscisic acid, ethylene, auxins and cytokinins with the latter two most commonly used in plant regeneration experiments. In tissue culture, PGRs influence

growth of explants in several ways but several aspects need to be established for them to be used successfully. They include endogenous levels present in explant tissues and the amount of exogenous levels supplied (Gahan and George, 2008).

#### **2.4.2.1.1 Gibberellins**

Gibberellins are involved in regulating cell elongation and important components in determining plant height and fruit-set. There are a number of known gibberellins but GA<sub>3</sub> is the most commonly used (Davies, 2004) and although their mode of action is not fully understood, they seem to have effects similar to auxin in tissue culture (Brian, 1959).

GA<sub>3</sub> is known to enhance growth of callus cells and prevent growth of somatic embryos but it has been documented to promote shoot formation once meristems are formed (Moshkov *et al.*, 2008).

#### **2.4.2.1.2 Abscisic acid and ethylene**

Abscisic acid inhibits cell division and most commonly used to promote developmental pathways such as those that are involved in somatic embryogenesis (Rai *et al.*, 2011).

Ethylene, on the other hand, is involved in controlling fruit ripening and leaf abscission; however it is not widely used in plant tissue culture media. Some plant cells produce ethylene and it has a tendency of building up in the culture container thus inhibiting growth and development of the cultured plant tissues (Kumar *et al.*, 1998).

#### **2.4.2.1.3 Auxins**

Auxins have many functions such as promoting cell elongation, adventitious root initiation, lateral root development, shoot elongation and root swelling (Treatise, 1972).

Auxins also promote differentiation of buds, roots, xylem and phloem; at higher

concentrations they stimulate ethylene production, combination with cytokinins they promote cell division in callus cultures and inhibit root growth due to ethylene (Machakova *et al.*, 2008). At low concentrations, the auxins promote growth of intact roots (Salisbury and Ross, 1986; Machakova *et al.*, 2008).

Some of the natural occurring auxins include Indole-3-acetic acid (IAA), 4-chloroindole-3-acetic acid (4-Cl-IAA) and 2-phenylacetic acid (PAA). The synthetic auxins include 2,4-Dichlorophenoxyacetic acid (2,4-D); indole-3-butyric acid (IBA);  $\alpha$ -naphthalene acetic acid ( $\alpha$ -NAA); 2,4,5-trichlorophenoxyacetic acid (2,4,5-T); 2-methoxy-3,6-dichlorobenzoic acid; 2-naphthylacetic acid (NOA); 4-amino-2,5,6-trichloropicolinic acid, (picloram); 4-fluorophenoxyacetic acid (4-FA) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) (Salisbury and Ross, 1986; Machakova *et al.*, 2008).

Auxins are primarily synthesized in young leaves, shoot and root apices (Chrispeels and Sadava, 2003). These regions have the highest concentrations of free auxin but they are widely distributed throughout the plant. The naturally produced auxins are synthesized through multiple pathways in plants thus suggesting their importance in plants (Salisbury and Ross, 1986). In young leaves, high concentrations of auxins occur along the leaf margins and then move towards the mid lamina through basipetal transport (Baker, 2000). This mode of transport from young leaves is known as polar transport, which is only known to occur for both natural and synthetic auxins (Salisbury and Ross, 1986). Those produced in roots move through a cell to cell pathway known as acropetal (Torrey, 1976). The mature leaves also synthesize IAA and then are transported to the rest of the

plant through non-polar transport via the phloem (Baker, 2000). Synthetic auxins like 2,4-D move quickly from leaves into roots (Torrey, 1976).

Auxins occur either as free or conjugated forms. The free form is active while conjugated form is inactive and is covalently bound to glucose, myoinositol, amide conjugates or glycol-proteins, which require activation by specific enzymes (Bajguz and Piotrowska, 2009). Free auxin is released through metabolism of the conjugated forms whereas the conjugated forms store and protect auxins against oxidative degradation.

In plant tissue culture, natural auxins have limited use due to instability. This is because they are easily destroyed by oxidation, high light intensity, hydrolase and/or oxidases enzymes (Dunlap and Robacker, 1988), and *in vitro* by plant pigments such as riboflavin (Ray, 1985). Synthetic auxins, on the other hand, are most commonly used and are effective in tissue culture since they are not quickly metabolized by the plant as compared to natural auxins (Salisbury and Ross, 1986). Since they are also not destroyed by oxidases they persist in plants (Salisbury and Ross, 1986). Synthetic auxins are widely used in sweetpotato regeneration and transformation (Oggema *et al.*, 2007; Santa-Maria *et al.*, 2009).

#### 2.4.2.1.4 Cytokinins

They stimulate cell division, morphogenesis, release apical dominance and enhance stomatal opening (Treatise, 1972). They also delay leaf senescence and root formation in the presence of auxins (Chrispeels and Sadava, 2003). In tissue culture the ratio of auxin to cytokinin affects differentiation of calli, where high auxin: cytokinin stimulates root formation while low auxin: cytokinin stimulates shoot formation (Gaspar *et al.*, 2003).

There are two kinds of cytokinins, the adenine-type and phenylurea-type. Although the root is the major site of synthesis, the adenine-types are also found in stems and leaves (Baker, 2000). Adenine-types include kinetin, zeatin and benzylaminopurine (BAP) while phenylurea-types include diphenylurea and thidiazuron (TDZ). Kinetin and BAP are most commonly used cytokinins in plant tissue culture media as opposed to zeatin because the latter is expensive and relatively unstable. Cytokinins are highest in meristematic regions and areas of continuous growth such as the roots, young leaves, developing fruits and seeds (Salisbury and Ross, 1986). Although cytokinins are high in meristematic regions, roots are the key sites for cytokinin synthesis (Baker, 2000).

Zeatin is a naturally occurring cytokinin (Miller, 1965) while kinetin is the synthetic analogue, which was discovered first and known to regulate cell division in presence of an auxin (Miller *et al.*, 1955). Zeatin occurs in cis-and trans- forms in higher plants, which can be interconverted by zeatin isomerase. The latter form of zeatin is more biologically active (free) than the cis form. The free forms are mainly synthesized in root apical meristems and move through the xylem into the shoot in form of zeatin ribosides (Baker, 2000). When they reach the leaf cells they are converted to both free-base form and glucosides (Saha and Sircar, 1990). Presence of high levels of free naturally occurring cytokinins, are regulated and limited by cytokinin oxidase unlike the synthetics (Mohr and Schopfer, 1995).

#### **2.4.2.2 Effect of explants used and genotype**

Explants play a big role in many plant tissue culture regeneration experiments. They have been shown to determine the ability to regenerate various species or genotypes such as

rice (Hoque and Mansfield, 2004). A similar scenario is also replicated in sweetpotato. Depending on the objective of the regeneration experiment, different types of explants such as leaf with petiole, leaf, petiole, stem, protoplasts and meristems can be utilised (Al Mazrooei *et al.*, 1997; Okada *et al.*, 2002).

Regeneration has been shown to be influenced by genotype. Same hormone combinations or any other treatment given to various genotypes will produce different results. This has also been demonstrated in other crops such as rice (Yookongkaew *et al.*, 2007). The same has been shown in sweetpotatoes (El Far *et al.*, 2009; Santa-Maria *et al.*, 2009).

### **2.4.3 Tissue culture techniques**

#### **2.4.3.1 Regeneration by organogenesis**

The process involves initiation of adventitious shoots either directly or indirectly from explants or callus, respectively. Direct organogenesis occurs by direct induction of plantlets from explants; while indirect happens when explant forms calli then to plantlets. Plants formed through organogenesis come from adventitious buds after initiation (Gahan and George, 2008). Organogenesis depends on plant plasticity as well as components in the media, for example, a high auxin to cytokinin ratio favours root formation while high cytokinin to auxin ratio favours shoot formation (Gahan and George, 2008). Plant regeneration by organogenesis is highly dependent on endogenous and exogenous levels of PGR (Gaspar *et al.*, 2003). It is therefore a prerequisite to empirically determine the optimum levels and combination of PGRs required for induction of organogenesis.

### **2.4.3.2 Regeneration by somatic embryogenesis**

Induction of embryos from non-zygotic cells with two major steps of initiation and maturation is referred to as somatic embryogenesis (Jimenez, 2001). Embryogenesis could take the direct or indirect route wherein the former embryos are produced directly from cells while the in the latter, callus is first induced before somatic embryos are initiated. Embryos progress through several stages from the globular to heart-shape to the torpedo shapes and finally to form the apical meristems (Von Arnold, 2008).

## **2.5 Plant genetic transformation**

Genetic transformation is the introduction and expression of foreign genes in a new host such as crop of interest to improve a certain trait(s) or simply for investigation of gene function (Birch, 1997). There are two methods of plant transformation namely, the direct and indirect transformation. The direct method includes electroporation (Hassanein *et al.*, 2007) and micro-projectile bombardment (Okada *et al.*, 2002). Indirect methods are through viral-mediated phage transfer (Chung *et al.*, 2005) and *Agrobacterium*-mediated transfer (Opabode, 2006). The techniques have been used in crop improvement programs such as banana (Tripathi *et al.*, 2010). Sweetpotato has been transformed using micro-projectile bombardment (Okada *et al.*, 2002) and *Agrobacterium*-mediated transfer (Song *et al.*, 2004).

## **2.6 Direct transformation methods**

### **2.6.1 Electroporation**

In electroporation, an electrical pulse is used to increase cell membrane permeability of the host increasing the chances of gene transfer. The advantages of this method are that

specialized vectors are not required and it works over a wide range of cells (Sambrook and Russell, 2001). The disadvantages are: low frequency of stable transformation, very high re-arrangement of DNA and for plants it can only be used in protoplasts.

### **2.6.2 Biolistics or micro-projectile**

Biolistics or micro-projectile bombardment involves rapid propelled tungsten or gold micro projectiles coated with DNA blasted onto cells, explants or calli. The advantages include exclusion of specialized vectors and could be used on a wide range of plants. One of the disadvantages include the foreign gene could be inserted within functional genes which could lead to gene silencing (Zawleski *et al.*, 2012).

## **2.7 Indirect transformation methods**

### **2.7.1 Virus-mediated gene transfer**

Viral-mediated transfer involves viruses such as retro-viruses and is mostly used in animal science. The advantage of viral vector is that it can be used with high efficiency; however, it is not practical in producing stable introgression of foreign genes in plants (Akuta *et al.*, 2002).

### **2.7.2 *Agrobacterium*-mediated gene transfer**

*Agrobacterium*-mediated transformation of plants involves transfer of genes into cells and subsequent regeneration of transgenic plants. *Agrobacterium* is a soil bacterium that naturally infects wounds and transfers part of its DNA (transfer DNA) into the host plant. The T-DNA is stably inserted into the host plant, which induces formation of crown gall tumours (Riva *et al.*, 1998). Scientists took advantage of its ability to transfer T-DNA into plant cells and replaced the oncogenes with genes of interest to enable artificial

transfer into plant cells (Riva *et al.*, 1998); this revolutionised crop improvement through gene transfer. The T-DNA transfer process is initiated when *Agrobacterium* detects presence of phenolics, which serve as inducers of virulence (*vir*) genes (Gelvin, 2003). The advantages of *Agrobacterium*-based method are low copy number of inserts, relatively efficient and huge foreign DNA can be inserted into plants (Gelvin, 2003). The disadvantage is a pre-requisite to develop a plant regeneration protocol that is amenable to this mode of transfer (Heeres *et al.*, 2002), which could be a challenge since most protocols are genotype dependent. *Agrobacterium*-mediated transfer has successfully been used in crops including rice (Ming-Xia *et al.*, 2005) and sweetpotato (Moran *et al.*, 1998).

## 2.8 Requirements for transgene expression

The requirements include a gene construct made up of various elements to enable transfer of genes into the target organism. The elements of vector constructs are candidate gene, promoter and polyadenylation signal sequence (Collier *et al.*, 2005).

### 2.8.1 Candidate genes

Candidate genes are genes of interest required to improve or worsen a certain trait(s) or specific to study gene function or placement within plant cells; for instance, inserting weevil resistance genes *cry3a* into a susceptible alfalfa plants to have in-built resistance against weevils (Tohidfar *et al.*, 2013). Reporter genes could also be candidate genes used to screen for presence of the introgressed gene (Jefferson *et al.*, 1987). Some widely used reporter genes include Green Fluorescent Protein (Jiang *et al.*, 2008) and  $\beta$ -glucuronidase, commonly known as GUS (Xing *et al.*, 2007).

### 2.8.2 Promoters

Promoters are used to regulate or drive genes (Jiang *et al.*, 2008). They are sequences of DNA called TATA box as binding sites for transcription factors in some promoters and in some classes of eukaryotes, they are usually located 30 bp upstream of transcription initiation sites and are conserved in most species (Pedersen *et al.*, 1999). Promoters could either be constitutive or inducible where constitutive promoters are typified by cauliflower mosaic virus (CaMV) 35S sequences (Yoo *et al.*, 2005) while some inducible promoters include sporamin (Wang *et al.*, 2002). CaMV-35S has been widely used in driving genes in plant species (Song *et al.*, 2004). Sporamin and  $\beta$ -amylase are storage proteins in sweetpotato roots and they make up between 60-80% of total soluble protein. Sporamin and  $\beta$ -amylase also occur in other parts of sweetpotato plants such as leaves in small amounts in presence of sugar (Maeo *et al.*, 2001). The sporamin and  $\beta$ -amylase promoters have been used in tobacco (Ohto *et al.*, 1992) and barley (Kihara *et al.*, 2000).

### 2.8.3 Selectable markers

These are genes expressing enzymes that are capable of metabolising certain substrates incorporated in selective media. Selectable markers are incorporated to the gene construct for successful identification of plants with integrated transgenes. Examples of selectable markers include those that encode for enzymes to metabolise agents that are toxic to plants such as kanamycin (Burgos and Albuquerque, 2003) and herbicide tolerance such as bar gene (Zang *et al.*, 2009).

## 2.9 Phytoalexins

Plants sacrifice tissues that are attacked by microbes, inorganic and organic chemical agents as defence mechanisms (Kuc, 2001) by eliciting production of a substance known as phytoalexins with known antimicrobial properties. The phytoalexins are produced in healthy tissues adjacent to necrotic tissues and they accumulate around both resistant and susceptible necrotic tissue (Agrios, 2005). Different plant families elicit production of different phytoalexins: dihydrophenanthrenes by orchidaceae family, sesquiterpenes and diterpenes by solanaceae family, polyacetylenes by compositae family, isoflavonoids by leguminosae and furanoterpenoids by convolvulaceae (Greisbach and Ebel, 1978). In addition, within these families, plants also produce specific phytoalexins, for example, Leguminosae family has alfalfa (*Medicago sativa*) that produces sativan, while soybean (*Glycine max*) produces glyceollin (Ahuja *et al.*, 2012).

Health related effects caused by the phytoalexins differ according to the plant species. For instance, garden pea (*Pisum sativa*) produces pisatin after fungal infection and it has been shown to have toxicity tendencies to mammals (Surak, 1978). Glycoalkaloids are associated with white potatoes (*Solanum tuberosum*) and are shown to cause birth defects in unborn children (Surak, 1978). On the other hand, furanoterpenoids are associated with sweetpotato and are known to cause lung oedema particularly 4-ipomeanol (Clark *et al.*, 1981), whereas ipomeamarone (*ipm*) causes hepatotoxicity and death to farm animals (Shen, 1997).

Microbes that elicit production of furanoterpenoids such as *ipm* have been identified, studied and classified. The fungi enter the host mostly through holes made by insects

(Moliszewska and Wisniewski, 2006) such as weevils which burrow through the storage roots. Infection of sweetpotato roots by microbes elicits production of phytoalexins at various levels. For instance, the low level inducers produce less than 100  $\mu\text{g/g}$  of the furanoterpenoid. These are namely *Streptomyces ipomoea* (bacterium) and *Meloidogyne incognita* (nematodes). The moderate level (100 to 1000  $\mu\text{g/g}$ ) inducers includes *Rhizopus stolonifer*, while high level inducers ( $>1000$   $\mu\text{g/g}$ ), which are mainly fungi include *Ceratocystis fimbriata* and *Fusarium oxysporum* (Clark *et al.*, 1981).

Identification of fungus infesting sweetpotato roots can be undertaken either through morphological or molecular characterization. Microscopes are used for distinguishing structures placed on a glass slides for morphological characterization. In the case of molecular characterization, some methods used for species identification include use of simple sequence repeats (SSR) and internal transcribed spacers (ITS). SSRs are repeat sequences which occur in tandem and are mostly flanked by conserved DNA (Scott and Chakraborty, 2008). ITS region, on the other hand, is located on ribosomal RNA (rRNA): the most conserved gene in cells and genes coding for rRNA occur as rDNA repeat units (Eickbush and Eickbush, 2007). ITS consists of ITS1, ITS2, 5.8S of about 700 bp long. ITS1 is located between the small sub-unit 16S-18S and 5.8S: ITS2 is located between 5.8S and large sub-unit 23S and 28S of the rRNA regions in fungi (White *et al.*, 1990). The ITS coding region is not translated into proteins but plays an important role in development of functional rRNA (Baldwin *et al.*, 1995; Liston *et al.*, 1996). It is shown to have a higher degree of variation compared to other regions of rDNA and many studies have been undertaken at this region hence used for fungal identification (Schoch *et al.*, 2012).

## CHAPTER THREE

### DEVELOPMENT OF A REGENERATION PROTOCOL THROUGH INDIRECT ORGANOGENESIS OF SELECTED SUB-SAHARAN AFRICAN SWEETPOTATO CULTIVARS

#### 3.1 INTRODUCTION

Organogenesis is a process through which cells are converted directly or indirectly into meristemoids from which organs are formed (Hicks, 1994). The main advantage of organogenesis is that it takes a relatively short time of 4-8 weeks to produce regenerants (Gosukonda *et al.*, 1995) when compared to somatic embryogenesis of about 8 months (Manrique *et al.*, 2013). Two methods of organogenesis include the direct and indirect, where in the former, plantlets are regenerated directly from explants while in the indirect method, explants produce calli first before regenerants are produced (Gahan and George, 2008). The indirect method was chosen for this study because the established protocol would be used in transformation experiments to generate transgenic events: when calli are produced after transformation, regenerants emerge from calli but the regenerants produced above the calli (direct organogenesis) are not transgenic.

Through indirect organogenesis, high regeneration efficiencies (RE) of between 50 to 80% have been reported for certain sweetpotato cultivars (Gonzalez *et al.*, 2008; Santa-Maria *et al.*, 2009; El Far *et al.*, 2009). However, there are also reports in literature indicating that attempts to regenerate sweetpotato through organogenesis failed to produce regenerants. For example, Porobo-Dessai *et al.* (1995) reported that out of 27 American cultivars, 9 failed to regenerate through organogenesis. Organogenesis has been reported mainly on cultivars from USA and one from Egypt; however, there are

very few reports on Sub-Saharan Africa (SSA) cultivars (Blasco, 2007; Ormachea, 2008). In another study by Kasili (1998) conducted using sweetpotato cultivars Kemb10, KSP20 and KSP11, node explants were reported as the best compared to leaves, internodes and petioles in shoot regeneration via direct organogenesis but they used different cultivars for indirect organogenesis. In the current study, an efficient regeneration protocol using leaf with petiole explants via indirect organogenesis of some SSA sweetpotato cultivars was established from a preliminary screen of 4 different explants including leaf, stem, petiole and leaf with petiole.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 Plant material**

A total of 32 sweetpotato cultivars were used in regeneration experiments. Out of the 32 cultivars, 30 SSA and 1 USA cultivar, Jewel, were acquired from the Gene Bank at the International Potato Centre (CIP) Lima, Peru, whereas Ukerewe cultivar was obtained from the Kenya Plant Health Inspectorate Service (KEPHIS), Nairobi, Kenya. Jewel cultivar was included as a control due to its ease in regeneration.

The cultivars were selected based on consumer preferences, long or short maturity period (Table 3.1) and susceptibility to weevil damage. Seven Kenyan cultivars were included including Kemb10, Gikanda, SPK004, SPK013, Muibai, KSP11 and Kemb37 where the first 4 cultivars are widely grown and have high consumer acceptability. Kemb10 is early maturing, has good taste but it is not drought tolerant and has poor in-ground storability. SPK004 cooks fast, has good orange flesh colour therefore liked by children, but has poor yield and poor in-ground storability. SPK013 is early maturing, has big roots and good taste but does not withstand drought as well (Ndolo *et al.*, 2001).

The study included 5 Tanzanian cultivars namely Budagala, Mwanamonde, Sinia, SPN/O and Ukerewe. Ukerewe produces large roots with high yields therefore accepted by farmers due to marketability (Masumba *et al.*, 2004). The research also included three Zambian cultivars namely, Luapula, Zambezi and Chingowva: Luapula had low acceptance and scarcity of planting material, Chingowva was preferred for boiling or eating raw while Zambezi for eating raw and making jams (Dr. Martin Chiona, *pers. comm*). Four Burundian cultivars namely Mohc, Rusenya, Luby and Imby were also selected. Mohc performs better than locally-grown cultivars while Rusenya was high yielding but abandoned in the 1990s due to infection by sweetpotato virus disease (SPVD). Luby is highly susceptible to SPVD and was therefore abandoned one year after its release, whereas Imby is not popular though virus resistant. Kamchiputu was a Malawian cultivar, popular in the 1980s due to good flavour but rarely grown currently due to late maturity and low yields (Michel Ntimpirangeza, *pers. comm*) (Table 3.1).

**Table 3.1: Characteristics of selected SSA sweetpotato cultivars used in regeneration experiments through organogenesis**

| Cultivar   | Country of origin | Leaf shape          | Flesh color | Rooting type | Resistance to SPVD | Maturity (mths) | DM (%) |
|------------|-------------------|---------------------|-------------|--------------|--------------------|-----------------|--------|
| Kemb 10    | Kenya             | Hastate 3 lobes     | Yellow      | Deep         | S                  | 4.5             | 28.5   |
| SPK004     | Kenya             | Moderate 4 lobes    | Orange      | Deep         | M                  | 3.5             | 30.6   |
| SPK013     | Kenya             | Triangular          | Yellow      | Deep         | S                  | 4               | 34.5   |
| Muibai     | Kenya             | Triangular 3 lobes  | Cream       | Deep         | M                  | 5.5             | 28     |
| Gikanda    | Kenya             | Triangular          | Cream       | Deep         | M                  | 5.5             | 31.5   |
| Mafutha    | Kenya             | Moderate 3 lobes    | Yellow      | Deep         | M                  | 5.5             | 27     |
| KSP11      | Kenya             | Triangular          | Cream       | Deep         | M                  | 5.5             | 29     |
| Kemb 37    | Kenya             | Triangular          | Cream       | ND           | ND                 | 4               | 28     |
| Zambezi    | Zambia            | Triangular          | Orange      | Deep         | M                  | 4               | 23     |
| Luapula    | Zambia            | Triangular          | White       | ND           | ND                 | 3.5             | 26     |
| Chingowwa  | Zambia            | 3 deep lobes        | White       | ND           | ND                 | 5.5             | 25     |
| Mugande    | Rwanda            | 3 very slight lobes | White       | Deep         | M                  | 4.5             | 29.7   |
| K51/3251   | Rwanda            | Triangular          | Cream       | ND           | ND                 | ND              | ND     |
| Rusenya    | Rwanda            | Triangular          | Cream       | ND           | S                  | ND              | ND     |
| Malawiala  | Rwanda            | Triangular          | Cream       | ND           | ND                 | 4               | ND     |
| Kawoga     | Uganda            | Triangular          | Cream       | Deep         | S                  | 4               | 32     |
| New Kawoga | Uganda            | Moderate 3 lobes    | White       | Deep         | HR                 | 4.5             | 32     |
| Bwanjule   | Uganda            | 5 very deep lobes   | White       | Deep         | R                  | 4               | 30     |
| Budagala   | Tanzania          | Moderate 3 lobes    | Orange      | ND           | ND                 | 4               | 30     |
| Mwanamonde | Tanzania          | Moderate 5 lobes    | White       | Deep         | ND                 | 4               | 32     |
| Sinia      | Tanzania          | Triangular          | Cream       | Deep         | ND                 | 4               | 34     |
| SPN/O      | Tanzania          | Moderate 3 lobes    | Yellow      | Shallow      | R                  | 3.5             | 32     |
| Ukerewe    | Tanzania          | Triangular          | Cream       | Shallow      | M                  | >7              | 35     |
| Kamchiputu | Malawi            | Triangular          | Orange      | Deep         | S                  | 4.5             | 35     |
| Chifukama  | Congo             | Triangular 3 lobes  | Yellow      | ND           | R                  | 5.5             | ND     |
| Chihongo   | Congo             | Triangular          | Yellow      | ND           | R                  | 4.5             | ND     |
| Chiuva     | Congo             | Triangular          | White       | ND           | R                  | 4.5             | ND     |
| Namagizi   | Congo             | Triangular          | Cream       | ND           | R                  | ND              | ND     |
| Rusenya    | Burundi           | Triangular          | cream       | ND           | S                  | ND              | ND     |
| Mohe       | Burundi           | Triangular          | Cream       | ND           | ND                 | ND              | 20     |
| Luby       | Burundi           | Moderate 3 lobes    | Yellow      | ND           | R                  | ND              | 22     |
| Imby       | Burundi           | Triangular          | Orange      | Deep         | R                  | >7              | ND     |
| Jewel      | USA               | Triangular          | Orange      | M            | S                  | 5               | 26     |
| Carrot     | Tanzania          | Moderate 3 lobes    | Orange      | M            | M                  | 4               | 33     |

ND means information unavailable: DM is dry matter: S is susceptible: R is resistant: M is moderate susceptible: SPVD is sweetpotato virus disease

### 3.2.2 Propagation and maintenance of sweetpotato stock plants

*In-vitro* cultured disease-free plantlets obtained from CIP Gene bank and KEPHIS were propagated. The propagation process was initiated using 5 to 6 week old plantlets as the source of explants; these plantlets were cut into 3 explants at the nodes and cultured in liquid propagation media consisting of Murashige and Skoog (1962) (MS) basal medium

containing sucrose (3% w/v) supplemented with ascorbic acid (23 mM), arginine (0.11 M); putrescine (0.03 M), gibberrellic acid (6 mM) and calcium pantothenate (0.8 mM) at pH 5.8. Four cuttings were placed per one Magenta<sup>®</sup> box containing 100 ml propagation medium and then incubated in a growth room at 27°C under 16 hours daylight (3000 lux) and 8 hours darkness for 3 to 4 weeks. To maintain a continuous supply of explants, 3 to 4 node explants were sub-cultured in fresh MS propagation medium after every 5 to 6 weeks as described by Nopo *et al.* (2005).

### **3.2.3 Regeneration through organogenesis**

Two preliminary screening experiments were conducted: (i) to confirm and establish the best explant for regeneration of SSA cultivars. To find out if leaf with petiole would be the best explant-type for SSA cultivars, a preliminary screen was conducted using cultivar Ukerewe to determine the influence of explant-type on regeneration efficiency (RE). Ukerewe cultivar was used due to its ease of acquisition. The explant-type tested were stem, leaf and leaf with petiole obtained from 3-4 week old *in-vitro* cultured stock plants. (ii) To determine the best kinetin concentration for induction of organogenesis in selected SSA sweetpotato cultivars. Regeneration through organogenesis was initiated using leaf with petiole explants based on a study by Gosukonda *et al.* (1995) and above-mentioned preliminary experiment conducted using cultivar Ukerewe. Determination of RE using leaf with petiole explant alone was conducted for 7 SSA cultivars namely Imby, Luapula, Ukerewe, Zambezi, Mafutha, Mugande and Carrot using different kinetin concentrations. The explants were subjected to a 2-step culturing process described by Gosukonda *et al.* (1995).

In step 1, explants were inoculated onto semi-solid MS medium containing sucrose (3% w/v), gelrite<sup>®</sup> (2 g/L) supplemented with 2,4-D (0.05 mg/L) for 2 to 4 days depending on cultivar. Then explants with swollen petioles were transferred to semi-solid MS media supplemented with kinetin at 3 concentrations 0, 0.2 and 0.4 mg/L. The explants were cultured for 60 days with 14-day sub-cultures in step-2 media or until they developed regenerants. The regenerants were then transferred to propagation media for 30 days and then transplanted to the greenhouse for storage root production. Based on the results obtained in the second preliminary screen, R.E of the remaining 26 cultivars (Table 3.1) was evaluated using 0.2 mg/L zeatin, kinetin and thidiazuron, each cytokinin separately, using leaf with petioles as explants. The RE were calculated as follows

$$\text{Regeneration efficiency} = \frac{\text{Number of explants producing regenerants}}{\text{Total number of explants}} \times 100$$

### 3.2.4 Data collection and analysis

In all the experiments conducted there were 4 petri-dishes per treatment, with each cultivar having 5 explants per petri-dish. Each experiment was repeated 3 times and a total of 60 explants per cultivar. A sample size of 60 was chosen because sweetpotato regeneration is genotype-dependent and a larger sample would increase chances of obtaining representative regeneration efficiency.

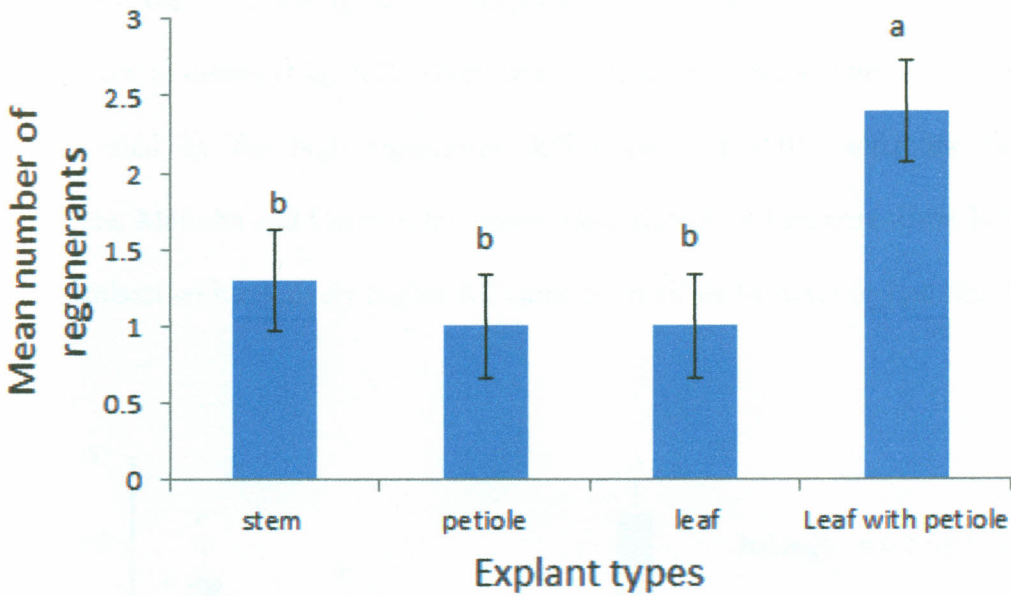
In each replicate experiment conducted data was collected at every 7 days after culture (DAC) for a period of 60 days. Depending on the DAC and experiment the data collected included number of explants with callus, callus colour, number of regenerants per explant and presence of roots and their length.

The data collected was subjected to analysis of variance (ANOVA) using the generalised linear models (GLM) by logistic regression analysis provided by Genstat version 14 (Payne *et al.*, 2011). Regression models are used to establish the relationship between response and explanatory variates of both hormone and cultivar variations. The GLM, on the other hand, are used to cover the normal regressions to cater for non-normal distributions such as binomial data recording (Payne *et al.*, 2011). Regression was therefore used in this study because not all explants in the petri-dishes produced regenerants and hence normal ANOVA was unfavourable. Post ANOVA analysis using Student Newman Keul's test.

### **3.3 RESULTS**

#### **3.3.1 Effect of explant-type on regeneration efficiency**

Four explants (leaf, petiole, stem and leaf with petiole) of Ukerewe cultivar were compared for RE: leaf with petiole explants showed the highest RE. The results obtained show that stem explants had RE of 1.7%, petiole alone 3.3%; leaf alone 1.7% and leaf with petiole the highest RE of 40%. There seemed to be some effect of explant-type on overall RE although the analysis of variance (ANOVA) was  $p > 0.174$ , which could have been due to high variations in explants that produced regenerants (Appendix 1). Leaf with petiole explants were therefore used in subsequent experiments due to their high RE and post ANOVA analysis using Student Newman Keuls test results showed it to be different from the other explants (Fig. 3.1).

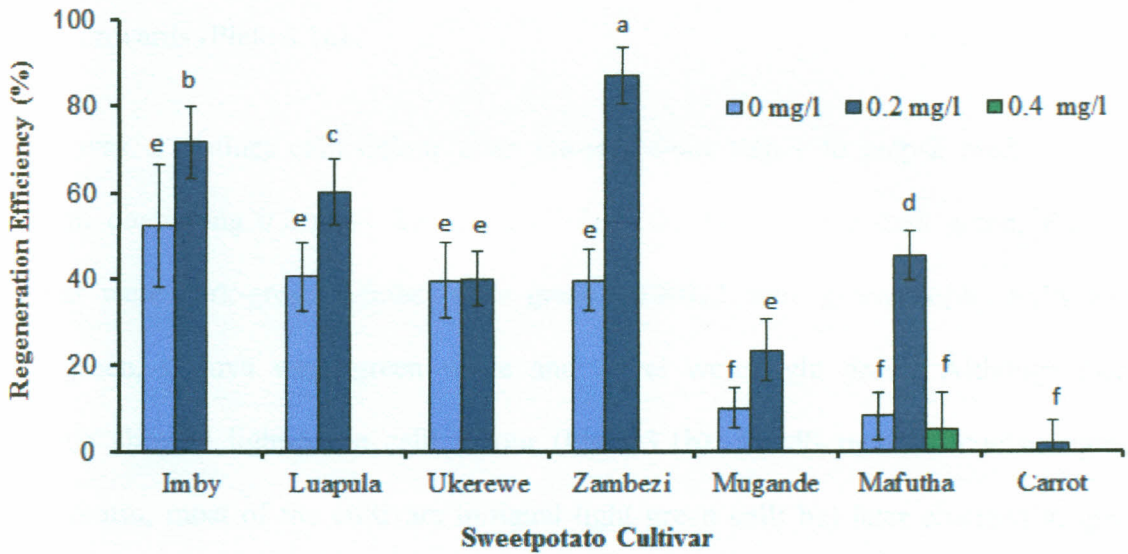


**Figure 3.1: Regeneration efficiencies of Ukerewe cultivar using 4 different explant-types.** The explants were first inoculated in step-1 medium and incubated for 2 to 3 days and then transferred to step-2 medium for 8 weeks. Values are means of 3 independent replicate experiments with 20 explants each and bars on the graphs represent standard error of means (SEM). The standard error of differences in means is 0.281 and used in classification of the different groups where 'a' had the highest values and was significantly different from b.

### 3.3.2 Effect of kinetin on RE for seven selected SSA cultivars

Preliminary experiments conducted using 3 kinetin concentrations (0, 0.2, 0.4 mg/L) on seven randomly selected SSA sweetpotato cultivars namely Ukerewe, Luapula, Zambezi, Mafutha, Mugande, Imby and Carrot led to identification of high and low regenerating cultivars. The highest RE was obtained when a concentration of 0.2 mg/L was used for all the cultivars under investigation: Zambezi cultivar had the highest RE of 86.7% while Carrot had the lowest RE of 1.7%. When no cytokinin was used, Imby and Ukerewe cultivars had highest RE of 36.7% and 35.1%, respectively. MS media with kinetin (0.4 mg/L) produced shoots for cultivar Mafutha alone, which resulted with an RE of 6.7% (Fig. 3.2). There was a significant difference between the concentrations and RE as

shown by the ANOVA ( $p < 0.01$ ) (Appendix 2) and post ANOVA for standard error differences in means (Fig. 3.2). There was some relationship between cultivar and origin as indicated by the high significant difference of  $p < 0.01$  (appendix 2): Ukerewe, Mugande, Mafutha and Carrot either came from Kenya or Tanzania while Imby, Luapula and Zambezi with relatively higher RE came from either Burundi or Zambezi (Table 3.1).



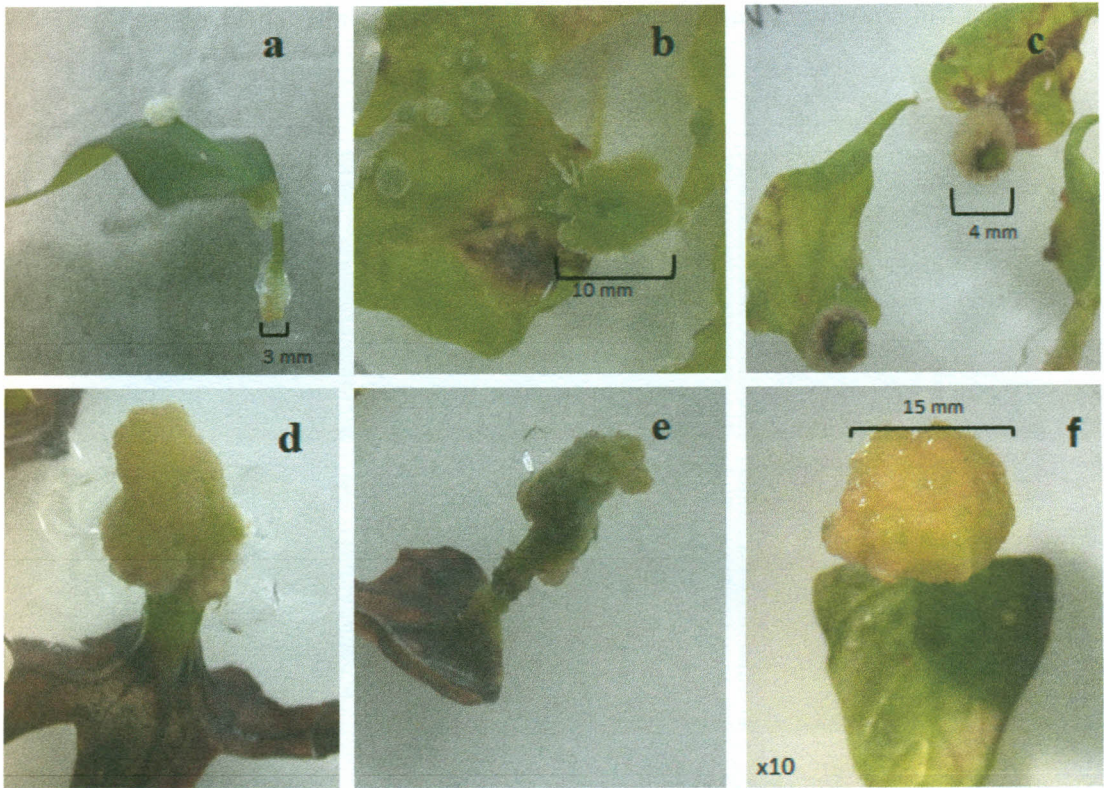
**Figure 3.2: Regeneration efficiencies of 7 SSA sweetpotato cultivars under varying kinetin concentrations.** Leaf with petiole explants were inoculated in step-2 medium consisting of MS basal salts supplemented with kinetin at 0, 0.2 and 0.4 mg/L for 8 weeks. Values are means of 3 independent replicate experiments with 20 explants each. Bars on the graphs represent standard error of means (SEM) of 2.86. The SEM was used in classification of the different means where ‘a’ had the highest values and was significantly different from b, c, d, e, f and g.

### 3.3.3 Effect of cytokinin on callogenesis of selected SSA cultivars

All the SSA cultivars in this study screened under 3 different cytokinins (kinetin, zeatin and thidiazuron) showed variations in calli formation. Swelling of petioles indicated initiation of callogenesis after culturing the explants in step-1 medium containing 0.05

mg/L 2,4-D (Plate 3.1a). Generally, petioles that were not swollen within 3 days in 2,4-D, did not produce any regenerants eventually. Observations made on swollen calli varied according to cultivar, for instance Sinia, Rusenya, Mwanamonde, Budagala, Gikanda, Kemb37 and KSP11 only had few swollen explants within three days of culture in step-1 medium. Transfer of the explants with swollen petioles to step-2 medium, resulted in increased calli size and also change in calli colour. The calli sizes started increasing from 2 days onwards (Plate 3.1a).

There was a distinct calli colour after transfer from step-1 to step-2 media. In MS medium containing 0.2 mg/L kinetin, calli for Kamchiputu were dark green, Zambezi cultivar were dark-green, Muibai were green, SPK013 were green/purple, Imby were light green, Chiuva were green white and Jewel were light green. Although many cultivars showed light-green calli colour (Plate 3.1b). In MS medium containing 0.2 mg/L zeatin, most of the cultivars initiated light green calli but later changed to green colour apart from Zambezi with friable purple/green calli while Budagala green/purple but spongy calli (Plate 3.1c). In MS medium containing thidiazuron (0.2 mg/L), most cultivars had light-green calli apart from Kemb10 and Zambezi whose calli were yellow, brown and necrotic 28 days of culture. Among the cytokinins used, after a period of 21 days in culture, leaves from explants for most cultivars started browning (Plate 3.1d,e) while some petioles developed relatively large-sized organogenic calli especially for media containing zeatin (Plate 3.1f).

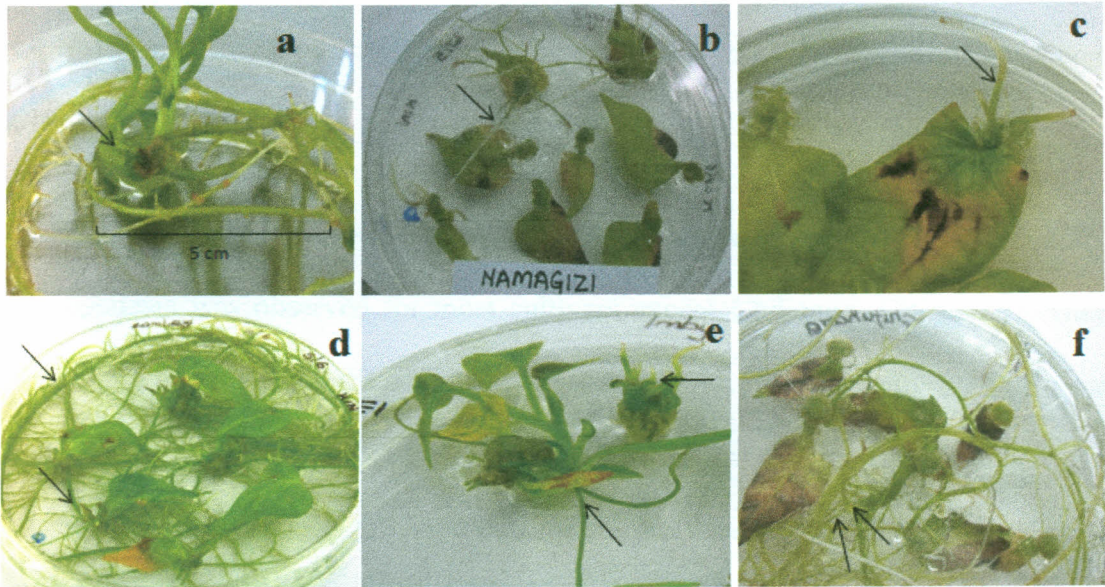


**Plate 3.1:** Variations in morphological display of calli in terms of size, colour and texture from sweetpotato cultivars (a) yellow green swollen petiole from leaf with petiole explant in 2 days in culture (b) Light green after 2 weeks (c) Spongy-looking calli with purple pigmentation after 3 weeks (d) Yellow-green with brown leaves after 4 weeks (e) Green-white with brown leaves (f) Organogenic calli after 4 weeks in culture.

### 3.3.4 Effect of cytokinin on rhizogenesis

Emergence of roots from calli that developed from leaf with petiole explants was observed during and after calli induction within 14 days of culture. The roots had similar colour to the callus produced (Plate 3.2a) and variations in root length were observed from those with no roots to those with long roots (Plate 3.2b). Some aerial root-like structures were also observed for some cultivars Malawiala, Luby and Gikanda cultivars (Plate 3.2c). The number of roots produced varied with cultivar such as Zambezi and

Imby with regenerants (Plate 3.2d,e) while Chifukama had long roots but no regenerants (Plate 3.2f).



**Plate 3.2:** Root production on calli and lengths of the SSA sweetpotato cultivars. **(a)** Root and callus with same color **(b)** Roots of less than 10 cm **(c)** Root-like structures growing upwards **(d)** Numerous long roots on explants that produced multiple shoots **(e)** Few long roots on explants that produced multiple shoots **(f)** Long roots but no shoots.

Different cultivars showed varying calli sizes but thidiazuron seemed to be consistently high for most of cultivars under investigation. For example, for media containing kinetin, Ukerewe cultivar showed mean sizes ranging between 0.15 to 0.2 cm; Imby had 0.25 to 0.3 cm while Luapula had 0.3 to 0.45 cm. for media containing zeatin, Ukerewe cultivar showed mean sizes ranging between 0.2 to 0.28 cm; Imby showed 0.4 to 0.45 cm while Luapula showed mean sizes ranging between 0.4 to 0.55 cm.

### **3.3.5 Caulogenesis on the SSA cultivars**

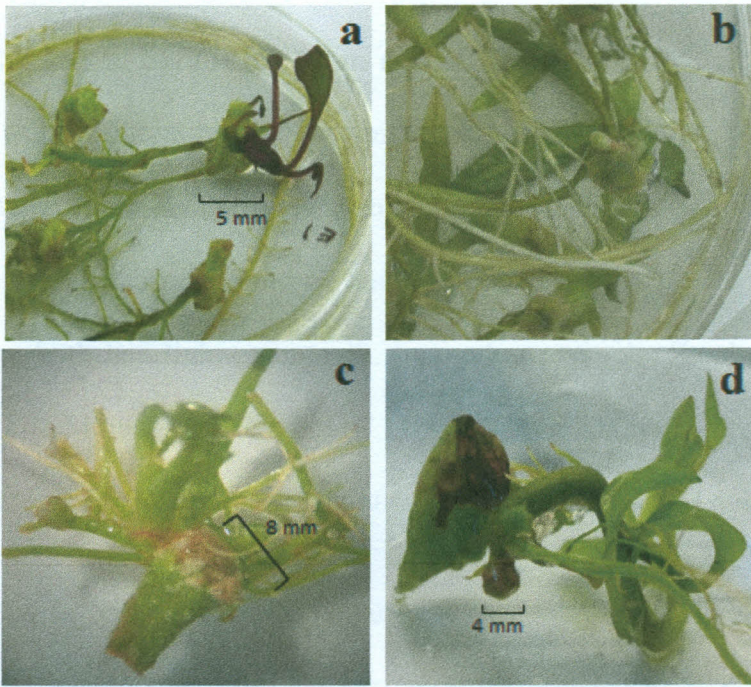
Caulogenesis is the production of regenerants from explants used in the tissue culture experiments. These regenerants were either produced on calli or from roots that emerged from the calli.

#### **3.3.5.1 Caulogenesis-types on the sweetpotato cultivars**

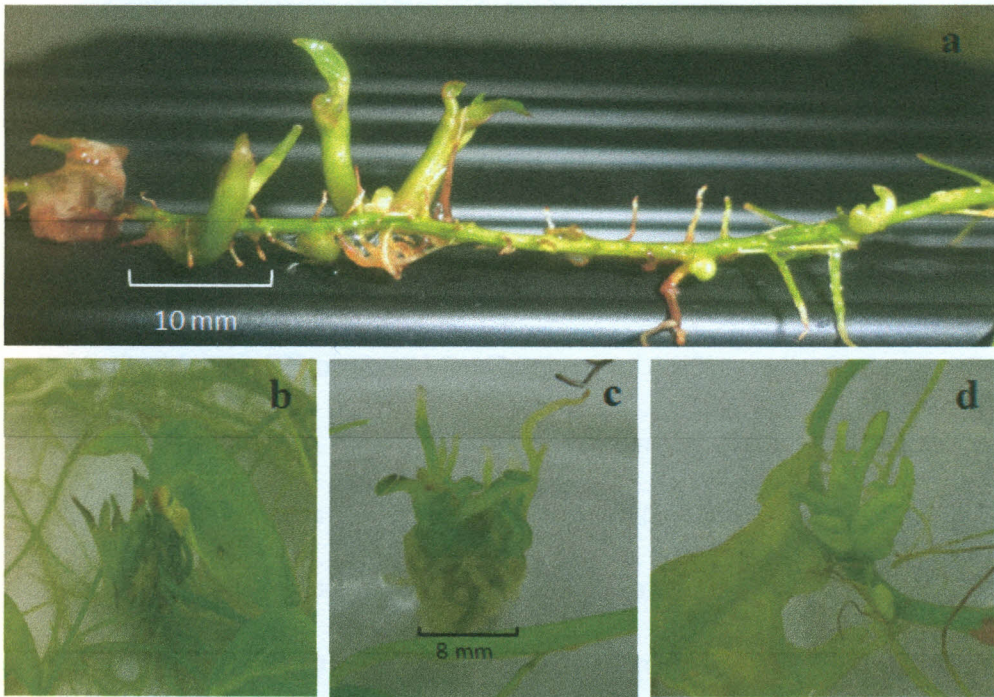
Caulogenesis was observed for most cultivars except Chihongo, Chingowva, Gikanda, Namagizi, Rusenya and SPN/O between 12 and 60 days of culture. Two types of regenerants were produced: deformed and normal. Deformed shoots were observed when regenerants were produced after 40 days of culture in MS medium containing cytokinin (Plate 3.3a); most of these plants reverted back to normal after transfer to propagation media.

Some cultivars started producing normal regenerants within 21 days of culture namely New Kawogo, Kawoga, Mugande, Luby, Luapula, Bwanjule and Chiuva (Plate 3.3b). The regenerants were produced at the base of callus in contact with medium (Plate 3.3c), on the newly-formed callus or just above the newly-formed callus (Plate 3.3d).

Multiple regenerants were also produced by calli of several cultivars, which emerged from roots that emanated from the callus (Plate 3.4a), directly from callus with many roots (Plate 3.4b) from callus with no roots (Plate 3.4c) and from roots that did not emerge from calli but from the mother plant (Plate 3.4d).



**Plate 3.3:** Shoot-type produced by calli of select SSA sweetpotato cultivars. **(a)** Deformed regenerants. **(b)** Single shoot emerging from callus. **(c)** Shoot produced at callus base. **(d)** Shoot produced above callus.



**Plate 3.4:** Multiple regenerants produced from SSA sweetpotato cultivars. **(a)** From roots emerging from calli **(b)** Production directly from callus **(c)** Shoot production on calli with no roots **(d)** Shoot emergence from roots but calli.

### 3.3.5.2 Effect of both callogenesis and caulogenesis on RE of the SSA cultivars

There were different observations made between calli colour and RE. Firstly, both high and low regenerating cultivars in some cases had the same calli colour but with different RE. For instance, Imby cultivar had the highest RE of 71.6% while Chihongo had 0%, but they both had light-green calli. In another example, Zambezi exhibited high RE of 86.7% and Muibai with low RE of 1.9% but both had dark-green calli but the difference was that Zambezi had organogenic friable calli while Muibai did not (Table 3.2).

Secondly, in other cases calli colour and high RE cultivars had some relationships. For instance, Luapula and Imby had very high RE with friable light-green calli; Kawoga and Mafutha had high RE with friable purple-green calli both on media containing kinetin. The GLM revealed that there were significant ( $p < 0.05$ ) differences of calli colour on RE for media containing kinetin (Appendix 3).

### 3.3.5.3 Effect of cytokinin-type on regeneration efficiency

The influence of 0.2 mg/L each of three cytokinins kinetin, zeatin and thidiazuron on RE of 32 cultivars was evaluated. The RE on MS medium containing kinetin ranged from 0 to 86%, the highest RE observed for Zambezi of 86.7% while the lowest RE was 0% for some cultivars such as Rusenya, Mohc, Chihongo, Namagizi, Gikanda and SPK013 (Table 3.2). There was significant difference ( $p < 0.05$ ) between RE for kinetin and cultivars screened (Appendix 4). Cultivar Zambezi produced the highest number of regenerants ranging from 2 to 9 per explant in media containing kinetin. Luapula had 2 to 6, Mafutha had 2 to 7, and the rest of the cultivars either had 1 or 2 regenerants per explant (Table 3.2).

**Table 3.2:** Regeneration efficiencies of selected SSA sweetpotato via organogenesis, 0.2 mg/L kinetin and leaf with petiole explant.

| <b>Cultivar</b> | <b>Calli color</b> | <b>Organogenic calli</b> | <b>RE (%)<br/>kinetin<br/>(mean<br/>differences)</b> | <b>Number of<br/>multiple shoots<br/>(explants with<br/>multiple shoots)</b> |
|-----------------|--------------------|--------------------------|--|--|
| Budagala        | Green white        | Few                      | 6.7 (g)  | 3(2)   |
| Bwanjule        | Green white        | Few                      | 11.7 (g)   | 4(2)   |
| Chifukama       | Green white        | Few                      | 5.3 (g)  | 0  |
| Chihongo        | Light green        | None                     | 0  | 0  |
| Chingowva       | Green white        | None                     | 0  | 0  |
| Chiuva          | Green white        | Few                      | 11.7 (g)   | 12(4)  |
| Gikanda         | Light green/white  | None                     | 0  | 0  |
| Imby            | Light green        | Friable                  | 71.6 (b)   | 43(30)   |
| Jewel           | Light green        | Friable                  | 50 (d)   | 18(10)   |
| K51/3251        | Green/white        | Few                      | 10 (g)   | 5(3)   |
| Kamchiputu      | Dark green         | Hardly any               | 1.7 (g)  | 0  |
| Kawogo          | Green/purple       | Friable                  | 48.3(d)  | 21(11)   |
| Kemb 10         | Light green        | Harldy any               | 1.9 (g)  | 0  |
| Kemb 37         | Dark green         | Few                      | 8.3 (g)  | 7(3)   |
| KSP 11          | Light green        | Hardly any               | 1.9 (g)  | 0  |
| Luapula         | Light green        | Friable                  | 60 (c)   | 86(34)   |
| Luby            | Green white        | Few                      | 13.3 (g)   | 4(2)   |
| Mafutha         | Purple/Green       | Friable                  | 45 (d)   | 76(21)   |
| Malawiala       | Light green        | None                     | 0  | 0  |
| Mohc            | Green/white/purple | None                     | 0  | 0  |
| Mugande         | Green/white/purple | Partly                   | 23.3 (f)   | 4(2)   |
| Muibai          | Green              | Hardly any               | 1.8 (g)  | 0  |
| Mwanamonde      | Light green/white  | None                     | 0  | 0  |
| Namagizi        | Dark green         | None                     | 0  | 0  |
| New Kawogo      | Green              | Few                      | 16.7 (g)   | 6(2)   |
| Rusenya         | Light green        | None                     | 0  | 0  |
| Rusenya         | Dark green         | None                     | 0  | 0  |
| Sinia           | Green white        | Hardly any               | 1.7 (g)  | 0  |
| SPK 004         | Green/purple/white | Few                      | 6.7 (g)  | 0  |
| SPK 013         | Green/purple       | None                     | 0  | 0  |
| SPN/O           | Light green        | None                     | 0  | 0  |
| Ukerewe         | Green/white        | Friable                  | 30 (e)   | 14(10)   |
| Zambezi         | Dark green         | Friable                  | 86.7 (a)   | 189(32)  |

Sample size of 60 for each cultivar was used. The means were generated by summation of the regenerants and dividing by the number of explants initially used. Regression analysis by GLM was used for ANOVA for the RE and regression factors in non-normal conditions. The standard error of differences of means is 2.65 and differences were observed between the cultivars with 'a' being the highest followed by b, c, d, e, f and g.

For media containing zeatin, RE ranged from 0 to 55% with Zambezi having the highest at 55%, then Mugande with 48% Luapula 40%, Kawogo 35% while the control cultivar Jewel had 28%. Those with an RE of 0% were Chihongo, Namagizi, Kemb 10, Gikanda, SPK013, Kamchiputu, K51, Rusenya, Malawiala, Sinia, SPN/O, Chingowva and KSP11 (Table 3.3). There was a significant difference ( $p < 0.01$ ) between cultivar and RE when explants were incubated on medium containing 0.2 mg/L zeatin (Appendix 5). However, there was no relationship between RE for high regenerating cultivars such as Mugande, Luapula and Zambezi and calli colour, which were green/white, light-green and purple green, respectively (Table 3.3). There was also no relationship between low RE cultivars namely Chihongo, Namagizi and Kemb10 and calli colour, with light-green, dark/green/white and green, respectively (Table 3.3). For medium containing zeatin, Luapula had the highest number of regenerants per explant (2 to 5 shoots), while the rest had 2 shoots on average (Table 3.3).

When media containing thidiazuron was used, RE ranged from 0 to 73%. Zambezi had the highest RE of 73% followed by Kawogo 45%, Luapula 31.7%, Imby 28.3% while Jewel control had 41.7% (Table 3.4). Cultivars with an RE of 0% RE were Chifukama, Chihongo, Chiuva, Namagizi, Kemb10, Kemb37, Gikanda, SPK013, Rusenya, Mwanamonde, Sinia, SPN/O, New Kawogo, Chingowva and Ukerewe. There was some significant difference ( $p < 0.05$ ) of cultivar-type on RE for media containing thidiazuron (Appendix 6). Media containing thidiazuron had fewer shoots per explant with cultivar Zambezi and Bwanjule having the highest number of regenerants (2 to 3) per explant (Table 3.4).

**Table 3.3:** Regeneration efficiencies of selected SSA sweetpotato via organogenesis, 0.2 mg/L zeatin and leaf with petiole explant.

| Cultivar   | Calli color        | Organogenic calli | RE (%)<br>zeatin (mean differences) | Number of multiple shoots<br>(explants with multiple shoots) |
|------------|--------------------|-------------------|-------------------------------------|--|
| Budagala   | Green/purple       | Few               | 13.3 (b)                            | 7(4)   |
| Bwanjule   | Light green/purple | Few               | 8.3 (b)                             | 4(2)   |
| Chifukama  | Light green        | Few friable       | 23.3 (b)                            | 5(2)   |
| Chihongo   | Light green        | None              | 0                                   | 0  |
| Chingowva  | Green              | None              | 0                                   | 0  |
| Chiuva     | Green              | Hardly any        | 1.7 (c)                             | 0  |
| Gikanda    | Green              | None              | 0                                   | 0  |
| Imby       | Light green        | Friable           | 26.7 (b)                            | 3(2)   |
| Jewel      | Green              | Friable           | 28.3 (b)                            | 19(9)  |
| K51/3251   | Green/yellow       | None              | 0                                   | 0  |
| Kamchiputu | Green              | None              | 0                                   | 0  |
| Kawogo     | Dark green         | Friable           | 35 (a)                              | 0  |
| Kemb 10    | Green              | None              | 0                                   | 0  |
| Kemb 37    | Green/purple       | Few               | 16.7 (b)                            | 3(2)   |
| KSP 11     | Light green        | None              | 0                                   | 0  |
| Luapula    | Light green        | Friable           | 40 (a)                              | 12(3)  |
| Luby       | Light green white  | Few               | 8.3(b)                              | 4(2)   |
| Mafutha    | Light green/purple | Few friable       | 18.3 (b)                            | 7(3)   |
| Malawiala  | Light green        | None              | 0                                   | 0  |
| Mohc       | Light green        | Few               | 8.3 (b)                             | 4(1)   |
| Mugande    | Green/white        | Friable           | 48.3 (a)                            | 5(2)   |
| Muibai     | Dark green/purple  | Hardly any        | 3.3 (c)                             | 0  |
| Mwanamonde | Green              | Few               | 6.7 (b)                             | 0  |
| Namagizi   | Dark green/white   | None              | 0                                   | 0  |
| New Kawogo | Light green/white  | Few               | 11.1 (b)                            | 9(3)   |
| Rusenya    | Green              | Hardly any        | 1.7 (c)                             | 0  |
| Rusenya    | Green              | None              | 0                                   | 0  |
| Sinia      | Green              | None              | 0                                   | 0  |
| SPK 004    | Green              | Few               | 11.7 (b)                            | 5(2)   |
| SPK 013    | Green              | None              | 0                                   | 0  |
| SPN/O      | Green              | None              | 0                                   | 0  |
| Ukerewe    | Green              | Friable           | 30 (b)                              | 6(3)   |
| Zambezi    | Purple/green       | Friable           | 55 (a)                              | 52(23)   |

Sample size of 60 for each cultivar was used. The means were generated by summation of the regenerants and dividing by the number of explants initially used. Regression analysis by GLM was used for ANOVA for the RE and regression factors in non-normal conditions. The standard error of differences of means is 4.813 and differences were observed between the cultivars with 'a' being the highest followed by b and c.

**Table 3.4:** Regeneration efficiencies of selected SSA sweetpotato via organogenesis 0.2 mg/L thidiazuron and leaf with petiole explant.

| Cultivar   | Calli color        | Organogenic calli | RE (%)<br>Thidiazuron<br>(mean differences) | Number multiple shoots (explants with multiple shoots) |
|------------|--------------------|-------------------|---|--|
| Budagala   | Green              | Hardly any        | 3.3 (b)                                     | 0  |
| Bwanjule   | Dark green         | Few               | 6.7 (c)                                     | 6(2)   |
| Chifukama  | Light green        | Hardly any        | 3.3 (c)                                     | 5(2)   |
| Chihongo   | Green              | None              | 0   | 0  |
| Chingowva  | Green brown        | None              | 0   | 0  |
| Chiuva     | Light green        | None              | 0   | 0  |
| Gikanda    | Light green        | None              | 0   | 0  |
| Imby       | Light green        | Few               | 28.3 (b)                                    | 18(9)  |
| Jewel      | Green/purple       | Friable           | 41.7 (b)                                    | 27(16)   |
| K51/3251   | Light green        | Hardly any        | 5   | 2(1)   |
| Kamchiputu | Green/purple       | Hardly any        | 1.7 (c)                                     | 0  |
| Kawogo     | Green/purple       | Friable           | 45 (b)                                      | 10(5)  |
| Kemb 10    | Yellow             | None              | 0   | 0  |
| Kemb 37    | Green              | None              | 0   | 0  |
| KSP 11     | Light green        | Hardly any        | 3.3 (c)                                     | 0  |
| Luapula    | Light green        | Friable           | 31.7 (b)                                    | 15(6)  |
| Luby       | Light green        | Few               | 13.3 (b)                                    | 0  |
| Mafutha    | Light green/purple | Hardly any        | 5   | 4(2)   |
| Malawiala  | Green              | Hardly any        | 1.7 (c)                                     | 0  |
| Mohc       | Green              | Hardly any        | 1.7 (c)                                     | 2(1)   |
| Mugande    | Green/yellow       | Hardly any        | 1.7 (c)                                     | 0  |
| Muibai     | Dark green         | Hardly any        | 1.7 (c)                                     | 2(1)   |
| Mwanamonde | Green              | None              | 0   | 0  |
| Namagizi   | Light green        | None              | 0   | 0  |
| New Kawogo | Light green        | None              | 0   | 0  |
| Rusenya    | Green              | Hardly any        | 1.7 (c)                                     | 2(1)   |
| Rusenya    | Green              | None              | 0   | 0  |
| Sinia      | Light green        | None              | 0   | 0  |
| SPK 004    | Light green        | Few               | 18.3 (b)                                    | 3(2)   |
| SPK 013    | Green              | None              | 0   | 0  |
| SPN/O      | Light green        | None              | 0   | 0  |
| Ukerewe    | Light green        | None              | 0   | 0  |
| Zambezi    | Yellow             | Friable           | 73.3 (a)                                    | 3(1)   |

Sample size of 60 for each cultivar was used. The means were generated by summation of the regenerants and dividing by the number of explants initially used. Regression analysis by GLM was used for ANOVA for the RE and regression factors in non-normal conditions. The standard error of differences of means is 4.68 and differences were observed between the cultivars with 'a' being the highest followed by b and c.

Generally multiple shoots per explant were produced for most SSA cultivars screened regardless of cytokinin type tested with the numbers ranging between 2 to 9 depending on cultivar and cytokinin. This notwithstanding, media containing kinetin supported the highest number of regenerants per explant, followed by zeatin and finally thidiazuron.

### **3.3.6 Effect of sweetpotato morphology on regeneration efficiency**

There was no relationship between the colour of the calli, RE (Tables 3.2, 3.3, 3.4) and root flesh colour of the cultivars (Table 3.1). There was some relationship between dry matter (DM) of storage roots and RE as evidenced by most cultivars with high DM (>30%) having low TE of less than 20%, including Kemb10, SPK004, SPK13, Gikanda, KSP11, New Kawogo, Bwanjule and Kamchiputu. Nevertheless, not all cultivars of high DM had low TE for example Ukerewe and Kawoga had a DM of greater than 30% and an RE of >40% RE (Tables 3.1, 3.2).

## **3.4 DISCUSSION**

### **3.4.1 Explant-type had some effect on regeneration efficiency**

Leaf with petiole explants produced the highest RE (40%) compared to other explant-types such as petiole alone (3.3%), leaf alone (1.7%) and stem alone (1.7%) in the preliminary experiment conducted using cultivar Ukerewe. These results obtained with Ukerewe were consistent with an earlier report, which demonstrated that leaves with petioles were effective in sweetpotato regeneration (Gosukonda *et al.*, 1995). Other studies however reported much higher RE using other explant-types for example 13-85% for leaves (Kasili, 1998) and stem (Gonzalez *et al.*, 1999), while 3-77% has been reported for petioles (Kasili, 1998) and 25-85% for lamina explants (Gosukonda *et al.*, 1995; 1995b). The high RE observed for leaf with petiole could be attributed to the age of

explant since only young leaves were used and young leaves are known to have high levels of auxin and cytokinin (Mohr and Schopfer, 1995). These auxins move acropetally through lamina to the petiole and the hormone levels (endogenous and exogenously applied) could have been adequate to induce regeneration (Baker, 2000). When young explants are cultured on media with cytokinins, the endogenous levels increase briefly before prompting cytokinin oxidase to regulate their levels (Gaspar *et al.*, 2003). Therefore, low RE obtained for leaf or petiole alone could suggest presence of high endogenous auxin thereby limiting regeneration; young leaves have high auxin levels occurring along leaf margins and these auxins move towards the mid lamina through basipetal transport (Baker, 2000). This suggests that the leaf alone without wounding to release the auxins could not have availed these hormones readily because mid lamina of leaf explants. In petiole or stem alone, the hormone source (leaf) was removed and exogenously applied hormones were insufficient for shoot induction. Nevertheless, the explant-type is dependent on the genotype.

#### **3.4.2 Kinetin concentration influences regeneration**

The highest RE was obtained on 0.2 mg/L kinetin for the 7 SSA cultivars screened in the second preliminary experiment. These included Zambezi with 86% at 0.2 mg/L compared to 40% on media with no kinetin; followed by Imby with 72% on media with 0.2 mg/L kinetin while 52% on media with no kinetin, the rest of the 5 cultivars followed the same trend. Furthermore, using 0.2 mg/L of zeatin and thidiazuron in subsequent experiments produced optimum RE for the other 25 SSA cultivars. Similar reports have been made for some American sweetpotato cultivars where the highest RE of 85% was reported on 0.2 mg/L, 19% for 0 mg/L and 36% for 0.4 mg/L in thidiazuron (Gosukonda *et al.*, 1995).

Since high cytokinin: auxin ratio forms shoots (Gaspar *et al.*, 2003; George *et al.*, 2008), it seemed that adding 0.2 mg/L of any cytokinin used tested for the SSA cultivars resulted in production of shoots. Production of regenerants on media with no cytokinin for some of the SSA cultivars clearly suggested that endogenous cytokinin levels play a role in shoot production. It seems that though regenerants were produced, the endogenous cytokinin levels may not have been sufficient enough to produce RE comparable to the media containing 0.2 mg/L cytokinins.

### **3.4.3 Formation of calli was affected by the hormone used**

Callus induction is an important step in regeneration of plants through indirect organogenesis. In all SSA sweetpotato cultivars tested in this study, calli production on leaf with petiole explants was initiated at 0.05 mg/L 2,4-D. There were differences in calli sizes between the cytokinins, for instance, for Ukerewe cultivar, the calli size ranged between 0.15 to 0.2 cm for kinetin; for zeatin, it ranged between 0.25 to 0.3 cm while 0.35 to 0.45 cm. Nevertheless, there were no relationships between calli size and regeneration potential. Media containing thidiazuron produced large-sized calli compared to media containing kinetin and zeatin. Zeatin, a natural cytokinin, has been reported to increase calli size in sweetpotato (Sihachakr and Ducreux, 1987). In the organogenesis study reported herein, however, several reasons could explain these variations: one, large-sized calli observed for media containing thidiazuron could have been due to the use of 2,4-D in step 1 that briefly increased endogenous levels of zeatin, thus enlargement of calli since auxins are known to elicit production of endogenous cytokinins (Pernisova *et al.*, 2009). Secondly, in media with thidiazuron (0.2 mg/L), production of small necrotic calli for some cultivars could have been due to increase in the natural endogenous auxin

levels thus stimulating ethylene production and hence reduction in cell growth. It has previously been reported that zeatin levels temporarily increase when plant tissues are treated with thidiazuron (Thomas and Katterman, 1986) and thidiazuron was shown to suppress conjugation of IAA while stimulating production of ethylene in mungbean (Yip and Yang, 1986). This suggests that when thidiazuron was added into step 2 media, the explants increased the endogenous zeatin levels and at the same time, the endogenous natural auxin (IAA) were increased due to suppression of the conjugation process of IAA. This in turn signaled production of ethylene, known to inhibit growth and development (Kumar *et al.*, 1998). Nevertheless, differences in calli size seemed genotype-dependent. Thirdly, increase in calli size on media with zeatin could have been due to addition of auxin for step-1 and cytokinins in step-2. Auxins are known to elicit production of endogenous cytokinins (Pernisova *et al.*, 2009) while combinations of auxin and cytokinin both endogenous and exogenous, stimulate cell division (Mohr and Schopher, 1995).

#### **3.4.4 Root formation did not influence regeneration efficiency**

The SSA cultivars produced roots under 2,4-D (0.05 mg/L) and the cytokinins used but there was no relationship with the RE. Root formation could have been due to presence of both auxin and cytokinin, both endogenous and exogenous, since they emerged from the second day in step-1 media. High auxin to cytokinin ratio is known to stimulate root formation (Machakova *et al.*, 2008). Secondly, the SSA cultivars could have had high endogenous auxin levels such that low levels of 0.05 mg/L 2,4-D could induce rooting. Root induction trend of the SSA cultivars are consistent with those reported by El Far *et al.* (2009) where the sweetpotato cultivars seemed to have high endogenous levels of

auxin indicated by the low 2,4-D levels added to the media for this study. In contrast, other crops have reported even lower 2,4-D levels of 0.005 mg/L for *Nicotiana tabacum* (Ruzicka *et al.*, 2008). The higher 2,4-D levels used in this study as compared to the study by Ruzicka *et al.* (2008) could have been due to genotype and species-dependence although the current study did not conduct experiments using lower 2,4-D concentration. Thirdly, low 2,4-D level of 0.05 mg/L used in this study was sufficient to induce rooting due to the regeneration method used. Use of methods such as somatic embryogenesis, higher auxin levels of 1 mg/L 2,4-D have been reported for rooting in 5 Kenyan sweetpotato cultivars (Oggema *et al.*, 2007). Furthermore, rooting during regeneration indicates the ease of root production on the regenerants eventually produced.

#### **3.4.5 Effect of cytokinin-type on number of regenerants**

The highest number of cultivars with regenerants was observed on step-2 media containing kinetin as opposed to either zeatin or thidiazuron. Similar reports have been made for kinetin in few SSA cultivars namely Wagabolige, Tanzania and SPK004 (Blasco, 2007). By contrast to kinetin as the best cytokinin with the highest number of cultivars with regenerants, MS media containing thidiazuron was documented as the best cytokinin, based on high RE in one cultivar (Gosukonda *et al.*, 1995). Interestingly, in the same study by Gosukonda *et al.* (1995) media with thidiazuron supported 2 out of 7 cultivars that produced shoots whereas media containing kinetin supported 6 out of the 7 cultivars. It was also reported that media with zeatin supported generation of high RE for 19 of 27 sweetpotato cultivars (Porobo-Dessai *et al.*, 1995). The gap between the study by Porobo-Dessai with the current study is that analysis for RE was conducted on media containing zeatin alone as opposed to this study where three hormones were used. This

indicates that for screening a number of cultivars for regeneration through organogenesis, kinetin could be used to eliminate low RE cultivars and proceed the high RE cultivars.

Some explanations for the differences in performance of the three cytokinins in the studies reported in the paragraph above could be attributed to cytokinin-type (natural or synthetic), where natural ones are naturally produced within a plant while synthetic ones are man-made, and sample size of cultivars screened. In the case of cytokinin-type, natural cytokinin levels are regulated by cytokinin oxidase but not the synthetics like kinetin (Mohr and Schopfer, 1995). This is due to the fact that natural cytokinins have -OH group on the side chain attached to glycosyl moiety and cleavage by highly specific cytokinin oxidase occurs while synthetic ones do not have the -OH group but they have an aromatic ring attached to the side chain inhibiting cleavage (Srivastava, 2002). The variations in performance of media with different cytokinins could have been due to the large number of SSA cultivars (32) screened in this study of 3 cytokinins, compared to those reported in previous studies. This suggests that previous conflicting results could have been as a result of fewer cultivars tested.

Multiple shoots (2 to 7) were produced per explant for several SSA cultivars and this varied with the cytokinin-type applied. These multiple shoots numbers exceeded those reported for 8 USA cultivars where only 3 multiple shoots per explant were reported (Gosukonda *et al.*, 1995b). This suggests that the auxin and cytokinin levels used in the current study were efficient in inducing generation of multiple regenerants per explant.

In this study, only the regenerants produced on the newly-formed calli from the explants were considered when calculating RE because the protocol was being optimized for

subsequent transformation experiments of selected cultivars with *A. tumefaciens*. Calli produced from the petiole had a high likelihood of being transformed due to the contact with *A. tumefaciens*, while regenerants produced above the newly-formed calli would not.

#### **3.4.6 Effect of sweetpotato phenotype and origin on regeneration efficiency**

For effect of phenotype on RE, most SSA cultivars with high DM had low RE including Mwanamonde, SPK004, SPK013, Gikanda, New Kawogo, Bwanjule and Budagala. Similar observations have been made for 7 USA cultivars which had high DM >30% and the highest DM of 34% had the lowest RE of 6.7% (Santa-Maria *et al.*, 2009). The influence of morphological traits and ability to regenerate is not only restricted to sweetpotato but has also been reported in wheat where spikelet number and calli formation had some relationship (Yildirim *et al.*, 2008). The results suggest that there could be some relationship between morphology and regeneration ability of sweetpotato cultivars but more research should be undertaken to verify this.

Secondly, the effect of cultivar or genotype on regeneration efficiency was also observed for cultivars Ukerewe, Mugande, Mafutha and Carrot, which were either from Kenya or Tanzania and had relatively low RE. These REs compared to those of Imby, Lupula and Zambezi, from Zambia and Burundi with high RE strongly indicated the effect of genotype on environment.

## CHAPTER FOUR

# DEVELOPMENT OF GENETIC TRANSFORMATION PROTOCOL OF SELECTED SUB-SAHARAN AFRICAN SWEETPOTATO CULTIVARS

### 4.1 INTRODUCTION

Sweetpotato transformation has been studied over decades with the aim of improving a number of agronomic traits. Several methods have been applied to insert new genes into sweetpotato germplasm but the most commonly used is through *Agrobacterium*-mediated transformation. This transformation method is widely used in sweetpotato experiments (Moran *et al.*, 1998) through somatic embryogenesis and organogenesis to produce the regenerated plants but transformation through organogenesis is not as commonly used. Organogenesis is a faster method of producing transgenic events as compared to somatic embryogenesis where the former takes between 30 to 60 days while the latter could take one year or more. Transformation via organogenesis has been reported in a few SSA cultivars but these protocols have been highly genotype-dependent. This chapter reports the results of transformation through organogenesis of 5 sweetpotato cultivars using *uidA* gene to evaluate transformability of the SSA cultivars while 3 out of the 5 cultivars were transformed using weevil resistance conferring genes, *Cry7Aa1* and *ET33-34*.

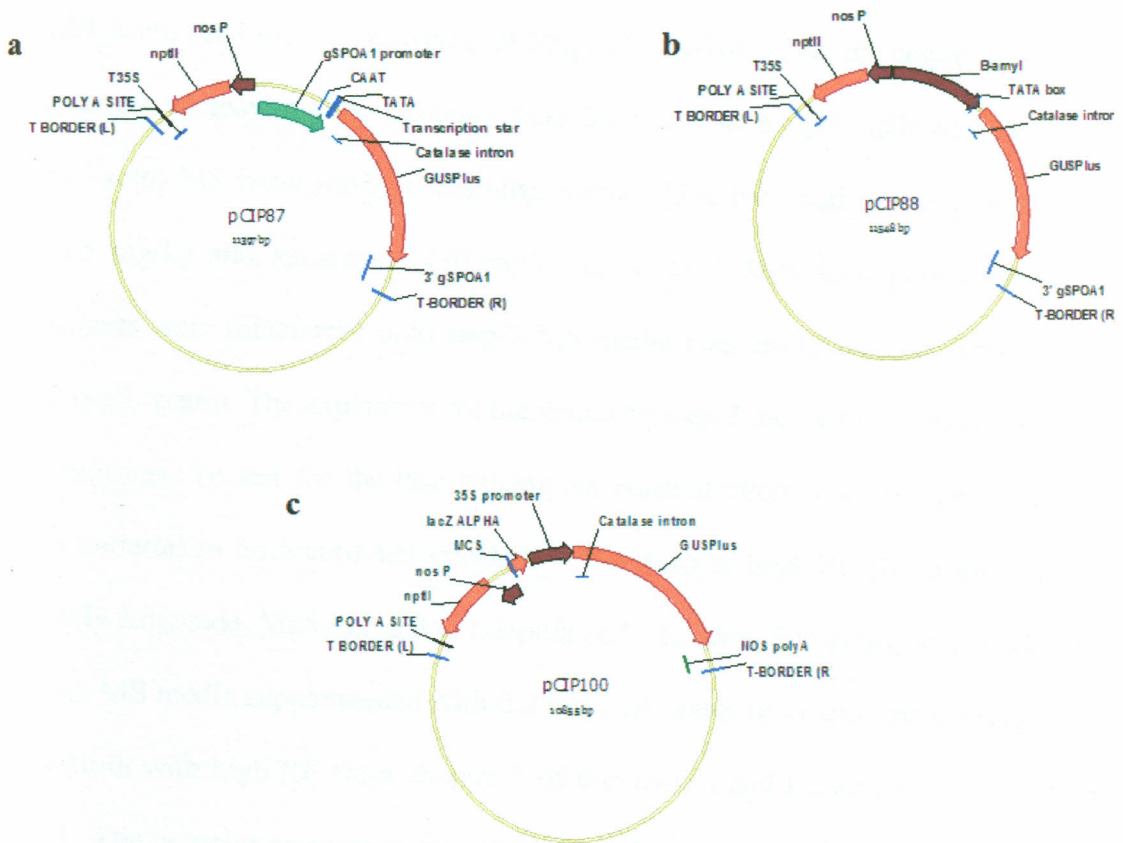
### 4.2 MATERIALS AND METHODS

#### 4.2.1 Transformation of the sweetpotato cultivars

##### 4.2.1.1 Transformation using *uidA* gene

The transformation experiments were conducted using cultivars Mafutha, Mugande, Imby, Luapula and Ukerewe with high RE of >40% reported in chapter 3 of this thesis.

The process followed the protocol described by Luo *et al.* (2006) with few modifications such as *A. tumefaciens* grown on semi-solid MS media as opposed to liquid medium, as described below. *A. tumefaciens* strain EHA105 was used harboring gene constructs containing the intron-GUSPlus gene (*uidA*) and neomycin phosphotransferase gene (*nptII*). *GUSPlus* gene was used because it is shown to have greater sensitivity than the normal GUS in plants and prolonged incubations do not tamper with non-destructive assays ([http://www.cambia.org/daisy/bioforge\\_gusplus/3850.html](http://www.cambia.org/daisy/bioforge_gusplus/3850.html)). The intron-gus gene is not expressed in bacteria, which is desirable when one uses it as a reporter gene for plant cell expression (Vancanneyt *et al.*, 1990). Three gene constructs were used harboring the *uidA* gene and all with different promoters. Different promoters were used to find out how *uidA* gene would be expressed in the sweetpotato transgenic calli and putative events for purposes of expression of the weevil resistance (WR) genes. The gene constructs were namely pCIP100, pCIP88 and pCIP87. Vector gene construct pCIP100 carried CaMV-*uidA* gene, pCIP87 carried SPOA1-*uidA* gene while pCIP87 carried the  $\beta$ -amy-*uidA* gene (Fig. 4.1). Sporamin and  $\beta$ -amylase proteins naturally occur in sweetpotato storage roots but occur in other plant parts in presence of sucrose hence the *SPOA1* and  $\beta$ -*amy* promoters being classified as induced promoters while CaMV-35S is constitutive and occurs all over the transgenic plant.



**Figure 4.1:** Gene constructs of *GUSPlus* gene with their respective promoters used for transforming selected SSA sweetpotato cultivars by organogenesis. (a) *GUSPlus* gene regulated by the sporamin promoter (*SPOA1*) and terminated by *SPOA1* for pCIP87 gene construct, (b) *GUSPlus* gene regulated by  $\beta$ -amylase promoter ( $\beta$ -*amy*) for pCIP88 and terminated by *SPOA1* (c) *GUSPlus* gene regulated by the CaMV-35S promoter and then terminated by nopaline synthase (*nos*) for pCIP100

The transformation process was initiated by growing *A. tumefaciens* containing the *uidA* gene on luria broth (LB) media supplemented with kanamycin (50 mg/L) followed by incubation at 28°C for 2 days. The young leaf with petiole explants were harvested and then the cut petiole-edge sparingly dipped onto *A. tumefaciens* culture for 1s and immediately inoculated onto previously described step-1 regeneration media comprising MS medium supplemented with 0.05 mg/L 2,4-D, acetosyringone (varying levels) and 2 g/L Gelrite<sup>®</sup>. In the first preliminary screen, 4 acetosyringone concentrations 1, 20, 30, 40

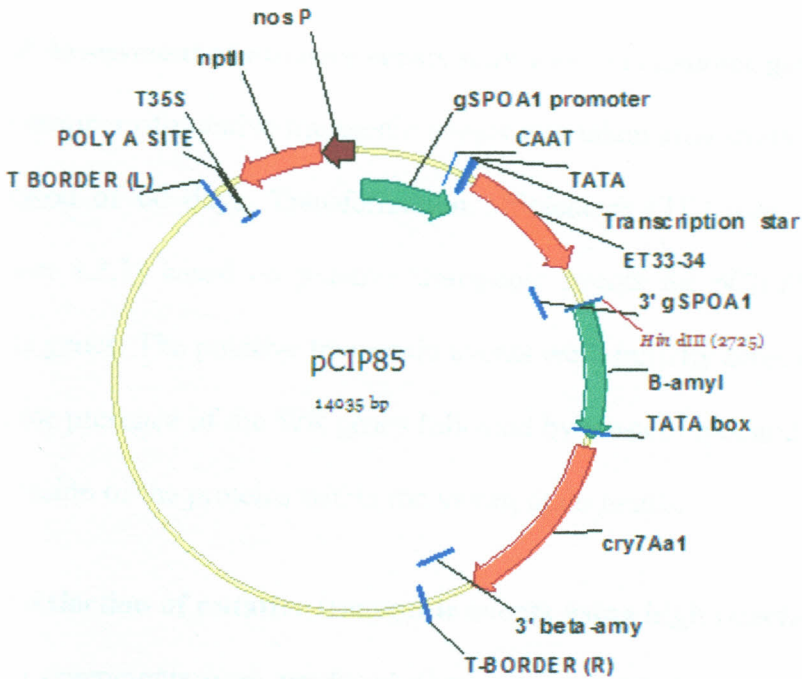
mg/L were used for TE evaluation of Mugande cultivar while the negative control did not have any acetosyringone. Explants were incubated overnight, followed by culture onto semi-solid MS basal media containing sucrose (3% w/v) and supplemented with 2,4-D (0.05 mg/L) and kanamycin (50 mg/L) for 2 to 4 days until petioles were swollen. Explants were transferred onto step-2 MS media containing sucrose supplemented with 0.2 mg/L zeatin. The explants were incubated on step 2 media for 30 days with bi-weekly subcultures. To test for the best kanamycin concentration, a second preliminary screen was undertaken on 5 cultivars selected on the basis of high RE (from previous chapter) namely Mugande, Mafutha, Imby, Luapula and Ukerewe. Screening was conducted using step-2 MS media supplemented with 0.2 mg/L of zeatin or kinetin (depending on the best cytokinin with high RE from chapter 3 of this thesis) and kanamycin 5, 25, 50 and 100 mg/L. The negative controls of the respective cultivars were not inoculated on media with kanamycin. After screening for the best concentration of kanamycin on the transformed calli, the best level was used in subsequent experiments for production of regenerants having the *uidA* gene. The negative control (Ukerewe) was not transformed using *A. tumefaciens* but went through step-1 and step-2 MS media as reported for the transformed explants.

#### **4.2.1.2 Transformation using the double gene construct (pCIP85)**

Transformation using the double gene construct was conducted using *A. tumefaciens* strain EHA105 harboring pCIP85 with weevil resistance (WR) genes (*cry7Aa1* and *ET33-34* in tandem) and neomycin phosphotransferase gene (*nptII* divergent to the genes of interest). The WR genes were driven by  $\beta$ -*amy* and *SPOA1* promoters, respectively, while *nptII* by *nos-P* for the double gene construct (Fig. 4.2).  $\beta$ -amylase and sporamin

were used because they are root-specific proteins but they occur in little amounts in other plant parts (Maeo *et al.*, 2001) while weevils cause damage on the storage roots hence the importance of localized expression of WR genes. Transformation using WR genes was conducted on 3 cultivars (Ukerewe, Imby and Luapula) selected from the 5 cultivars (Zambezi, Mafutha, Mugande, Ukerewe, Imby and Luapula) that were initially transformed by the *uidA* gene. The selection was based on availability of the germplasm due to technicality of acquiring all the 5 cultivars. Jewel was used as the control cultivar because it had previously been shown to be relatively easy to transform through organogenesis using *Agrobacterium tumefaciens* (Luo *et al.*, 2006).

In addition to the transformed explants, negative controls were also included in the experiment and they were not transformed but went through step-1 and step-2 media as the transformed explants.



**Figure 4.2:** A double gene construct with *cry7A1* and *ET33-34* genes driven by  $\beta$ -amy and SPOA1, respectively while *nptII* is driven by nos P. The gene construct was used to transform 3 SSA cultivars from the initial list of five.

### 4.3 Assessment of the transformed transgenic calli and events

#### 4.3.1 Assessment of GUS expression on calli and regenerants

Determination of transformed calli was undertaken using non-destructive GUS staining following a protocol described by CAMBIA ([www.cambia.org](http://www.cambia.org)). 5-bromo-4-chloro-3-indolyl glucuronide cyclohexylammonium salt substrate (Duchefa Biochemie) was made by dissolving 20 mg of the substrate in 1 ml of dimethylsulphoxide (DMSO). A final concentration of 0.2 mg/ml was used in 20 mM sodium phosphate buffer pH 7 in 1 litre as the working solution in the GUS-assays. Each of the one-month old callus or regenerant were placed in GUS solution for 12 to 72 hours. Transformation efficiency (TE) was determined using the GUS-stained calli and calculated as follows:

$$\text{Transformation efficiency} = \frac{\text{Number of stained calli}}{\text{Total number of calli}} \times 100$$

### **4.3.2 Assessment of putative events with weevil resistance genes (pCIP85)**

The number of putative transgenic events was taken after every seven days in culture for a period of 60 days. Transformation efficiencies (TE) were calculated (as shown in section 4.3.1) based on putative transgenic events for pCIP85 with weevil resistance (WR) genes. The putative transgenic events were initially screened by PCR and southern blot for presence of the WR genes followed by western blot and DAS-ELISA to confirm expression of the proteins within the sweetpotato plants.

### **4.3.3 Selection of putative transgenic events using high selection pressure**

When regenerants were produced, they were subjected to high selection pressure using 100 mg/L kanamycin using a protocol described by Nopo *et al.* (2005). This was done by placing leaf samples of the putative transgenic events and non-transgenic control on petri dishes with callus test media containing MS basal salts, MES (2.56 mM), mannitol (109.77 mM), glucose (110.9 mM), L-glutamine (1.37 mM), adenine sulphate (0.22 mM), Naphthalene acetic acid (NAA) (1 mg/L), benzylamino-purine (0.1 mg/L), kanamycin (100 mg/L), phytigel (3 g/L) and cefotaxime (100 mg/L).

The putative transgenic events were cultured on the kanamycin test media for a period of between 30-60 days depending on calli formation or necrosis. The putative events were sub-cultured bi-weekly. True events produced small calli while escapes showed necrosis.

## **4.4 DNA analyses of the putative transgenic events**

### **4.4.1 DNA extraction**

DNA extraction for both polymerase chain reaction (PCR) and southern blot were done using CTAB extraction method described by Nopo *et al.* (2005). The DNA samples were

then stored at 20°C. PCR was done to screen for presence of the inserted genes of interest in the transgenic events generated while southern blot was done to confirm the inserted genes were present but also the number of copies inserted.

#### 4.4.2 Polymerase chain reaction (PCR)

PCR was conducted using a reaction volume of 15 µl consisting of PCR buffer (1x), MgCl<sub>2</sub> (1.5 mM), dNTPs (0.1 mM), forward primer (0.25 µM), reverse primer (0.25 µM) and Taq polymerase (0.1 U). The primer pairs used were specific for *nptII*, *Cry7* and *ET33-34* genes. *VirD2* primer pairs were used to check for presence of *A. tumefaciens* contamination from the transgenic events generated (Table 4.1). The PCR reactions were conducted using the following program, initial denaturation 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 30s, annealing at 58°C for 30s, extension at 72°C for 45s. The final extension was 72°C for 10 min.

**Table 4.1:** Primer pairs and their respective sequences for PCR

| Primer           | Sequence (5' – 3')        | Amplicon size (bp) | Amplification type |
|------------------|---------------------------|--------------------|--------------------|
| <i>Cry7</i> F    | ACAACTCATCACCATACCAAAC    | 608                | PCR                |
| <i>Cry7</i> R    | AAGAGCAAGATGCAAGTTTG      |                    |                    |
| <i>ET33-34</i> F | CACCTATAGTAAAACCATTGGACAC | 465                | PCR                |
| <i>ET33-34</i> R | AGTGGATTTGACGGTTGTTG      |                    |                    |
| <i>NptII</i> F   | TCGGCTATGACTGGGCACAACAGA  | 722                | PCR                |
| <i>NptII</i> R   | AAGAAGGCGATAGAAGGCGATGCG  |                    |                    |
| <i>VirD2</i> F   | ATGCCCGATCGCGCTCAAGT      | 338                | PCR                |
| <i>VirD2</i> R   | CCTGACCCAAACATCTCGGCT     |                    |                    |

#### 4.4.3 Southern blot analysis

##### 4.4.3.1 Genomic DNA restriction and transfer of resolved DNA

The extracted genomic DNA for southern blot was quantified and then 30 µg digested using a reaction mix containing Hind III restriction enzyme (10U), enzyme buffer (1x),

DNA (30 µg) using a protocol described by Nopo *et al.* (2005). Resolving of the gel was done at 60 V. Transfer of the resolved gel was conducted by preparing a sandwich as described by Nopo *et al.* (2005).

#### 4.4.3.2 Labelling, membrane hybridization and processing

PCR DIG Synthesis kit (Roche®) was used to prepare probes for *cry7* gene that was eventually used for hybridization of the resolved genomic DNA transferred on the nylon membrane. The probe was prepared and the membrane processed according to Roche manufacturer's instructions.

The wrapped nylon membrane was then placed in a Hyper-cassette, Amersham® and Kodak® film placed over the membrane in a dark room. The cassette was covered with foil and incubated at room temperature overnight. After incubation, the nylon membrane was then washed using a Kodak® GDX developing solution followed by rinsing briefly in water and then washed with Kodak® GBX fixer. The film was then dried and scanned. The events with a band signified that the gene was successfully inserted into the plant's genome while those with no band, meant that they were not transgenics.

#### 4.5 Protein analyses

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was done according to Nopo *et al.* (2005). Reading was then done on a photometer (Biotek Synergy HT) at 405 nm wavelength using Gen5 1.06® software. Protein positive plants had their absorbance values higher than the negative control while the negative plants had values lower than the negative control.

Western blot analysis was initiated by extracting protein from leaves and root samples of the SSA putative transgenic events and the 2 Jewel control events since leaf samples have little amounts of both sporamin and  $\beta$ -amylase in presence of sucrose as described by Nopo *et al.* (2005). The extracted proteins were run on resolving gel at 10% and stacking gel at 4% (w/v), followed by development and processing of the nylon membrane as described by Nopo *et al.* (2005).

#### **4.6 Efficacy of the transgenic events on weevils (*Cylas puncticollis*)**

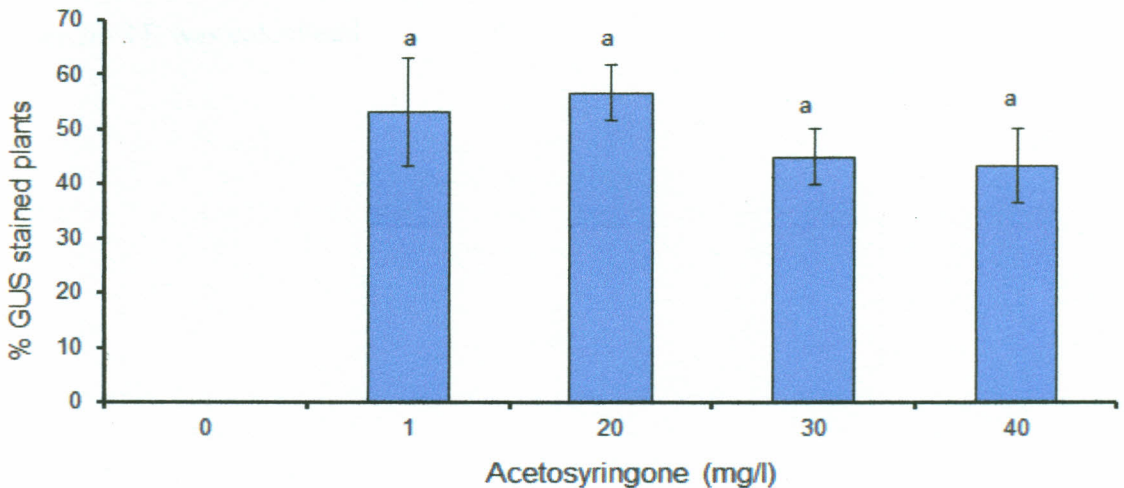
Sweetpotato storage roots from transgenic events and Jewel negative control were harvested from the greenhouse and then washed. The roots were then separately placed in 2-liter plastic jars of dimensions 20.5 cm height x 10.5 cm diameter covered with a net. This was done in three replicates. Sexing of the weevils was then conducted to separate males from females followed by separation of newly emerged females from older ones. The newly emerged females were starved for 3 days before introducing them into jars containing these events and their negative control samples. Ten newly emerged female weevils (*C. puncticollis*) were introduced into the plastic container with the sweetpotato samples: observations for mortality were undertaken after every 2 days for a period of 60 days. The number of feeding holes, deaths and survival were reported for these events with comparison to the negative controls.

## 4.7 RESULTS

### 4.7.1 Screening for transformability of selected SSA cultivars with the *uidA* gene

#### 4.7.1.1 Influence of acetosyringone level on TE of Mugande cultivar

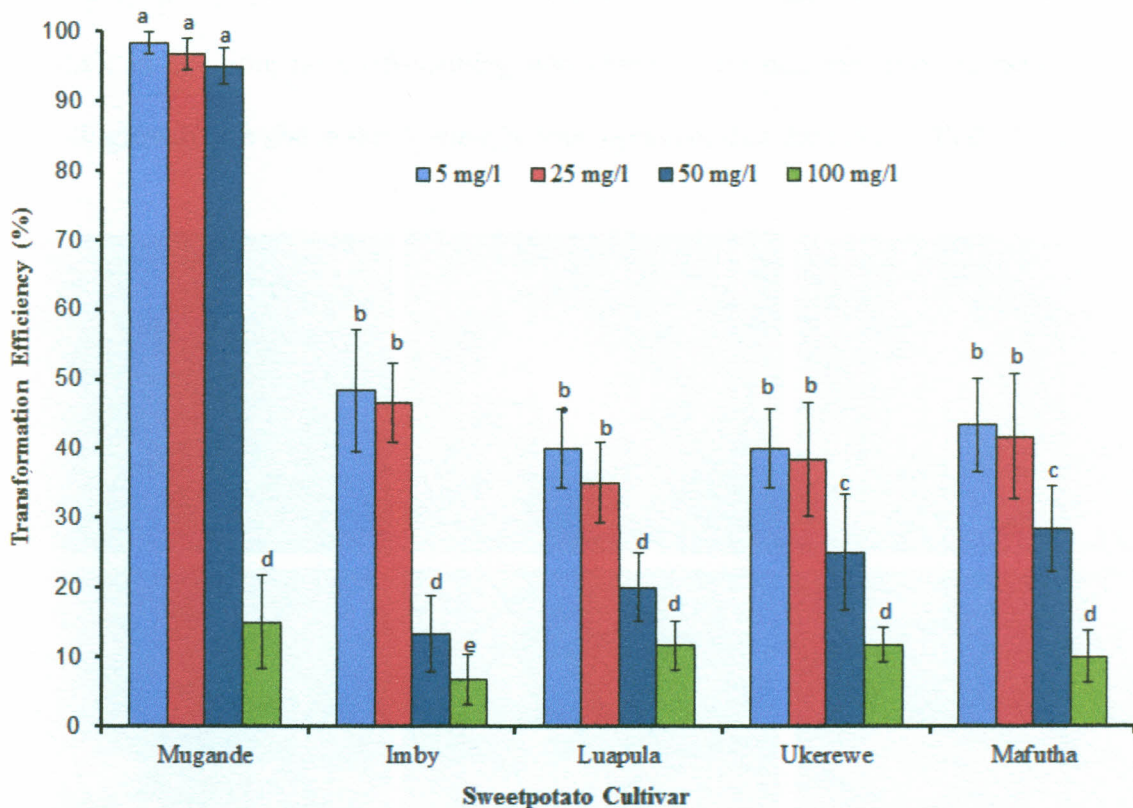
Effect of acetosyringone on TE of Mugande cultivar was evaluated using 1, 20, 30 and 40 mg/L concentrations for the first preliminary screen. Mugande cultivar was initially used due to ease of acquisition. The highest TE of 57% was observed when 20 mg/L acetosyringone was used while the lowest TE (1.67%) was observed when none was applied (Fig. 4.3). There were significant differences ( $p < 0.05$ ) when acetosyringone used on TE for the 5 concentrations (0 mg/L) but no significant differences ( $p > 0.05$ ) when 0 mg/L was removed from the analysis (appendix 7). Post anova analysis done using Student Newman Keuls test also showed no difference between the means (Fig. 4.3).



**Figure 4.3:** Transformation efficiency of Mugande cultivar on GUS-stained calli with vector construct pCIP100 on media with different acetosyringone levels. Values are means of 3 independent replicates and bars on the graphs represent standard error of means (SEM). The standard error of differences of means was 2.98 for the percentages of GUS-stained calli and there were no differences hence all classified as 'a'

#### 4.7.1.2 Influence of kanamycin concentration on transformation efficiency

There was an effect of kanamycin concentration on TE for the GUS-stained calli for the second preliminary screen (Fig. 4.4). There was reduction in TE with the increase in kanamycin concentration in step 2 media for the GUS-stained calli produced irrespective of the sweetpotato cultivar. Higher TE were obtained on MS media supplemented with 5 and 25 mg/L kanamycin except for Mugande where TE was high for 5, 25 and 50 mg/L (Fig. 4.4). Low kanamycin concentration of 5 mg/l resulted in high TE for the cultivars screened, for example, Mugande had the highest TE of 98.3%, followed by Imby (48.3%), then Mafutha with 43.3%, Luapula and Ukerewe were both 40%. Lowest TE was 6% for Imby followed by Mafutha (10%) when 100 mg/L of kanamycin was used (Fig. 4.4). There were significant ( $p < 0.05$ ) effects of cultivar type and kanamycin levels ( $p < 0.05$ ) on TE (appendix 8). The negative control showed no staining of calli produced hence no TE was calculated.

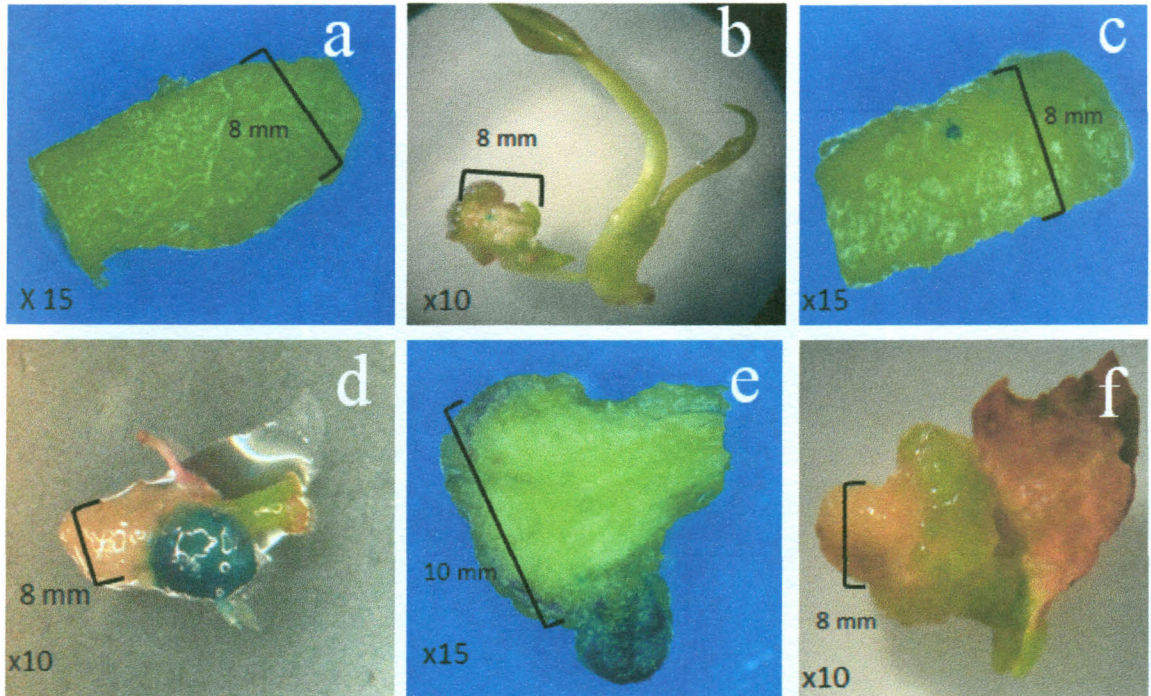


**Figure 4.4: The effect of kanamycin concentration on TE of Mugande, Luapula, Imby, Ukerewe and Mafutha based on GUS-assays on calli.** Transformation efficiency of 5 SSA sweetpotato cultivars was determined based on the number of GUS stained calli obtained from step-2 regeneration media containing 0.2 mg/L kinetin supplemented with kanamycin at 5, 25, 50 and 100 mg/L. The values are means of 3 independent replicate experiments and bars on graphs represent standard error of means (SEM). The standard error of differences of means was 2.4 and used to calculate the differences between TE of the cultivars where 'a' has the highest values and is significantly different from b, followed by c then d then e.

#### 4.7.1.3 Variations in GUS-stained calli and regenerants of the *uidA* gene

There were 5 SSA cultivars (Imby, Luapula, Ukerewe, Mafutha and Mugande) screened for TE using GUS assays after transforming with pCIP100 and generally, calli screened on MS media with low kanamycin levels had larger regions of GUS-staining than those on media with higher levels. Observations made on GUS-stained calli or regenerants was varied where some calli had no GUS-staining (Plate 4.1a), some regenerants had partial

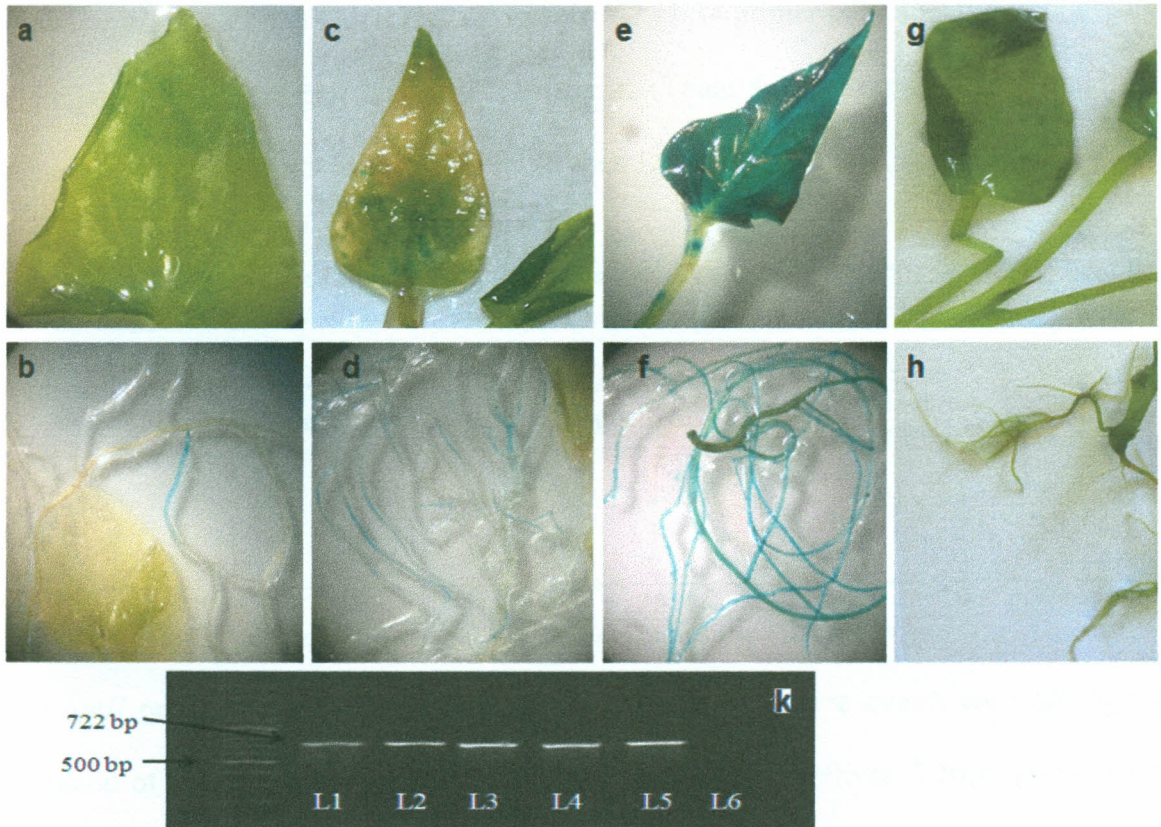
GUS-staining on a part of shoot (Plate 4.1b), small part of the callus was stained (Plate 4.1c,d,e). In addition, no GUS-staining was observed on non-transformed explants and the calli growth was above the *A. tumefaciens* dipped end of the petiole (Plate 4.1f).



**Plate 4.1: GUS staining on calli generated after transformation** using the pCIP100 vector construct. TE was evaluated on the basis of presence or absence of GUS-staining on the calli **(a)** No GUS-staining. **(b)** Chimeric regenerating. **(c)** Spot stained sections. **(d)** GUS-stained outcrops. **(e)** A cut section of the outcrop. **(f)** Non-transformed control with no GUS staining and calli did not grow from petiole tip like the other transformed explants.

Ukerewe cultivar was successfully transformed with 3 vector systems namely *βamy-uidA* gene for pCIP88, *SPOA1-uidA* gene for pCIP87 and *CaMV35S-uidA* gene for pCIP100 and produced regenerants. Regenerants produced using *β-amy-uidA* showed less GUS-staining on the roots and leaves (Plate 4.2a,b) compared to those transformed with *SPOA1-uidA* (Plate 4.2c,d) but highest expression was observed on those transformed with *CaMV-35S-uidA* gene (Plate 4.2e,f). Nevertheless, staining on plant organs such as roots, leaves or calli was not evenly spread (Plate 4.2). The negative control did not show

any GUS-staining (Plate 4.2g,h). PCR conducted on DNA extracted from different plant parts, either stained or unstained showed presence of *nptII* (Plate 4.2k). When these putative transgenic events were subjected to the callus test at high selection pressure of 100 mg/L for a period of 30 days, they produced small calli while the negative control produced necrotic tissue.



**Plate 4.2: Images of GUS-staining on regenerants after transformation** (a) Less staining on leaf transformed using pCIP87 (b) Partial staining on roots transformed using pCIP87 (c) Spatial staining on leaf transformed using pCIP88 (d) More staining on roots for explants transformed with pCIP88 (e) Whole leaf staining on leaf transformed with pCIP100 (f) Whole root staining on root transformed using pCIP100 (g) Non-transformed leaf with no GUS-staining (h) Non-transformed root with no GUS-staining (k) PCR amplicons of GUS stained and unstained organs from events positive for *nptII* primer pairs 722 bp (L1) pCIP87 root with no visible GUS-stain (L2) pCIP88 root with GUS-stain (L3) pCIP88 leaf with GUS-stain (L4) pCIP88 leaf with no visible GUS-stain (L5) positive control (L6) negative control

## 4.7.2 Screening of putative events produced with the double gene construct

### 4.7.2.1 Putative transgenic events observed for *cry7Aa1*, *ET33-34* and *nptII* genes

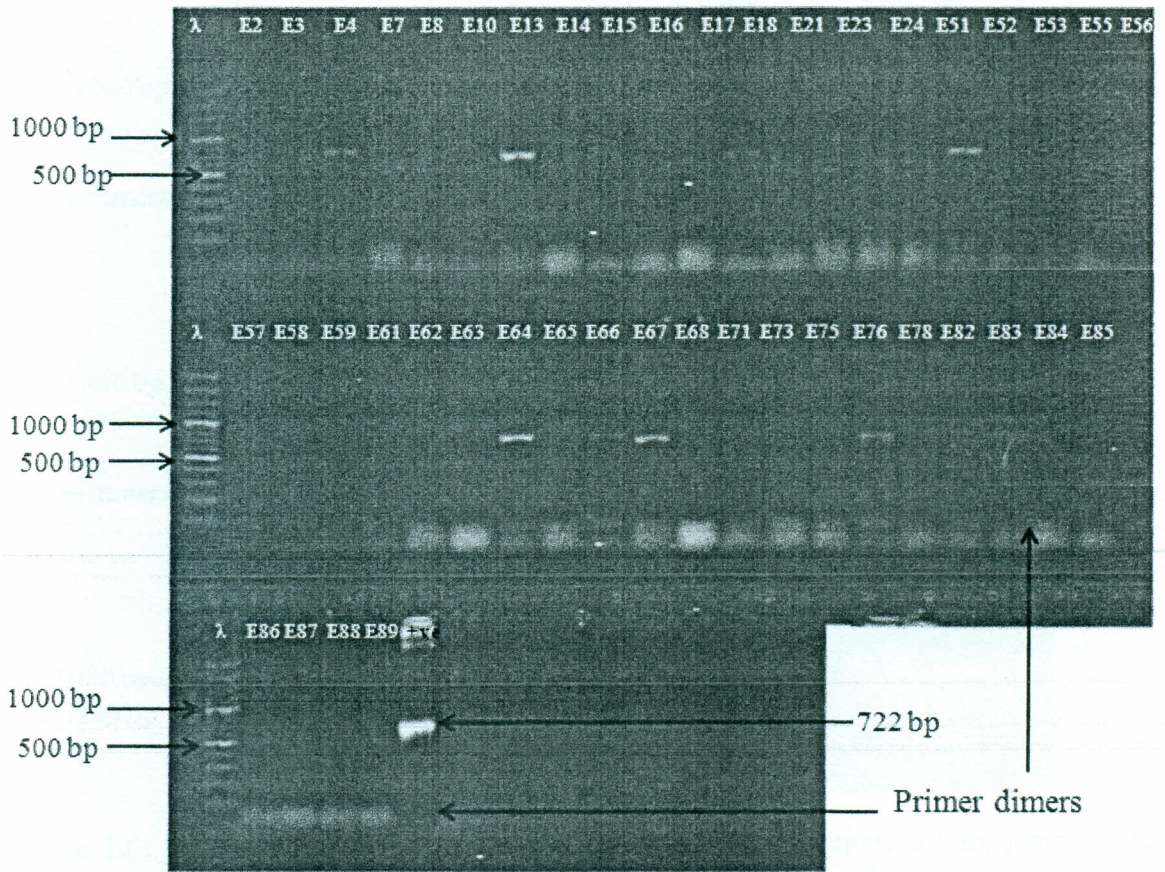
A total of 45 putative transgenic events for SSA cultivars and 2 for Jewel control were produced, which were screened for presence of the 3 genes namely *cry7Aa1*, *ET33-34* and *nptII*. The respective primer pairs led to identification of the following putative events: *nptII* primer pairs identified 12 events; *ET33-34* primer pairs identified 25 events while *cry7Aa1* primer pairs identified 15 events (Table 4.2). Ukerewe cultivar had the highest number of events followed by Jewel then Luapula and Imby (Table 4.2).

**Table 4.2:** Cummulative transgenic events produced with pCIP85 gene construct bearing tandem copy of *cry7Aa1* and *ET33-34* genes

| Cultivar     | Total explants | Putative events | <i>nptII</i> positive | <i>ET33-34</i> positive | <i>cry7Aa1</i> positive | Total confirmed events | TE (%) |
|--------------|----------------|-----------------|-----------------------|-------------------------|-------------------------|------------------------|--------|
| Ukerewe      | 660            | 36              | 7                     | 16                      | 10                      | 5                      | 0.01   |
| Luapula      | 906            | 6               | 2                     | 4                       | 2                       | 1                      | 0.001  |
| Imby         | 624            | 3               | 1                     | 3                       | 1                       | 1                      | 0.001  |
| Jewel        | 554            | 2               | 2                     | 2                       | 2                       | 2                      | 0.004  |
| <b>Total</b> | <b>2744</b>    | <b>47</b>       | <b>12</b>             | <b>25</b>               | <b>15</b>               | <b>9</b>               |        |

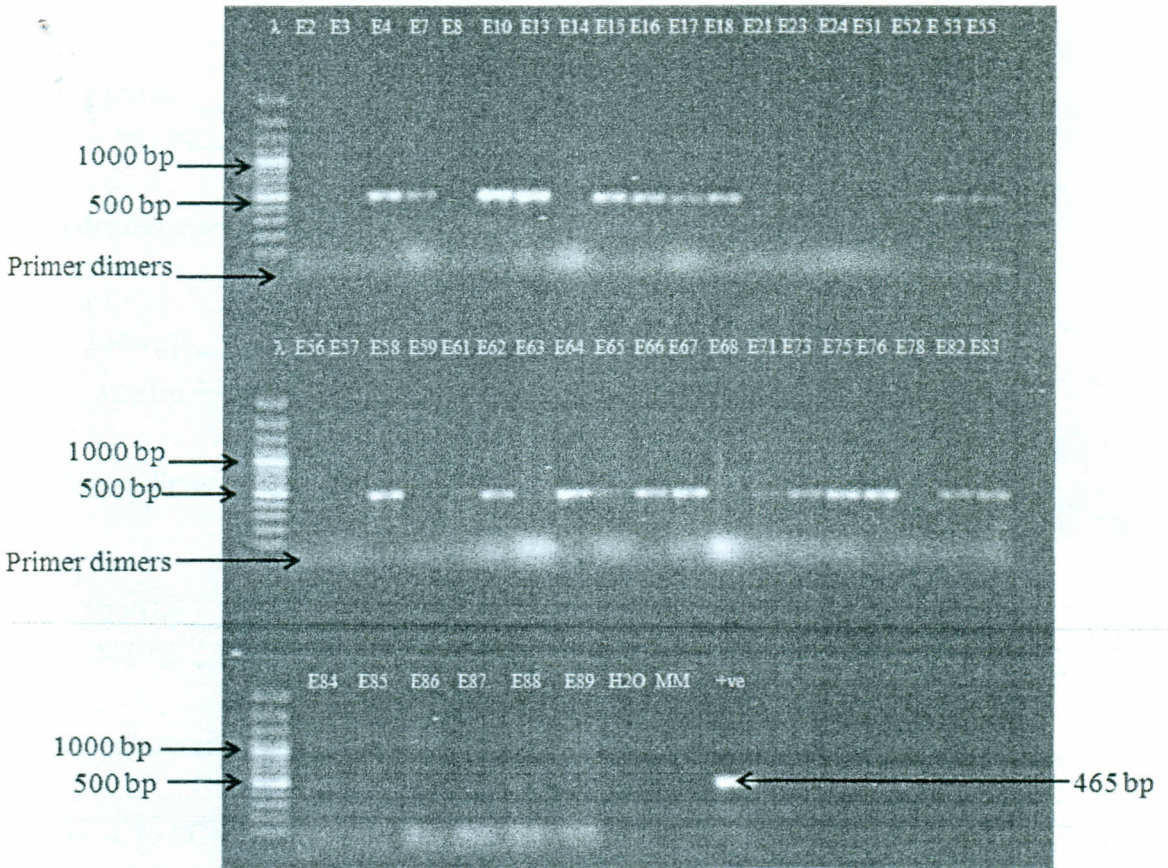
Total confirmed events were those events that showed positive bands from the southern blot

The *nptII* primer pairs led to identification of 10 PCR-positive events each having an amplicon of 722 bp (Fig. 4.5), which were 5 from Luapula cultivar, 3 from Imby and 7 from Ukerewe.



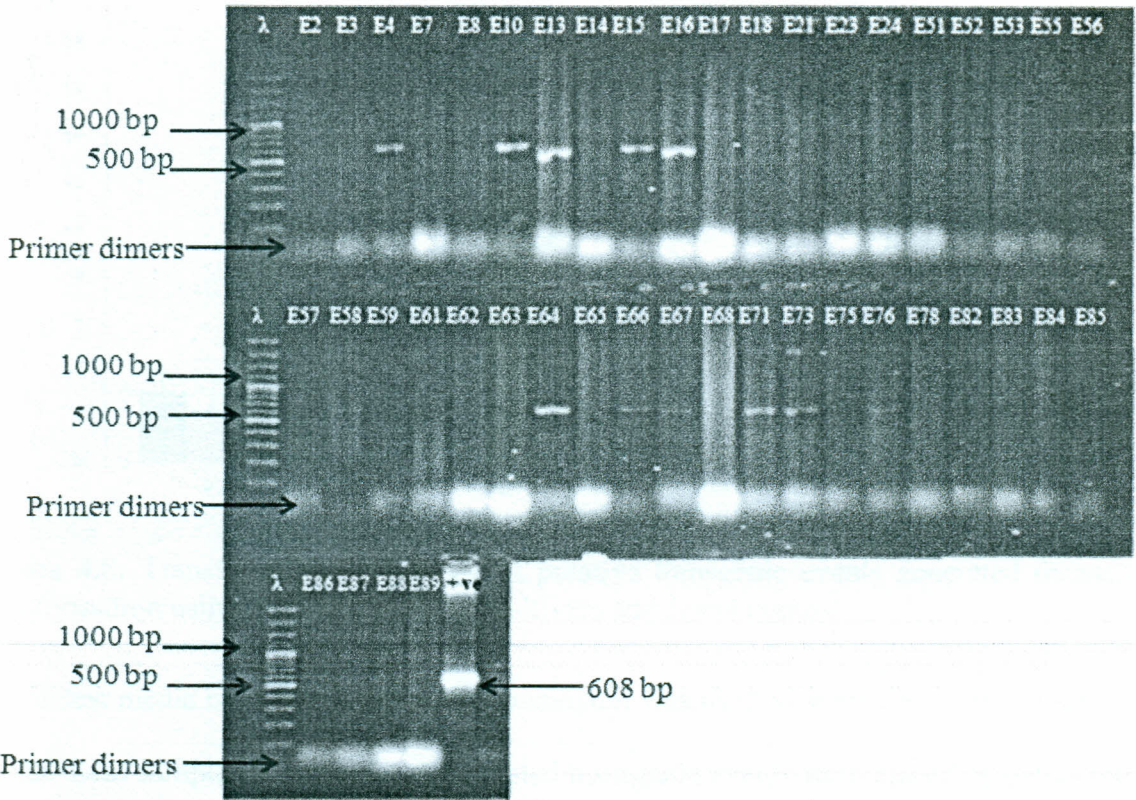
**Figure 4.5:** PCR-based gel image showing putative SSA sweetpotato events identified using *nptII* primer pairs with amplicon size of 722 bp. E2 to E89 are the putative events generated after transformation experiments using the WR genes. All the events showed presence of primer dimers indicating the necessity of further optimization

The second set of primer pairs *ET33-34*, led to identification of 23 transgenic events with 465 bp, which was the same size as the positive control amplified from the pCIP85 plasmid (Fig. 4.6). There were 2, 5 and 15 positive events of Imby, Luapula and Ukerewe cultivars, respectively (Table 7). In addition, all PCR-positive transgenic events screened using *ET33-34* primer pairs were PCR-positive for *nptII* primer pairs apart from E71.



**Figure 4.6:** PCR based gel image showing putative SSA sweetpotato transgenic events identified using *ET33-34* primers of the expected amplicon sizes of 465 bp. E2 to E89 are the putative events generated after transformation experiments using the WR genes

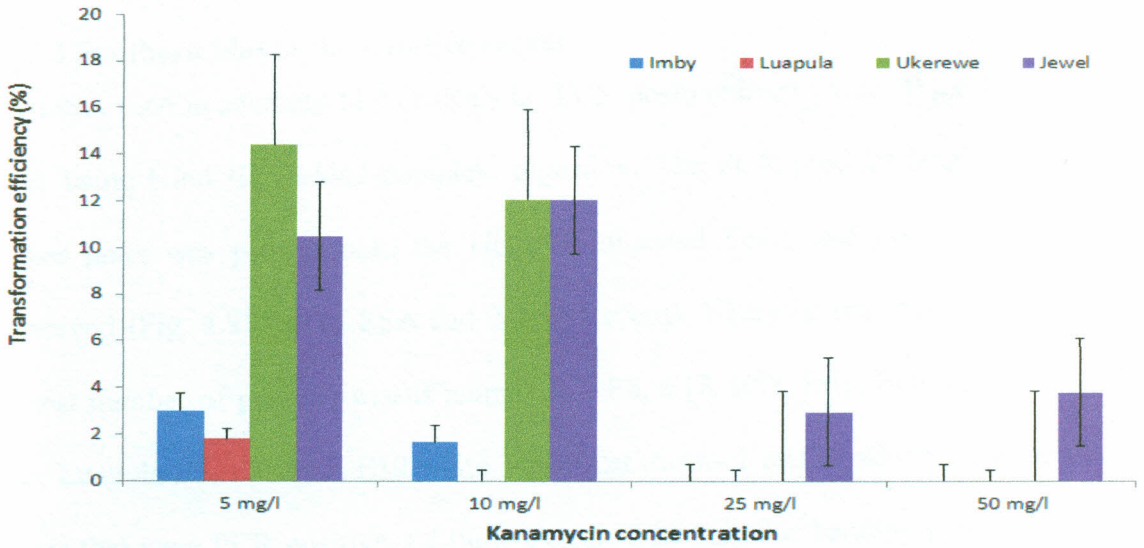
The third set of primer pairs was for *cry7Aa1* gene, which was used to identify 13 PCR-positive transgenic events with the expected amplicon size of 608 bp (Fig. 4.7). The number of events was 2, 4 and 7 for Imby, Luapula and Ukerewe cultivars, respectively (Table 7). Additionally, all the PCR-positive events using *cry7Aa1* primer pairs were also positively identified in both *ET33-34* and *nptII* primer pairs.



**Figure 4.7:** PCR based gel image of 89 putative events showing presence of *cry7A1* in the 13 events with amplicons of the expected 608 bp, comparable to the positive control. E2 to E89 are putative events generated after transformation using WR genes

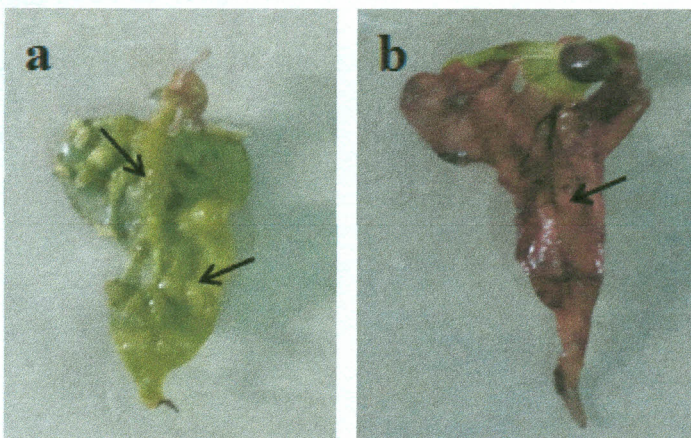
#### 4.7.2.2 Influence of kanamycin level on transformation efficiency for pCIP85 events

Different kanamycin levels were used to screen for TE of 3 cultivars (Luapula, Imby and Ukerewe) for events produced using weevil resistance genes (*cry7Aa1* and *ET33-34*) and some regenerants were produced. At 5 mg/L, Ukerewe cultivar had TE of 12.08% while Luapula had TE of 1.81%: at 10 mg/L Ukerewe had TE of 14.4% while Imby had TE of 3.01% (Fig. 4.8). Kanamycin concentrations of 25 and 50 mg/L produced no regenerants for the 3 SSA cultivars. The control, Jewel cultivar showed TE of 10.5% and 22.05% for 5 and 10 mg/L, respectively but at 50 mg/L it had a TE of 3.8% (Fig. 4.8).



**Figure 4.8:** Transformation efficiency of putative transgenic events generated through transformation using pCIP85 for 3 SSA cultivars and Jewel control

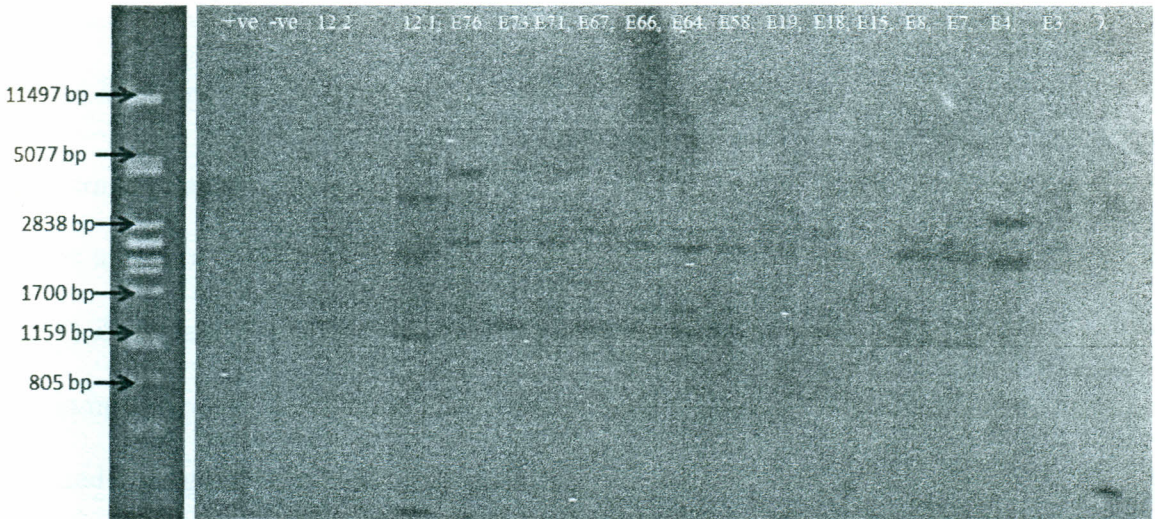
Callus test media containing 100 mg/L kanamycin was used to screen for true transgenic events. Leaf samples from the 14 regenerated transgenic events were placed on callus test media for 30 days and results showed small calli growth on some transgenic events: E4, E13, E18, E51, E64, E66, E67, and E76. These events were positive for nptII PCR. The negative controls on callus test media developing necrotic tissue after 2 weeks and they eventually died within the 4 weeks of culture (Plate 4.3).



**Plate 4.3:** Transformed and non-transformed leaf samples on callus media having 100 mg/L kanamycin for selection after 30 days in culture (a) calli growth on leaf sample from a transformed event (b) Necrosis and death of a non-transformed leaf sample

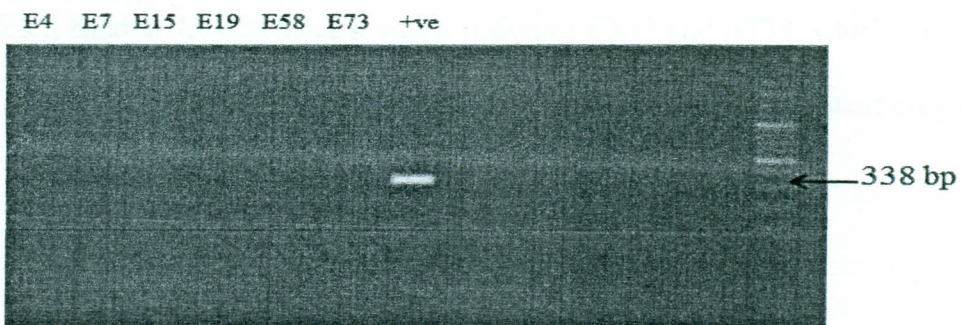
#### 4.7.2.3 Southern blot of the putative events

The DNA used in southern blot analysis for PCR-positive events was 30 µg. Digestion of DNA using Hind III yielded complete digestion. The PCR product from the *cry7Aa1* primer pairs was probed onto the Hind III digested DNA and copy numbers were observed (Fig. 4.9) for 13 SSA and 2 Jewel events. Ukerewe transgenic lines had the highest number of positive events namely E7, E8, E18, E58, E64, E66, E67, E71, E73, E76; Luapula E3, E4, E15, E19 while Jewel had events 1 and 2, which made up the 15 events that were PCR positive. Of these events, 7 had similar banding pattern of 4 copy numbers each for events E7, E8, E18, E64, E66, E67 and E71 all from Ukerewe cultivar. The pure plasmid (pCIP85) had the complete size of the plasmid of 14 kb while the expected band size of *cry7Aa1* was 3545 bp, which included the HindIII digestion site from the insert, promoter and terminator. Due to this, bands from the events were expected to be of a similar size of 3545 bp or bigger. Event E76 had one band of about 3454 bp for one copy while the other samples either had darker shades but smaller sizes. Nevertheless, most bands generated from these events, produced had sizes that ranged between 1000 and 3000 bp (Fig. 4.9). Some of the events had darker shades than that of the control like 12.1, E76 and E4 while others like E19, E18, E3 and 12.2 had lighter shades (Fig. 4.9).



**Figure 4.9:** Southern blot analysis showing *cry7Aa1* gene in 13 SSA (E76, E73, E71, E67, E66, E64, E58, E19, E18, E15, E8, E7, E4, E3) and 2 Jewel (12.1, 12.2) sweetpotato cultivar events with several copies each.

The positive events by southern blot were checked for presence of *A. tumefaciens* using *VirD2* primer pairs within interstitial tissues and the six events (E4, E7, E15, E19, E58, E73) showed no contamination (Fig. 4.10).

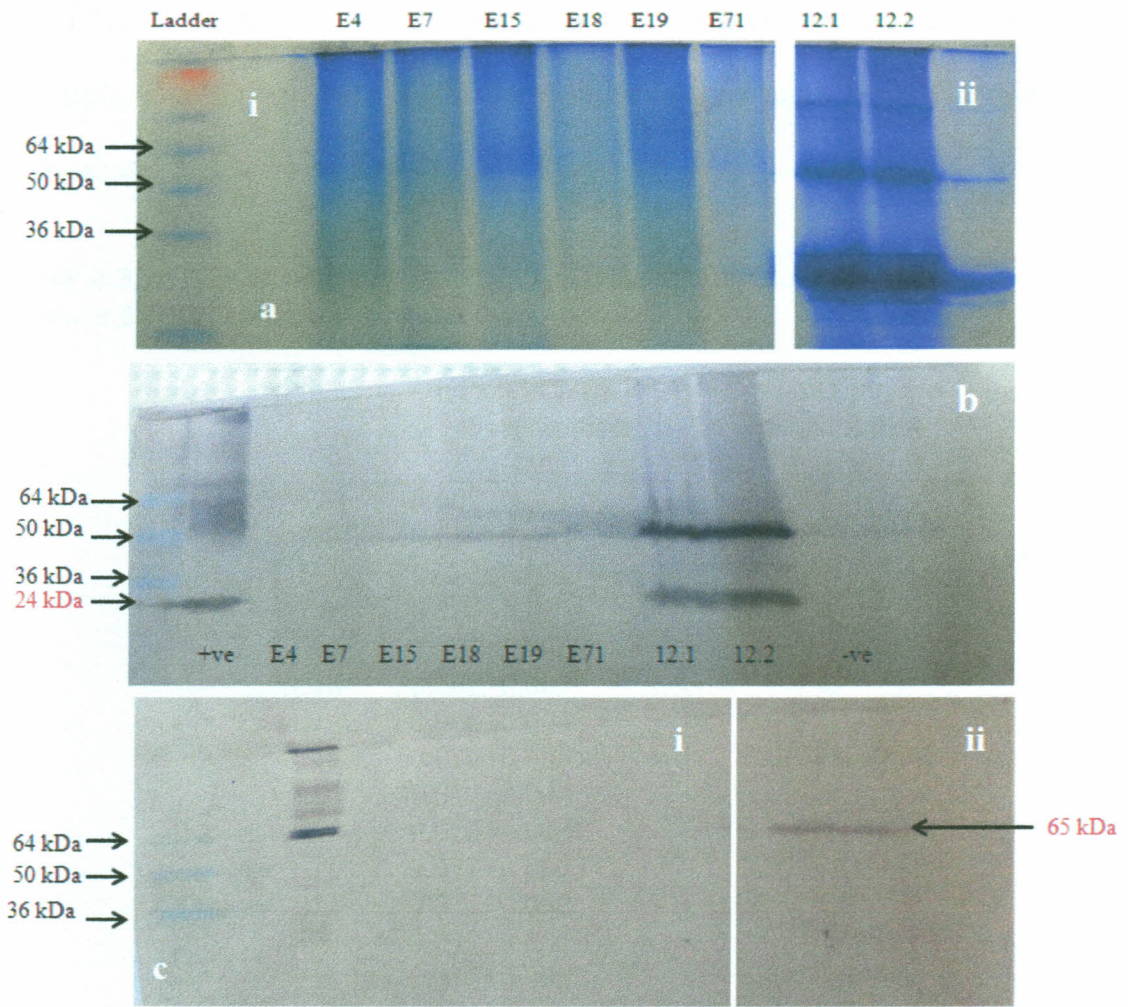


**Figure 4.10:** PCR based gel image of the generated events showing no *A. tumefaciens* contamination for the samples but the pure plasmid (+ve) using *VirD2* primer pairs with an amplicon of the expected 338 bp

### **4.7.3 Screening for protein quality and quantity of the putative transgenic events**

#### **4.7.3.1 Western blot of Cry7Aa1 and ET33-34 for protein expression**

Western blot analysis confirmed the presence of Cry7Aa1 and ET33-34 proteins in the leaves and root extracts of the positive transgenic events. The SDS-PAGE image (Fig. 4.11a) shows a profile of the total soluble proteins from an equal volume of 30  $\mu$ l loaded per sample, present in sweetpotato lines and untransformed control. Extracts from both leaf and storage root samples had a number of proteins observed for events 12.1, 12.2 root extracts, which were more compared to those observed in the leaf extracts (Fig. 4.11a). When the specific polyclonal antibodies to both proteins were used to identify ET33-34 proteins in the samples, transgenic events Jewel 1 and 2 showed darker bands, which could indicate higher protein levels compared to the leaf samples E4, E7, E15, E18, E19 and E71 of ET33-34 (Fig. 4.11b). Nevertheless, extracts from both storage roots and leaf samples had the expected protein of 43 kDa. The pure protein did not have the expected band at 43 kDa since it was a fusion of ET33 and ET34, which were 16 and 24 kDa, respectively, separately (Fig. 4.11b). Cry7Aa1 protein was identified only for root samples but not leaf samples with the expected 65 kDa size (Fig. 4.11c).



**Figure 4.11:** Proteins observed in leaf samples of SSA events and roots from Jewel control events. **a)** SDS-PAGE of total protein i) for leaf samples ii) for root samples **b)** Western blot of ET33-34. This protein was a fusion of ET33 and ET34 that had different weights of 16kDa and 24kDa, respectively, and the antibodies were specific for both ET33 and ET34, separately. **c)** Western blot of Cry7Aa1 i) leaf samples showed no detection of the protein. ii) The root samples have a band, which was expected due to the root specific promoters used. The negative control (non-transformed) is -ve while the positive (pure Cry7Aa1 and ET33-34 protein) +ve: the ladder (LD) used was SeeBlue® (Invitrogen)

#### 4.7.3.2 Protein expression levels of the putative events

Double antibody sandwich ELISA was used to determine ET33-34 protein concentration of the transgenic events in both SSA cultivars and Jewel control. Protein content of ET33-34 in roots of 15 transgenic events namely, E3, E4, E7, E8, E15, E18, E19, E64,

E67, E71, E73, E76, Jewel 1 and 2 was quantified: the protein levels varied between 0 to 0.03  $\mu\text{g/g}$ . Cry7Aa1 protein concentration levels were much lower than the ET33-34 with values ranging from 0 to 0.018  $\mu\text{g/g}$  (Table 4.3).

**Table 4.3.** ET33-34 and Cry7Aa1 protein concentrations in roots of SSA transgenic events and Jewel control

| Events  | ET33-34 protein concentration ( $\mu\text{g/g}$ ) | Cry7Aa1 protein concentration ( $\mu\text{g/g}$ ) |
|---------|---|---|
| E3      | 0.008+0.0004                                      | 0   |
| E4      | 0.019+0.005                                       | 0.002+0.001                                       |
| E7      | 0.020+0.004                                       | 0   |
| E8      | 0.020+0.05  | 0   |
| E15     | 0.005+0.0004                                      | 0.001+0.005                                       |
| E18     | 0.002+0.007                                       | 0   |
| E19     | 0.008+0.003                                       | 0.002+0.0009                                      |
| E58     | 0.020+0.005                                       | 0.003+0.0006                                      |
| E64     | 0.012+0.004                                       | 0   |
| E67     | 0.026+0.003                                       | 0.001+0.0002                                      |
| E71     | 0.021+0.0003                                      | 0.001+0.0002                                      |
| E73     | 0.021+0.002                                       | 0   |
| E76     | 0.017+0.006                                       | 0   |
| Jewel 1 | 0.010+0.002                                       | 0.008+0.003                                       |
| Jewel 2 | 0.025+0.003                                       | 0.018+0.004                                       |
| UK      | 0   | 0   |

#### 4.7.4 Effect of feeding transgenic events on *Cylas puncticollis*

The experiment involved storage roots of the transgenic events and Jewel. Ten weevils were placed in plastic jars (as described in section 4.5 of this chapter). The experiment was conducted for a period of 60 days, with refreshing the roots once they dried out until all the weevils died. Observations made on feeding and mortality of the weevils showed similar low mortality and variation in number of feeding holes for Jewel 1 and Jewel 2 events and negative control (Table 4.4). There were high significant differences ( $p < 0.05$ ) between the number of feeding holes of the 3 samples used (Appendix 9).

**Table 4.4.** Feeding assay and mortality of *Cylas puncticollis* on storage roots

| Sweetpotato     | Number of feeding holes |     |     | Number dead |   |   |
|-----------------|-------------------------|-----|-----|-------------|---|---|
|                 | a                       | b   | c   | a           | b | c |
| Jewel 1         | 119                     | 147 | 113 | 0           | 1 | 2 |
| Jewel 2         | 97                      | 101 | 70  | 3           | 1 | 0 |
| Jewel (control) | 100                     | 89  | 115 | 1           | 2 | 0 |

There was a significant difference ( $p < 0.05$ ) between the samples (appendix 9)

## 4.8 DISCUSSION

### 4.8.1 Effect of acetosyringone on transformation efficiency

Calli produced after transforming leaf with petiole explants using pCIP100 and incubated on medium containing different acetosyringone levels indicated that increasing it had no overall influence on TE except when it was absent. These results are consistent with those reported for Chinese sweetpotato where only acetosyringone applied between 20 to 100 mg/L indicated a statistically insignificant influence on TE than when none was applied (Xing *et al.*, 2007). In this study, influence of acetosyringone on TE was not dependent on genotype since the addition of 1 to 40 mg/L to medium led to production of transformed events.

### 4.8.2 Effect of kanamycin on TE for calli of transformed explants

The effect of kanamycin on transformed calli was evaluated for different cultivars. A concentration of 100 mg/L kanamycin in medium seemed to inhibit callus induction in some cases whereas those that developed calli either had none or minimal GUS-staining for the SSA cultivars, which indicated that either the plant cells were killed or unable to multiply since they were not transformed to resist kanamycin. Calli screened on medium containing kanamycin at 50 mg/L had a wide range of TE from 13 to 95% depending on cultivar while prolific calli formation was observed at low kanamycin levels of 5 mg/L

resulting to high TE (40 to 98%). The non-transformed controls formed calli above the petiole-end but not in contact with MS media indicating there was no cell multiplication for the petiole in contact with the media. Prakash and Varadarajan (1992) reported lower TE of 0.02% for GUS-stained calli for Jewel sweetpotato cultivar using 50 mg/L kanamycin while Yu *et al.* (2003) used 50 mg/L kanamycin and reported 48% TE while 5 mg/L was reported to have a TE of 88% in papaya.

From these studies, higher kanamycin levels exhibited higher inhibitory effects on transformed explants compared to lower kanamycin levels. A possible explanation of high levels inhibiting growth is shown by the fact that kanamycin inhibits protein synthesis in non-transformed cells, in turn, these dying non-transformed cells possibly secret inhibitors, therefore, preventing transport of essential nutrients to the living transformed cells (Joersbo and Okkels, 1996). Non-transformed controls formed good calli at low kanamycin concentration but higher concentrations either did not have calli or there was cell death. Nevertheless, the non-transformed did not have GUS-staining. However, the exact levels varied with the genotype as reported for rose species where explants cultured on media with 50 mg/l kanamycin concentrations and higher did not develop calli (Li *et al.*, 2002).

#### **4.8.3 Partial GUS-staining was not associated with genetic chimeras**

Transgenic events were produced with 3 gene constructs namely pCIP88 with the  $\beta$ -*amy-uidA* gene, pCIP87 with *SPOA1-uidA* gene and pCIP100 with *CaMV35S-uidA* gene; all showing variations in GUS-staining. Humara *et al.* (1999) reported similar observations of GUS-stained spots for *Pinus pinea* cotyledons transformed using constructs bearing

sunflower ubiquitin-*uidA*, maize alcohol dehydrogenase-*uidA*, rice actin-*uidA* or CaMV-*uidA* genes. Some of the reasons explaining these variations could be as follows: firstly, in apples such variations were attributed to genetic chimera due to presence of both transformed and non-transformed cells (Ko *et al.*, 1998). This was unlikely for the events produced in this study because PCR results showed presence of the *nptII* gene even in sections that had no GUS-staining, even in the meristematic regions of the plant. Secondly, Day *et al.* (2000) reported partial GUS-staining in tobacco attributing it to low *uidA* gene expression, DNA-methylation and gene silencing. Genetic chimeras was ruled out from the observations made in this study, DNA-methylation could have been another factor although it requires further analysis to determine the exact cause.

#### **4.8.4 Effect of promoter-used on GUS-staining among putative transgenic plants**

The *uidA* transgenic events showed uneven display of GUS-staining on roots and leaves of the regenerants produced using pCIP87, pCIP88 and pCIP100. Events with the highest GUS-stained regions were transformed with *CaMV-uidA* followed by those with *SPOA1-uidA* and then  *$\beta$ -amy-uidA* genes. Events with *SPOA1* and  *$\beta$ -amy* promoter showed less staining probably because they root-specific proteins but found in other plant parts in small amounts (Maeo *et al.*, 2001) where total protein of sporamin accounts for 60 to 80% while  $\beta$ -amylase for 2% in storage roots (Maeshima *et al.*, 1985). Other studies have reported using *SPOA1* and  *$\beta$ -amy* separately (Wang *et al.*, 2002; Morikami *et al.*, 2005; Maeo *et al.*, 2001), but not comparing the 2 as in this study. GUS-staining on events with 35S was spread throughout the plant organs, which is consistent to those reported by Yang *et al.* (2011) and Binka *et al.* (2012).

#### 4.8.5 Physiological factors that seemed to contribute to the observed GUS-staining

The main observations made for GUS-staining of transgenic events produced using the 3 gene constructs (pCIP87, pCIP88 and pCIP100) included the following. Firstly, uneven GUS-staining was evidenced on roots and leaves of regenerants. A previous study reported higher GUS-staining in apical cells of the root and attributed it to high cell meristematic population in that region (Jefferson *et al.*, 1987) and Srivastava (2002) reported that meristems are spread across the plant and not confined to specific sections. This suggests that uneven GUS-staining in this study, were contributed by the meristematic cell population. Secondly, there was more GUS-staining along the base of mid-lamina close to the petiole compared to leaf tip for *SPOA1*. A similar reported by Ichihashi *et al.* (2011) showed active cell proliferation and strong expression at leaf blade/petiole junction in *Arabidopsis* when *CaMV-35S* promoter was used. From this study, *SPOA1* showed GUS-staining on leaves because sporamin is normally found in small amounts in leaves (Maeo *et al.*, 2001) in addition to high cell multiplication compared to the leaf tip. Thirdly, the variation in GUS-staining was also observed on the roots. It has previously been reported that development of storage and fibrous roots in sweetpotato could be identified from the time plants are young (Villordon *et al.*, 2012) and an up-regulation of sporamin and  $\beta$ -amylase in storage roots compared to fibrous roots as reported by Firon *et al.* (2013). From these reports, the uneven GUS-staining on the roots could be due to several factors including separation of fibrous from storage roots and higher staining at meristematic regions.

#### 4.8.6 Optimal kanamycin levels for selection of putative pCIP85 transgenic events

A kanamycin concentration of 5 to 10 mg/L produced the highest number of putative events for the double gene construct with *cry7Aa1* and *ET33-34* while at higher levels of above 50 mg/L; it seemed to inhibit production of regenerants for Ukerewe, Imby and Luapula cultivars. However, when the transgenic plants and non-transgenic controls leaf samples were subjected to high selection pressure of 100 mg/L kanamycin for 30 days, calli were produced for the kanamycin positive events while the negative controls died. Other studies have reported production of transgenic events at relatively high levels of 50 mg/L (Moran *et al.*, 1998; Gonzalez *et al.*, 2008) but production of transgenic events at low kanamycin levels (10 mg/L) has also been reported (Xing *et al.*, 2007).

Low kanamycin levels reported herein could suggest either one of the following. Firstly, different genotypes respond differently to varying kanamycin levels, for instance, SSA cultivars transformed in this study seemed to be affected by kanamycin toxicity at relatively high levels. Secondly, although low kanamycin levels were used in selection and regenerants were produced, most of them (80%) were not transgenic events and other methods could be exploited to reduce the number of escapes generated. Thirdly, high kanamycin levels of 50 mg/L is widely used as optimal for generation of sweetpotato events (Song *et al.*, 2004) nevertheless, at this concentration, escapes have also been reported (Gonzalez *et al.*, 2008). This indicates that at low and high kanamycin levels escapes are generated. Several methods could be used to address this, such as increasing the number of explants at the beginning of the experiments, subjecting the putative events to a progressive increase in kanamycin levels over several sub-cultures and exploring other methods of regeneration.

Variations in number of transgenic events produced in varying kanamycin concentrations could be attributed to factors such as reduced cell viability. This has been reported by Shin *et al.* (2007) where there was gradual reduced cell viability in sweetpotato when kanamycin levels were increased from 0 to 100 mg/L. The reduced cell viability could be explained by the fact that kanamycin inhibits protein synthesis in non-transformed cells, in turn, these dying non-transformed cells possibly secret inhibitors, therefore, preventing transport of essential nutrients to the living transformed cells (Joersbo and Okkels, 1996). Secondly, since kanamycin was the only selection agent for both *A. tumefaciens* and the sweetpotato explants, other selection methods could be exploited. Okada *et al.* (2001) reported that kanamycin was ineffective for screening transgenic events due to the high number of escapes when 0 to 50 mg/L were used compared to hygromycin. Thirdly, although *A. tumefaciens* is documented to insert long T-DNA into the genome, more experiments should be done to confirm the efficacy of inserting the 8 kb fragment into sweetpotato cultivars in relation to kanamycin concentration levels. Fourthly, a catalase intron could be included in synthesis of new gene constructs as those for the *uidA* gene constructs since it has been reported to improve on transformation efficiencies 50 to 100 times (Callis *et al.*, 1987).

#### **4.8.7 Gene insertion using the double gene construct for the transgenic events**

Southern blot analysis confirmed *cry7Aa1* gene to be stably integrated into the genome but the copy number varied between events. Due to the possibility of gene truncation of the transgenes, there could have been multiple insertions on a single chromosome for dark bands or part of a gene as visualized for the lighter bands since all the events on the gel had equal amounts of DNA of 30 µg. A similar deduction would be made for lighter-

shaded bands than the positive control, which could be that parts of the genes were lost due to gene truncation after transformation. This is also explained by presence of smaller sized bands than 3454 bp, which were present in all the events. Presence of multiple insertions for genes of interest has been documented to result to gene silencing especially for higher copy numbers from 3 and above (Tang *et al.*, 2007) and this could have contributed to the low gene expression levels documented under protein expression. Similar reports were documented for sweetpotato with high copy numbers of 1-7 using *Agrobacterium*-mediated transformation (Kreuze *et al.*, 2008). Such high copy numbers are normally generated when indirect transformation techniques are used, including microprojectile bombardment (Okada *et al.*, 2002; Shin *et al.*, 2007) but not through *Agrobacterium*. High copy numbers reported in these studies could not be explained since *A. tumefaciens*-mediated transformation is known to generate low copy numbers. It is therefore speculated they were not all copies of the gene but truncated genes (Ford *et al.*, 1994) or parts of the gene hence visualized as separate copies on the membrane.

The southern blot also revealed that 7 transgenic events had similar banding patterns and copy numbers meaning that the gene was inserted at same locations on the chromosome, which is highly unlikely but they were the same event, which emerged from a single transformed cell or a couple of cells hence the similarities. Luo *et al.* (2006) reported similar observations where events produced from a single callus were identical. The southern blot confirmed integration of the gene into the sweetpotato plants but not *A. tumefaciens* contamination due to high amounts of plant DNA (30 µg) that was used for southern blot analyses hence disqualified presence of *A. tumefaciens* in interstitial spaces of these events; the transgenic events were screened for presence *A. tumefaciens* by PCR

but there was none and low kanamycin concentrations were initially used for selection of transgenic events of 5 and 10 mg/L but a higher level of 100 mg/L was applied to these events to eliminate the escapes.

#### 4.8.8 Protein expression of Cry7Aa1 and ET33-34

The protein profile from the western blot showed few proteins were visualized on SDS-PAGE gel, which could have been attributed to the protein extraction method used where these extracted proteins were saturated ammonium sulphate and then dialysis. During dialysis, as the ammonium sulphate is washed out, some small-sized proteins could have been removed as well hence few protein profiles on the membrane. The western blot results for ET33-34 showed presence of 2 bands, of 43kDa and 24kDa. This is because it is a fused protein of 16 kDa for ET33 and 24 kDa for ET34 as separate entities but fused together with 6 amino acids. The 43 kDa product could have been a mixture of the two proteins while the 24 kDa product could either have been ET34 or an epitope since they were polyclonal antibodies.

When DAS-ELISA was undertaken to estimate protein content of ET33-34 using the polyclonal antibody, the roots had levels between 0 and 0.03  $\mu\text{g/g}$  but Cry7Aa1 had lower levels from 0 to 0.018  $\mu\text{g/g}$ . The levels are below those reported in an American sweetpotato cultivar, Jewel, which had CryIIIa protein driven by 35S promoter with levels from undetectable to 0.5-1.5  $\mu\text{g/g}$  in roots (Moran *et al.*, 1998). The high ET33-34 levels in the roots was expected since it is driven by *SPOA1*, which is not only root specific but shown to have better expression than  $\beta$ -amylase, as was observed for *uidA* gene in this study. The relatively low protein levels observed for these events suggested

other factors such as post-transcription and post-translation could have affected them. Secondly, the genes could have been introgressed in regions of genome with low activity and thirdly, modification of the gene construct to increase gene expression could be done such as including translation enhancers.

#### **4.8.9 Effect of feeding the transgenic plants on *C. puncticollis***

The results obtained in the bioassay indicated no difference in number of feeding holes and mortality of weevils in both transgenic and non-transgenics. This was expected since the pure Cry proteins (Cry7Aa1 and ET33-34) in artificial diet had previously been tested against *Cylas* species and mortality was greater than 90% where the concentration used was 1 µg/g (Ekobu *et al.*, 2010). In contrast to this study with levels from 0 to 0.03 µg/g, Moran *et al.* (1998) reported CryIII A protein levels from 0 to 0.5 µg/g conferring resistance to *C. formicarius* and non-transgenics had 5 times more infestation than the transgenic roots. Reasons for the low expression of Cry proteins could have been low toxicity to *Cylas* spp. therefore the need to develop and screen for higher expressing transgenic events; the inducible promoters (*SPOA1* and *β-amy*) were used in this study but constitutive promoters should be exploited and lastly, there could have been conformity of the protein due to post-translation modification as reported by Seo and Lee (2004) hence inability to bind to the midgut of the weevils.

## CHAPTER FIVE

### DETERMINATION OF IPOMEAMARONE LEVELS PRODUCED DUE TO SECONDARY FUNGAL INFECTION FOUND ON WEEVIL-INFESTED SWEETPOTATO ROOTS

#### 5.1 INTRODUCTION

In sweetpotato production, *Cylas puncticollis* and *C. brunneus* weevils result into high production losses ranging between 28-100%, depending on season. The larvae stage of the weevils attack sweetpotato through storage roots, making holes which act as avenues for secondary infection by microbes (Akazawa and Uritani, 1960). In response to secondary infection, sweetpotato, like many other plants produces phytoalexins and are known as furanoterpenoids (Greisbach and Ebel, 1978). These furanoterpenoids include dehydro-ipomeamarone, 4-ipomeanol, ipomeamarone (*ipm*), ipomeamanorol, ipomeanine, 1-ipomeanol and 1,4 ipomeadiol and are of significant economic importance (Pandey, 2008; Moss, 2008; Shen, 1997; Beier, 1990; Clark *et al.*, 1981). Secondary infection by microbes is not the only way furanoterpenoids are elicited but also through mechanical injury but the latter elicits production in small quantities (Coxon *et al.*, 1975).

It is documented by previous researchers (Clark *et al.*, 1981) that microbes such as fungi elicit production of *ipm* but at varying levels. For instance, moderate level inducers such as *Rhizopus stolonifer* elicit *ipm* levels between 100-1000 µg/g while high level inducers such as *Ceratocystis fimbriata* and *Fusarium oxysporum* elicit more than 1,000 µg/g (Clark *et al.*, 1981). Information on toxicity levels of *ipm* shown to affect experimental rats has been reported ranging between 250-500 mg/kg (Pandey *et al.*, 2006; Pandey,

2008). Hepatotoxicity and deaths in cattle due to furanoterpenoids has also been reported in other countries (Donovan, 2011; Figuera *et al.*, 2003).

In relation to SSA, there could be hepatotoxicity or even cattle deaths due to consumption of furanoterpenoids present in storage roots fed to farm animals but no documentation is available. This is a concern because it is a common practise that the infested and infected sweetpotato storage roots are harvested alongside healthy ones in SSA. The infected parts are removed and fed to farm animals while the remaining 'healthy-looking' parts are consumed by humans. Feeding farm animals with the infected roots is of economic importance because animals maybe subjected to intoxication by furanoterpenoids. These animals could develop complications on consumption of the roots and yet the farmers may not have knowledge on the cause, therefore continue feeding them with infected roots, especially during drought. There is also a possibility that humans who consume the healthy-looking parts may be affected but no documentation is available. The main objective of the study was, therefore, to determine the ipomeamarone levels produced due to secondary infection by a fungus isolated from weevil-infested storage roots. This involved identification of any fungus that emerged from the weevil-infested storage roots since different fungi are reported to elicit different ipomeamarone concentrations.

## **5.2 MATERIALS AND METHODS**

### **5.2.1 Sampling and incubation of weevil-infested sweetpotato for fungus isolation**

Three sites were selected for sampling of weevil-infested sweetpotato storage roots since they are known sites for weevils, including Siaya, Busia and Marigat. The former 2 sites did not have weevils during that season but Marigat had some weevils. Sampling was done on a relatively small piece of land (about 1-acre) on Kenya Agricultural Research

Institute (KARI) Marigat farm since they had already harvested most roots from a trial. Sweetpotato samples from this field were from a family of known lineage from breeding experiments but not screened for consumer preference yet, hence not used in subsequent experiments as a source of healthy sweetpotato for inoculation purposes. The sampling was done on 4 blocks and randomly selected roots from different regions of the blocks.

Many samples had weevil damage (Plate 5.1) but no obvious microbial growth, probably due to low moisture associated with very high temperatures in very dry seasons. The sampled sweetpotato were carefully placed in covered but well aerated plastic jars (20.5 cm height x 10.5 cm diameter) immediately after harvest. The jars containing weevil infected sweetpotato storage roots were taken to the lab and then incubated at room temperature (25 and 28°C) for 14 days to promote growth of microbes. These microbes were subsequently used for fungal isolation.



**Plate 5.1:** A sweetpotato root heavily infested with weevils and burrowing through by larvae. The burrowing of sweetpotato storage root by weevils makes them unsuitable for human consumption and sales.

## 5.2.2 Fungal isolation from weevil-infested sweetpotato

After incubating the weevil-infested sweetpotato for 14 days, fungal isolation was conducted in a laminar flow-hood in the lab. The old necrotic tissues were not used to isolate the fungus due to heavy infection. Pin isolation method (use of a sterile needle to isolate mycelia and spores) was conducted from masses of mycelia and spores on these weevil-infested storage roots. Since the main focus of this study was to isolate any fungus that emerged from weevil-infested storage roots and use it to infect the healthy roots, antibiotics were used in the media. Isolation of a fungus was necessary because it has been reported that different fungi elicit varying concentrations of ipomeamarone (Clark *et al.*, 1981). Isolates were inoculated on petri plates containing potato dextrose agar (PDA) media supplemented with chloroamphenicol (25 µg/ml) and streptomycin sulphate (50 µg/ml) antibiotics to inhibit bacterial growth and then incubated overnight at 25°C. Several colonies emerged and further purification of the isolates was undertaken by isolating a single colony from the emerging colonies and then sub-cultured on fresh PDA media without antibiotics. There was a colony that was consistently observed on most PDA plates and this colony was further purified and named Marigat Isolate-1 (MI-1).

## 5.2.3 Identification of Marigat Isolate-1 (MI-1)

### 5.2.3.1 Morphological identification of MI-1

The morphological identification process was initiated by sterilizing forceps and needles over a flame briefly in a laminar flowhood. Structures from the colony were picked out using the sterile forceps and mounted onto glass slides. The structures on the slide were then stained using lactophenol aniline blue before being covered by cover slips. The glass slides were then mounted on a light microscope (Leica EZ4D) and observed at

magnification of between x8 and x35. Some of the expected structures for fungi include mycelia, spores, sporangia, rhizoids and collumela.

### 5.2.3.2 Molecular characterization of MI-1

MI-1 cultures were grown on PDA plates for 2 days. This was followed by addition of distilled water into the plates to facilitate harvest of mycelia and spores. The mycelia with spores suspension were used as DNA template for PCR. Initially, PCR was conducted using SSR primer pairs (Table 5.1). Each SSR reaction volume consisted of 20  $\mu$ l of PCR buffer (1x), forward primer (0.2  $\mu$ M), reverse primer (0.2  $\mu$ M), dNTPs (0.2 mM),  $MgCl_2$  (4 mM), Taq polymerase (0.5 U) (Thermo Scientific). The SSR-PCR was conducted using the following program, initial denature 95°C for 95 sec followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 60 sec, elongation at 72°C for 30 sec and a final extension of 72°C for 10 min. Since the SSR primers did not produce amplicons of expected size, new sets of primers were identified to help classify this fungus.

New sets of primer pairs were sourced from <http://www.qbol.org/UK/>. The primers have been developed for the identification of fungi-based conserved rRNA region of fungal genomes. One such region is the internal transcribed spacers (ITS) and several primers specific for this region have already been developed and were selected for use in identification of MI-1 (Table 5.1). Some primer sequences used for identification of MI-1 were not included on the primer list on table 12 due to restrictions imposed on publishing these primer sequences: they include EFCF1, EFCF2, EFCF6, EF1-728 and EF-2 (Qbol, 2011). For fungal identification using ITS specific primers, a 25 $\mu$ l reaction volume was set up consisting of PCR buffer (1x), forward primer (0.2  $\mu$ M), reverse primer (0.2  $\mu$ M),

dNTPs (0.06 mM), MgCl<sub>2</sub> (2 mM), Taq polymerase (0.5 U) (Thermo Scientific). The mixture was thereafter subjected to the following PCR program, initial denaturation 94°C for 5 min, followed by 40 cycles of denaturation 94°C for 45 sec, annealing temperature (55 to 62°C) for 30 sec, elongation 72°C for 90 sec and a final elongation of 72°C for 6 min. The PCR products were resolved on 1% agarose gel in TBE for 1 hour at 100V.

Band PCR was performed for non-specific amplifications (Bjourson and Cooper, 1992). The non-specific PCR amplicons close to the expected size was cut from the agarose gel using a clean razor blade under UV and then used as DNA template for the second round PCR using the same program cycle described earlier for ITS primer pairs.

**Table 5.1:** Primer sets and sequences used for molecular characterization of MI-1 isolate

| Primer name    | SSR or ITS* | Primer sequence (5' – 3')                 | Amplicon size (bp) | Source                       |
|----------------|-------------|---|--------------------|------------------------------|
| CfCAA9 (F)     | SSR         | GGCTGGTTCATCATGATGTT                      | 253                | Steimel <i>et al.</i> , 2004 |
| CfCAA9 (R)     |             | CTATGGCACCTAAGCAATCT                      |                    |                              |
| CfCAA10 (F)    | SSR         | TGACACGCGCTTCACTAACAG                     | 129                | Steimel <i>et al.</i> , 2004 |
| CfCAA10 (R)    |             | TGCACCATACCCAGGGGACA                      |                    |                              |
| CfCAA15 (F)    | SSR         | GCTACAGCAGCCGCAGTG                        | 342                | Steimel <i>et al.</i> , 2004 |
| CfCAA15 (R)    |             | GATTGGCGTTAGTGTTAGGT                      |                    |                              |
| CfCAA38 (F)    | SSR         | AATTCGGGAGCTGCTGTGAG                      | 240                | Steimel <i>et al.</i> , 2004 |
| CfCAA38 (R)    |             | GAGCCCCAGCCTCAAACCTCA                     |                    |                              |
| CfCAA80 (F)    | SSR         | ACCCGTCTCGTATTGGCTAT                      | 311                | Steimel <i>et al.</i> , 2004 |
| CfCAA80 (R)    |             | AATCGTTCGCATTCAGGTGG                      |                    |                              |
| CfCAG15 (F)    | SSR         | GGGCTAGTAGCAGAGTTGG                       | 269                | Steimel <i>et al.</i> , 2004 |
| CfCAG15 (R)    |             | GCCAATGTCTTCACACCAC                       |                    |                              |
| ITS1           | ITS         | TCCGTAGGTGAACCTGCGG                       | 700-1400           | White <i>et al.</i> , 1990   |
| ITS4           |             | TCCTCCGCTTATTGATATGC                      |                    |                              |
| ITS C. fimb. 2 | ITS         | GAGCGTCATTTCACTCA<br>CCTACCTGATCCGAGGTCAA |                    | q-bank                       |

\*sequences for ITS primers EFCF1, EFCF2, EFCF6, EF1-728 and EF-2 are not included in the table due to restrictions imposed on publishing their sequences

After band PCR, the amplicons were purified using QIAquick PCR purification kit while gel extraction was conducted using QIAquick Gel Extraction Kit from Qiagen. The purified samples were sequenced using Sanger-dideoxy method on ABI 3730 Genetic

Analysed and further analysis for consensus sequences conducted using MEGA5 program (Tamura *et al.*, 2011). Consensus sequences show similarities between two or more sequences from an alignment after sequencing the fragments. The databases used as blast search tools in identification of MI-1 consensus sequences include National Centre for Biotechnology Information (NCBI), Bar Coding of life Data systems (BOLD) and q-bank. In addition to the BLAST search, phylogenetic analysis was conducted to determine fungal strains or isolates related to the MI-1 isolate. The evolutionary analyses were conducted in MEGA5 and generated using Neighbour-Joining (N.J.) method. The bootstrap approach was used and the consensus tree inferred from 500 replicates was to represent the evolutionary history of taxa analysed with confidence limits (Felsenstein, 1985). Evolutionary distances were computed using the Maximum composite Likelihood method (Tamura *et al.*, 2004). All positions with less than 95% site coverage were eliminated. When identification of MI-1 fungus was complete, it was used to inoculate consumer-preferred sweetpotato roots to establish the concentration levels produced both as a whole and in sections away from the visibly infected region.

## **5.2.4 Analysis of the furanoterpenoids**

### **5.2.4.1 Plant material and inoculation of healthy sweetpotato**

Healthy-looking sweetpotato storage roots (for inoculation of the identified fungus, MI-1) were acquired from different markets due to consumer preferences. Sweetpotato roots used in these experiments were farmer grown and consumer preferred cultivars for both the rural and urban households in these regions. These cultivars included Kemb, Naspot, Bungoma and Nyawo, according to the vendors.

Two inoculation experiments were conducted on healthy sweetpotato roots to establish which method would effectively infect them, as follows. In the first experiment, agar plugs containing MI-1 were placed on healthy sweetpotato samples to enable full infection after 30 days at 25°C. After full infection, these samples were analysed for furanoterpenoid concentrations. In the second experiment, inoculation with both agar plugs and suspensions of mycelia with spores on healthy sweetpotato was conducted but the samples were then covered with polythene bags and elastic thread either fully or partially in order to refrain the fungus from outgrowing into over the whole sweetpotato sample. Infection by MI-1 fungus was done between 14 and 30 days and furanoterpenoid analysis undertaken at 1 cm intervals from infected to healthy tissues for samples with progressive infection.

#### **5.2.4.2 Preparation of sweetpotato samples for furanoterpenoid analysis**

The samples were prepared using the method described by Clark *et al.* (1981) with a few modifications. The sweetpotato roots were washed, peeled, surface sterilized for five minutes using 0.5% w/v sodium hypochlorite and then rinsed in three changes of distilled water inside the laminar flow hood. The samples were then cut into halves and one half was placed in clean sterile plastic containers before inoculating with actively growing fungal isolate (MI-1) using agar plugs whereas the second half were the controls, which were not inoculated with the MI-1 isolate. Each cultivar had 3 samples for analysis but some of samples dried out during infection period and could therefore not be used in subsequent analysis.

The inoculated and control root samples were incubated at 25°C between 14 to 30 days to allow for full infection of MI-1 on the healthy sweetpotato. After this, agar plugs with

MI-1 sweetpotato samples were removed, then samples were weighed and blended with 100 ml methanol and 3g of sodium chloride for 3 min; sodium chloride was used to reduce solubility of organic compounds in aqueous layer and preferentially dissolve in methanol which is less polar. The extracts were then filtered using Whatman filter paper No. 4 and then concentrated in a rotary evaporator (Heidolph, Laborota 4000 efficient HB digital) to remove methanol. The remaining solution was then transferred into a separatory funnel and 50 ml of dichloromethane (DCM) added. After vigorously shaking the mixture, organic and aqueous phases were separated; organic phases were combined and concentrated to dryness in a rotary evaporator and the weight recorded. The extracts were dissolved in 1 ml DCM, dried over anhydrous  $MgSO_4$  then analysed by thin layer chromatography (TLC) and coupled-gas chromatography-mass spectrometry (GC-MS).

#### **5.2.4.3 Thin layer chromatography (TLC) analysis of extracts**

The extracted samples were initially analysed using TLC to confirm presence of organic components. It was carried out on a silica gel plate impregnated with fluorescent material PF254, thickness 0.2 mm; 20x20 cm and aluminium cards (Fluka) as stationary phase. The standard chromatograms were prepared by applying 20  $\mu$ l of the extracts to the TLC plates using hexane: ethyl acetate 9:1 system as the mobile phase. After air drying and UV observation of the plates, they were sprayed with a mixture of 98% w/v methanol with 2% sulphuric acid and oven activated for 10 mins.

#### **5.2.4.4 Coupled gas chromatography-mass spectrometry analysis of extracts**

Extracts were further analysed by coupled Gas Chromatography-Mass Spectrometry (GC-MS) to detect presence of furanoterpenoids. GC-MS analysis was carried out on a 7890A gas chromatograph (Agilent Technologies, Inc., Beijing, China) coupled to a 5975

C mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, USA) using the following conditions: inlet temperature 270°C, transfer line temperature of 280°C, column oven temperature programmed from 35 to 285°C. The initial temperature was maintained for 5 min then 10°C/min to 280°C held at this temperature for 10.5 min and finally 50°C /min to 285°C and held at this temperature for 9.9 min. The GC was fitted with a HP-5 MS low bleed capillary column (30 m × 0.25 mm i.d., 0.25 µm) (J&W, Folsom, CA, USA). Helium at a flow rate of 1.25 ml/min served as the carrier gas. The Agilent 5973 mass selective detector was maintained at ion source temperature of 230°C and a quadrupole temperature of 180°C. Electron impact (EI) mass spectra were obtained at the acceleration energy of 70 eV. One µl aliquot of the extract was injected in the split/splitless mode using an 7683 auto-sampler (Agilent Technologies, Inc., Beijing, China). Fragment ions were analysed over 40 to 550 m/z mass range in the full scan mode and the filament delay time was set at 3.3 min. GC-MS results showed consistent peaks, the best and characteristic peaks, for furanoterpenoids detected in the samples.

### **5.2.5 Isolation of pure furanoterpenoid standards for analyses**

The process of isolation of pure furanoterpenoid standards was then initiated. The process was initiated by loading 1 ml of the crude extract (from Section 5.2.4.1) on to a separating column with 50 g silica gel (32-63 µm Riedel-De Haen 31607) and 100 ml hexane: ethyl acetate (HE) 9:1 as a developing solvent. The extract was repeatedly subjected to the column over silica gel using the HE developing solvent system for further purification using fresh HE solvent. The composition of the resulting 15 tubes of 5 ml fractions were initially examined by TLC followed by GC-MS to confirm presence of furanoterpenoids and then HPLC to collect pure fractions.

HPLC analysis was performed on a Shimadzu VP series using an ACE Q C-18 column (205 mm x 10 mm i.d. 5  $\mu$ M) and the following conditions: oven temperature 30°C, maximum temperature 85°C, injection volume 40  $\mu$ l, total flow rate of 1 ml/min, pump A pressure acetonitrile of 14.6 MPa, pump A Degas -90 KPa, low pressure gradient system mode. The mobile phase used a gradient program initially 95.5 (A:B), to 85.15 at 3 min, 75.25 at 13 min, 70.30 at 25 min, 45.55 at 35 min, 45.55 at 45 min, 5.95 at 46 min, 5.95 at 58 min, 95.5 at 60 min (Ferrerres *et al.*, 2003). Purification was done as described above using a mobile phase comprising acetonitrile: water (40:60, v/v). Detection was by UV absorption at 270 nm for 20 min running time. The HPLC results were expected to show several peaks containing pure furanoterpenoids and collected for further analyses using nuclear magnetic resonance (NMR).

On confirmation of the pure furanoterpenoids by HPLC and GC-MS, calibration curves were developed using 5 different concentrations namely 0, 25, 50, 75 and 100 ng/ $\mu$ l in three replicates. Calibration curves were used for analysis of furanoterpenoid levels in the sampled sweetpotato extracts in subsequent experiments.

### **5.2.6 Nuclear magnetic resonance (NMR) analysis of pure furanoterpenoids**

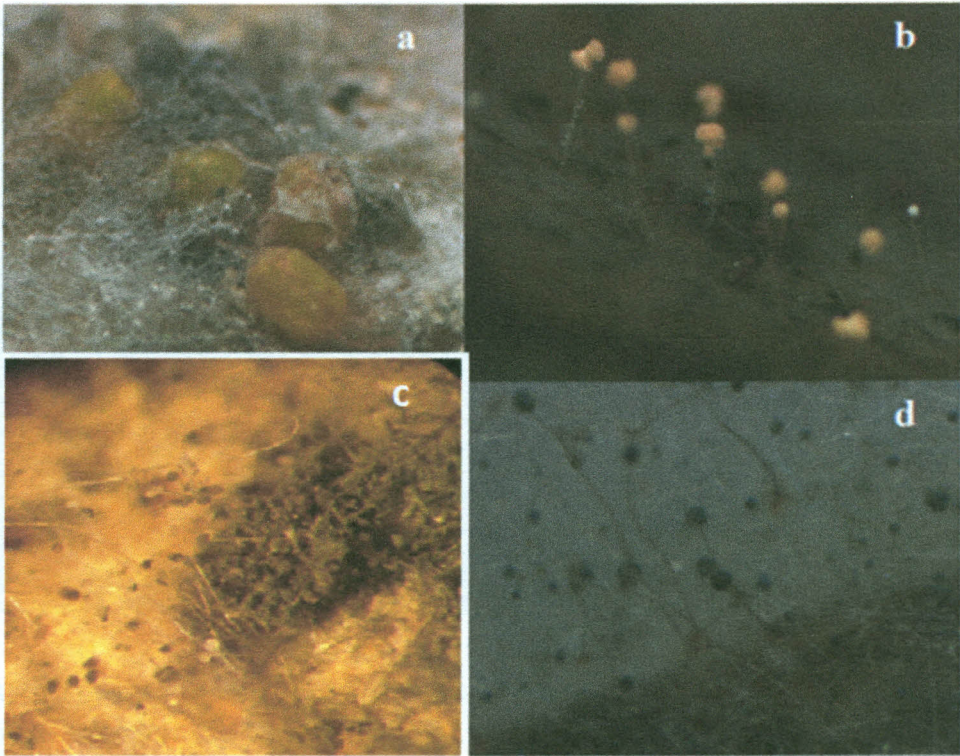
When the isolation of pure furanoterpenoids (*ipm* and dehydro-*ipm*) was completed using GC-MS and HPLC techniques, they were further tested for structure similarity via NMR. The structures of the isolated furanoterpenoid (*ipm* and dehydro-*ipm*) were determined by NMR; ( $^1\text{H}$ -NMR,  $^{13}\text{C}$ -NMR). Exactly 5 mg of dried isolated compounds, *ipm* and dehydro-*ipm* were each re-suspended in  $\text{CDCl}_3$  (Cambridge Isotope Laboratories, Inc.) and placed in 2.5-mm NMR tubes (Norell). 1D and 2D,  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy, including correlation spectroscopy, hetero-nuclear single quantum coherence, HMBC

was used for verification. 2D NOESY data was collected and analysed for structural verification of dehydro-IPM. All NMR spectra were acquired at 22°C using a 5-mm TXI cryo-probe (Bruker Corporation) and a Bruker Avance II 600 console (600 MHz for  $^1\text{H}$  and 151 MHz for  $^{13}\text{C}$ ) except for the 2D NOESY data which was collected at 25 °C. Residual  $\text{CHCl}_3$  was used to reference chemical shifts to  $\delta (\text{CHCl}_3) = 7.26$  ppm for  $^1\text{H}$  and  $\delta$  of *ipm* was referenced to 72.6 ppm for  $^{13}\text{C}$  according to Sugimura and Tai (1994). NMR spectra were processed using Bruker Topspin 2.0 and MestReNova (Mestrelab Research) software packages. Both *ipm* and dehydro-*ipm* were numbered according to Schneider *et al.* (1984).

## 5.3 RESULTS

### 5.3.1 Growth of the microbes in culture

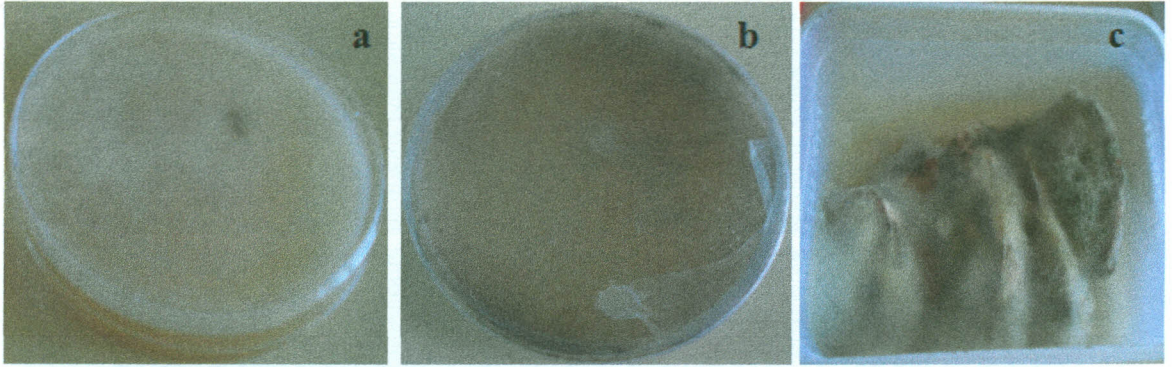
Initially, weevil-damaged sweetpotato developed visible microbial structures within seven days after incubation at room temperature. Presence of yellow/green or brown/black microbial structures on the surface of weevil-damaged sweetpotato were observed (Plate 5.2a). White mycelia were initially observed within 14 days followed by yellowing and finally turning to darker shades of black or grey (Plate 5.2b). The structures observed also revealed that each sporangia was held on a single sporangiophore and was either yellow, white or black in colour (Plate 5.2b,c,d).



**Plate 5.2:** Fungal structures on weevil-damaged sweetpotato root samples, 7 to 14 days after incubation. **(a)** Unknown (green/yellow colour) on sweetpotato surface, **(b)** Yellow/white sporangia on single sporangiophores, **(c)** Maturing mycelia with spores, **(d)** Black/grey coloured sporangia on single sporangiophores.

Secondly, single spores isolated from sweetpotato that were previously inoculated with MI-1 produced mycelia after 1 day of culture on PDA media while sporulation occurred after 7 days. Prior to sporulation, white mycelia were produced, which covered the PDA plate within 2 days of inoculation (Plate 5.3a) and later changed colour to grey/black when sporangia were produced after 14 days. It was also observed that when MI-1 was cultured on PDA media, mycelia and spores started multiplying and growing towards the edge of the parafilm-sealed petri dish with parafilm (Plate 5.3b).

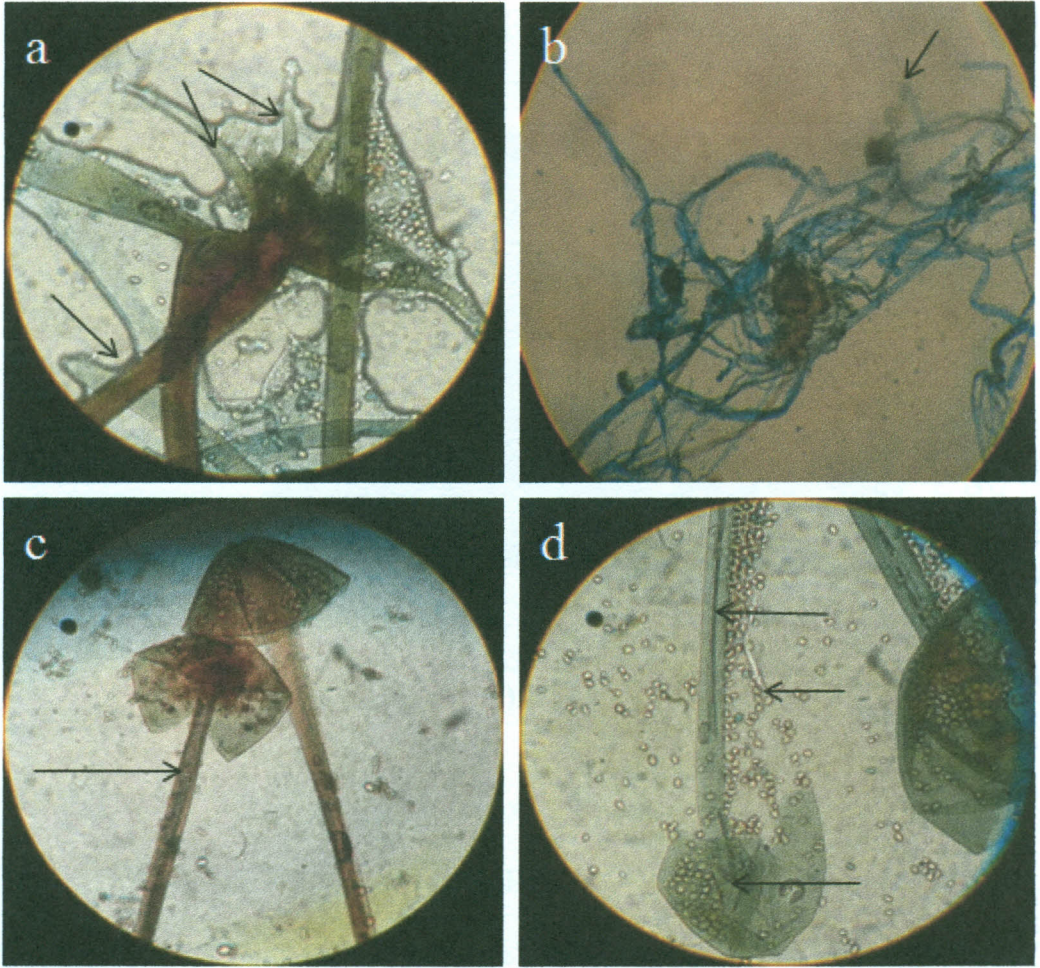
Bungoma and Kemb cultivars produced white mycelia in 7 days after inoculation while Nyawo and Naspot produced them after 14 days. When sweetpotato roots were fully infected by MI-1, they had a characteristic unpleasant acidic rotting smell (Plate 5.3c).



**Plate 5.3:** Growth of MI-1 fungal isolate on PDA media and surface sterilized sweetpotato samples. **(a)** Growth of MI-1 starting with white mycelia on PDA media within 12 hours after inoculation, **(b)** Maturation of mycelia and production of sporangia with spores (black colour) on PDA media after 7 days in culture, **(c)** Growth of MI-1 fungus on sweetpotato to full infection under *in-vitro* conditions (30 days of incubation).

### 5.3.2 Morphological characteristics of MI-1

Results from observations of MI-1 under the microscope (Leica EZ4D) revealed several structures typical of fungi such as sporangia, sporangiophores, spores, collumella, rhizoids and stolons. Sporangiophores arose from intersections with rhizoids and stolons. The columella was dome-shaped and did not fall off when the sporangium dried-out (Plate 5.4).

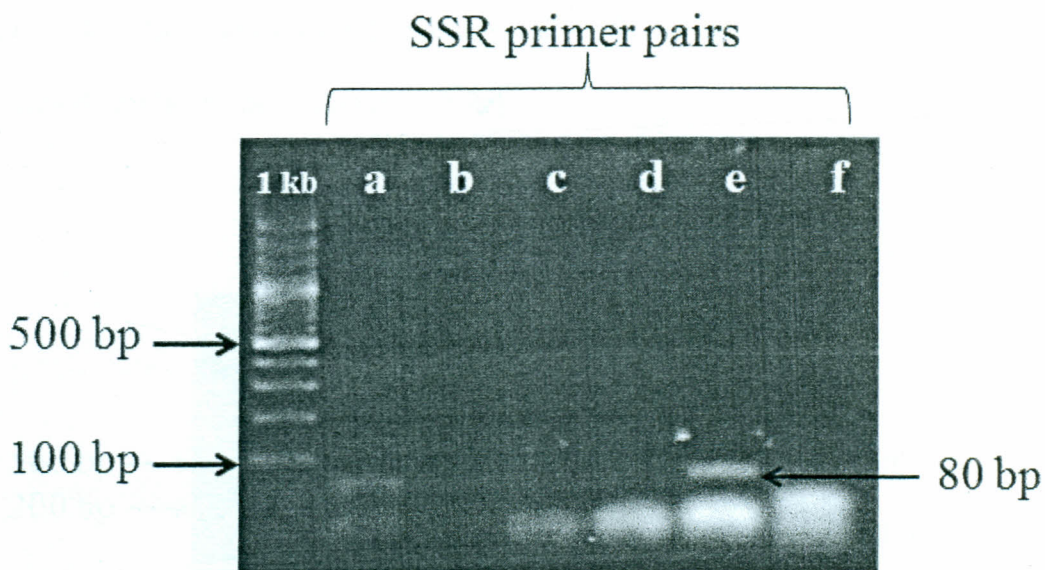


**Plate 5.4:** MI-1 structures visualised under a microscope at 100x magnification. (a) Presence of rhizoids located opposite the sporangiophore at intersection with the stolon. (b) Dome-shaped collumella attached to stolons. (c) Sporangia losing spores. (d) Sporangium, sporangiophore and spores

### 5.3.3 Molecular identification of MI-1

PCR amplification of MI-1 using SSR primers produced amplicons (80 bp) with only two primer pairs, CfCAA15 and CfCAA10, respectively out of the 6 primer pairs (Fig. 5.1).

However, expected amplicons for the primer pairs was 129 and 342 bp. The SSR results showed that MI-1 was not *C. fimbriata* prompting further molecular characterization using ITS primer pairs.

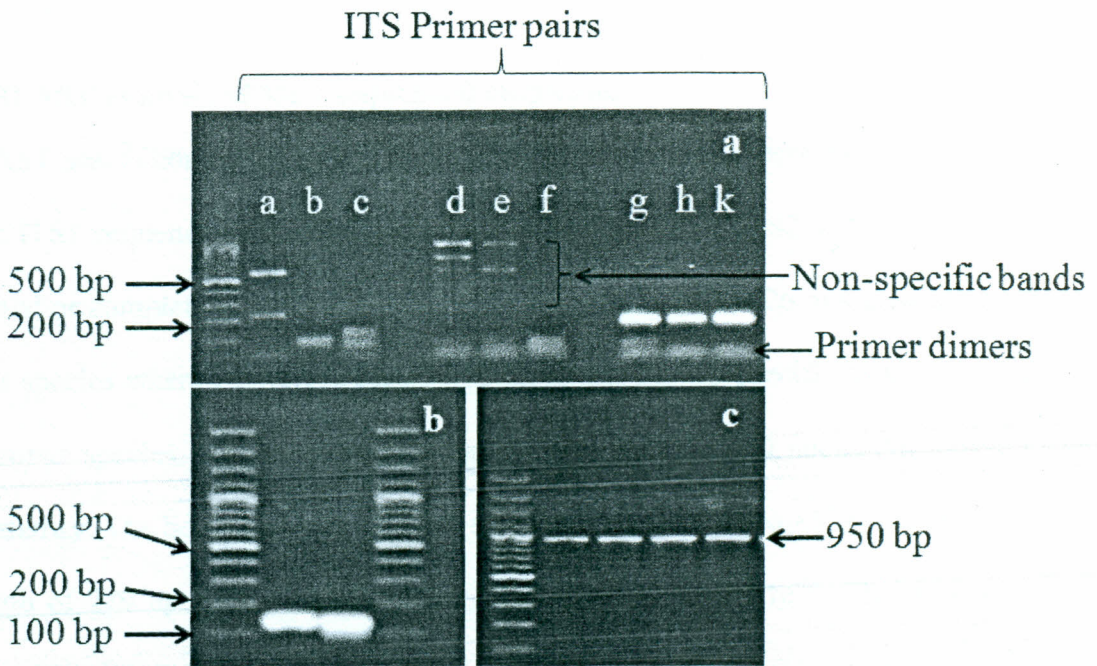


**Figure 5.1:** PCR-based identification of MI-1 fungal isolate using the SSR primer pairs. (a) CfCAA15 (b) CfCAA38 (c) CfCAA9 (d) CfCAG15 (e) CfCAA10 (f) CfCAA80

PCR-amplification of MI-1 ITS isolate region from MI-1 generated products ranging from 100 to 950 bp (Fig. 5.2a). Primer pairs EF1-728 with EF-2 generated two amplicons of 220 and 600 bp (Figure 5.2a lane a) while the expected size was 700-1400 bp. This was followed by primer pairs EFCF1 and EFCF2 with an amplicon of 110 bp (Fig. 5.2a lane b) and EFCF1 with EFCF6 primer pairs produced an amplicon of 120 bp (Fig. 5.2a lane c) but not of the expected size of 430 bp. Another PCR was conducted using primer pairs ITS1 and ITS4 (Fig. 5.2a lane d) and EF1-728 with EF-2 under 2 annealing temperatures of 58 and 60°C but none produced a one single amplicon (Fig. 5.2a lanes e,f). In addition, ITSb primer pairs had an amplicon of 120 bp at annealing temperatures of 55, 58 and 60°C although the expected size was between 400 bp (Fig. 5.2a lanes g,h,k).

Band PCR was undertaken for non-specific bands produced after doing the normal PCR for primer pairs ITSb and EFCF1 with EFCF6 (Fig. 5.2b). This was also conducted on

ITS1 and ITS4 primer pairs since they had several bands instead of a single amplicon and they generated amplicons of 950 bp (Fig. 5.2c).



**Figure 5.2:** Images of gel-red stained agarose gels showing DNA fragments from amplification of MI-1 using ITS primer pairs. **(a)** EF1-728 on lane a; ECFCF1 and ECFCF2 on lane b; ECFCF1 and ECFCF6 on lane c; ITS1 with ITS4 on lane d; EF1-728 primer pairs on lanes e,f; ITSb on lanes g,h,k using 3 temperatures **(b)** Band PCR product of ITSb ECFCF1 and ECFCF2 resulting to production of one amplicon **(c)** ITS1 with ITS4 primer pairs under 4 temperatures of 58, 60, 62 and 63°C

### 5.3.3.1 Determination of consensus sequences

Sequencing of MI-1 amplicons for the primer pairs (ITS1: ITS4, EF1-728: EF-2 and ITSb forward with reverse) was conducted using Sanger method (ABI 3730 Genetic Analyser) followed by analysis for consensus sequences using MEGA5 program (Tamura *et al.*, 2011). Consensus sequences were produced for EF1-728: EF2 and ITSb primer pair having 256 and 141 bp, respectively, with the latter having numerous gaps. ITS1 and ITS4 primer pairs, on the other hand, did not have a consensus sequence but one

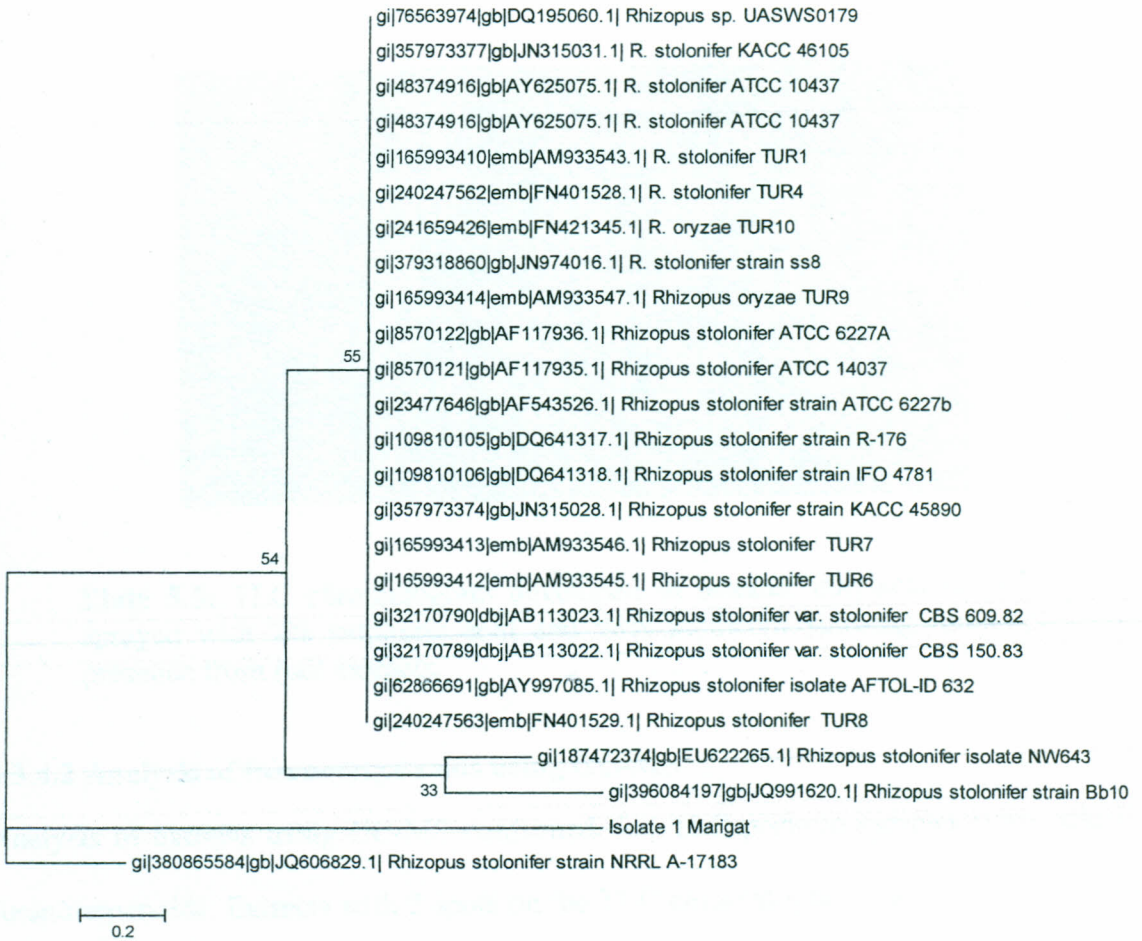


Another BLAST search was conducted for the consensus sequence from ITSb primer pairs of MI-1 and it generated hits for several species including *Dothideomyces* sp., *Cladosporium* sp., *Fusarium* sp., *Pteris* sp. and uncultured soil fungus. All the species had identities of 96%, an e-level of  $1e^{-52}$  with query coverage of 100% and lengths ranging from 218 to 1167 nucleotides. One uncultured soil fungus (clone BL39) had an e-level of  $2e^{-53}$ .

Finally, a BLAST search was done for the consensus sequence from EF1-728 and EF2 primer pairs on MI-1 and it generated many hits of many species. Some of these species were *Corynespora cassicola*, *Dendryphion penicillatum*, *Microcyclosporella*, *Davidiella* sp., *Gloniopsis subrugosa*, *Pseudocercospora* sp., *Herpotrichia juniper*, *Dendryphiella* sp. among others. All the species had a range of identities between 85 to 100% but the e-level were high from  $2e^{-16}$  to  $9e^{-15}$ . The query coverage also ranged between 19 and 41% while the lengths ranged from 273 to 1769.

### 5.3.3.3 Phylogenetic analysis of MI-1 sequenced amplicon

When the BLAST search was completed, phylogenetic analysis using neighbour-joining method was conducted using all the 26 sequences generated alongside MI-1 sequences generated by ITS1 primer on the NCBI database. The results showed clustering of many *Rhizopus* species isolates in one branch. Of the 26 sequences, 4 separate clades were produced where 21 were clustered in one clade, isolate NW643 and strain Bb10 were in the second clade, isolate 1 Marigat (MI-1) was in the third clade while strain NRRL A-17183 was in the fourth clade (Fig. 5.4).

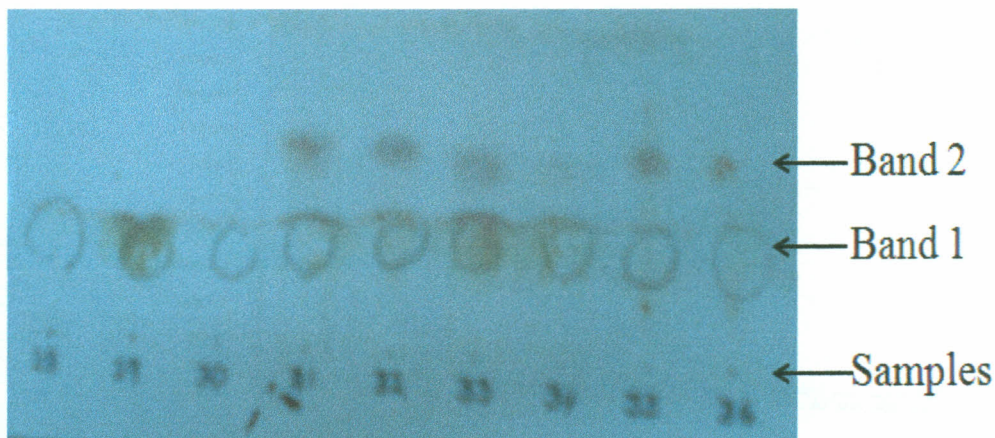


**Figure 5.4:** Phylogenetic tree based on the nucleotide sequences from NCBI database of ITS region from 25 *Rhizopus* species and Isolate-1 Marigat (MI-1). The percentage of replicate trees in which the associated taxa clustered together in bootstrap test are shown next to the branches. The tree was drawn to scale with branch lengths in same units as those of the evolutionary distances used to infer the tree.

### 5.3.4 Analysis of furanoterpenoids extracted from MI-1 infected samples

#### 5.3.4.1 Analysis of furanoterpenoids using thin layer chromatography

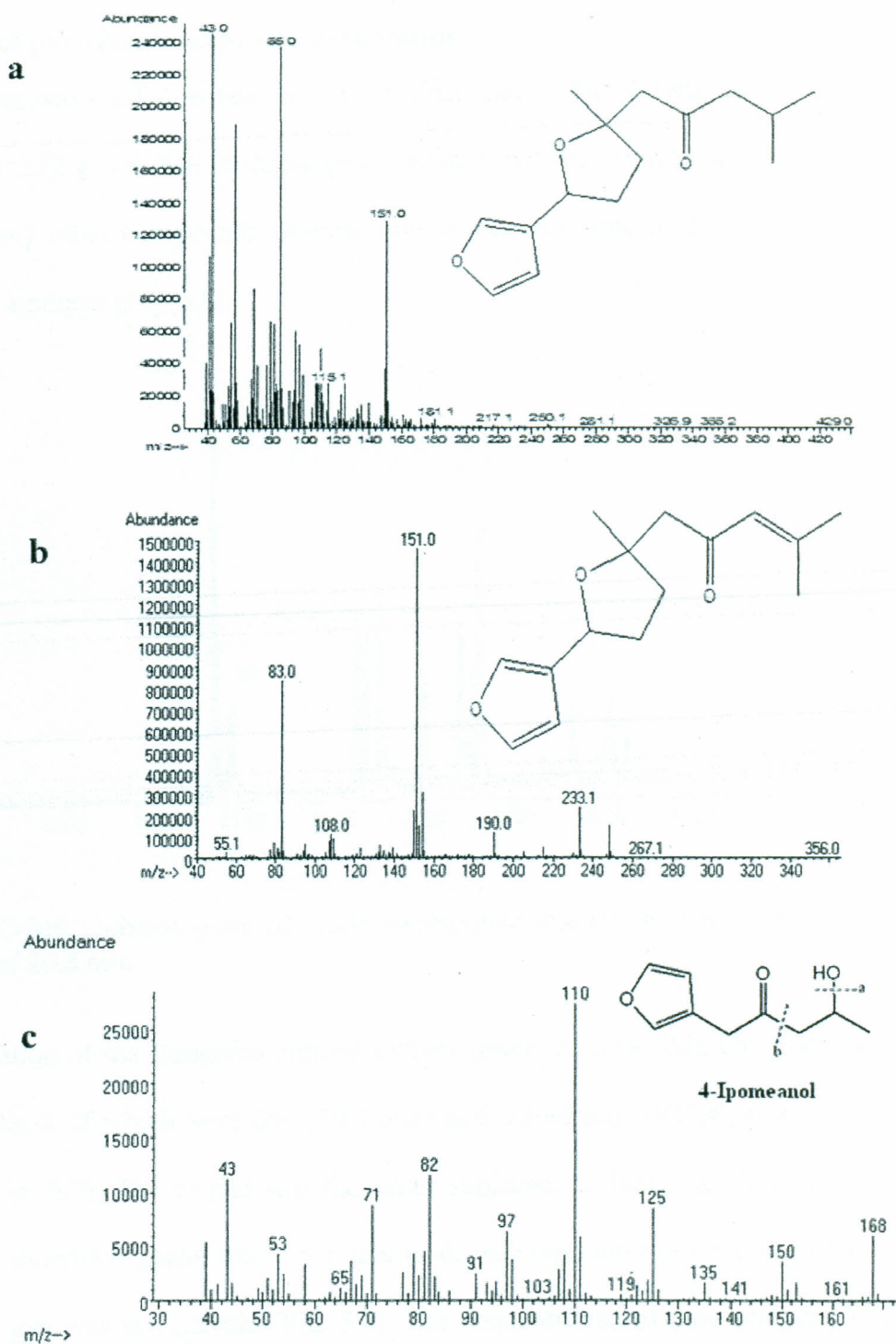
Analysis of furanoterpenoids production using TLC in sweetpotato inoculated with MI-1 revealed 1 to 2 bands (Plate 5.5). The banding pattern did not give information on which furanoterpenoids were present but 2 bands could mean they were different metabolites.



**Plate 5.5:** TLC chromatogram developed in hexane: ethylacetate (9:1) system sprayed with 2% sulphuric acid and 98% methanol showing furanoterpenoids presence from root extracts.

#### 5.3.4.2 Analysis of furanoterpenoids using GC-MS

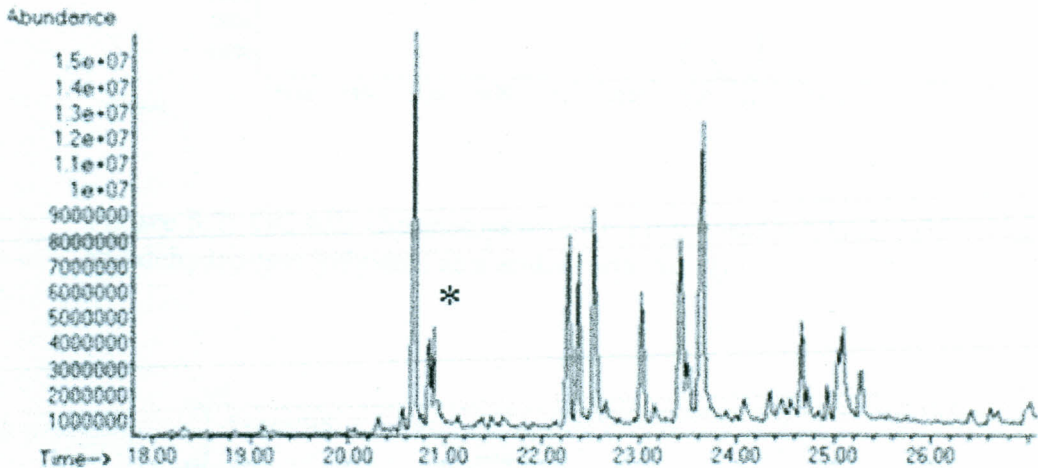
Analysis of extracts using GC-MS confirmed that TLC-positive samples were actually furanoterpenoids. Extracts with 2 spots on the TLC chromatogram had *ipm* and dehydro-*ipm*, while single spots had either. GC-MS mass spectra of furanoterpenoid extracts (Fig. 5.5) showed several peaks but 2 main ones (characteristic and best) were represented by  $m/z$  85, 151 for *ipm* (2-Pentanone, 1-[5-(3-furyl)tetrahydro-2-methyl-2-furyl]-4-methyl-) with a molecular of weight 250 (Fig. 5.5a); dehydro-*ipm* (3-Penten-2-one, 1-[5-(3-furanyl)tetrahydro-2-methyl-2-furanyl]-4-methyl-) was represented by  $m/z$  83, 151 of MW 248.14 (Fig. 5.5b). The third furanoterpenoid, 4-ipomeanol (1-(3-furyl)-4-hydroxy-1-pentanone) was also present and it had major peaks at  $m/z$  110, 150 (Fig. 5.5c) of MW 110 but the levels were negligible. On comparison of mass spectra of compounds in the NIST library from the GC/MSD Chemstation software, there was no match hence the need for pure standards.



**Figure 5.5:** Mass spectrum and structures of furanoterpenoids identified by GC-MS sweetpotato extracts. (a) *ipm* had 151 and 85 as the main peaks (b) Dehydro-*ipm* 151 and 83 as the main peaks (c) 4-ipomeanol had 110 and 168 peaks

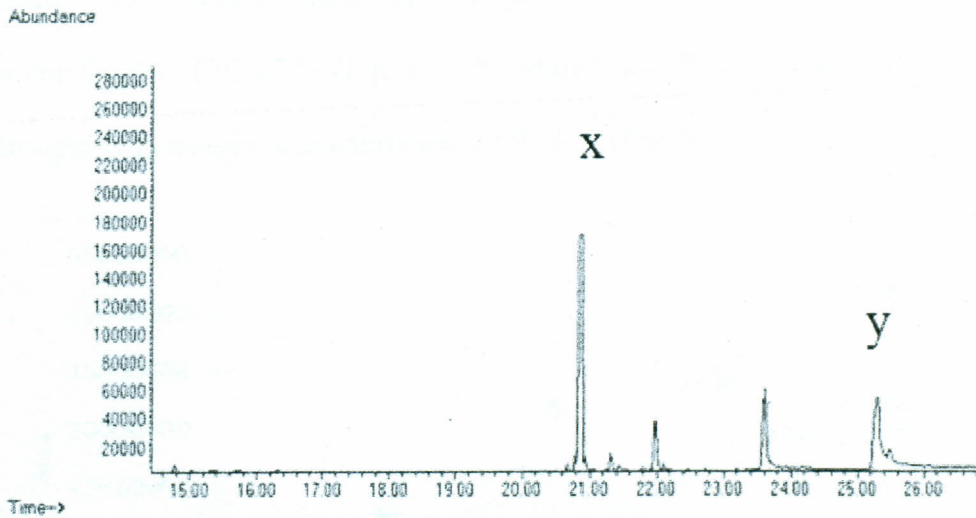
### 5.3.5 Isolation of pure furanoterpenoid compounds

Bungoma cultivar was used to isolate the pure standard since it had the highest amount of crude extract of 0.72 g. GC-MS chromatogram showed that the extract had many peaks representing many other compounds whereas *ipm* at retention time of 20.8 min was in relatively small amounts (Fig. 5.6).

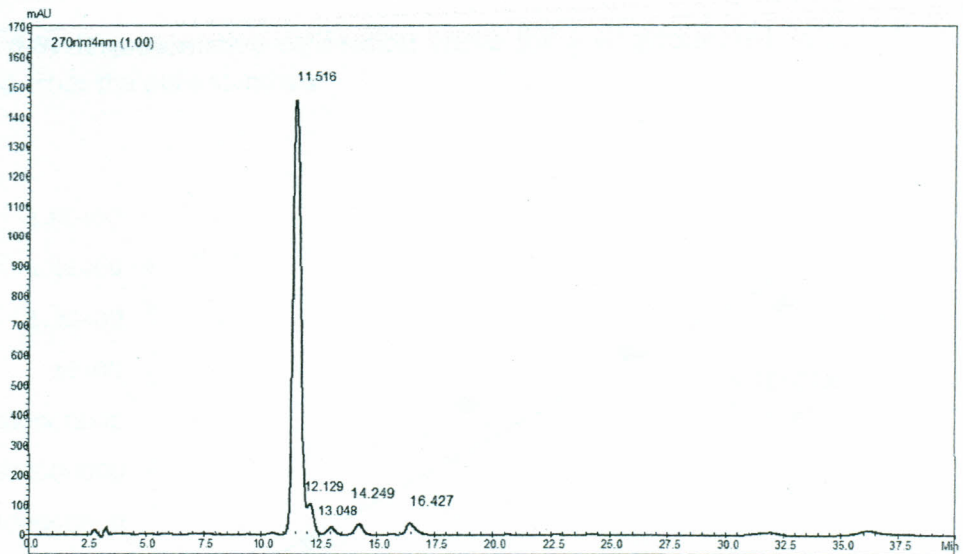


**Figure 5.6:** GC-MS chromatogram of crude sweetpotato extract showing *ipm* (\*) at a retention time of 20.8 min

Further purification of the Bungoma cultivar extract generated a GC-MS chromatogram showing 6 peaks, 2 of which were *ipm* (20.8 min) and dehydro-*ipm* (21.4 min), *x* and *y* respectively (Fig. 5.7). The extract was thereafter subjected to HPLC analysis and the chromatogram showed 6 peaks where the *ipm* peak was baseline resolved at 16.4 min while dehydro-*ipm* was at 11.5 min (Fig. 5.8). The concentration of pure *ipm* was 3.75 mg/ml while pure dehydro-*ipm* was 1.17 mg/ml.

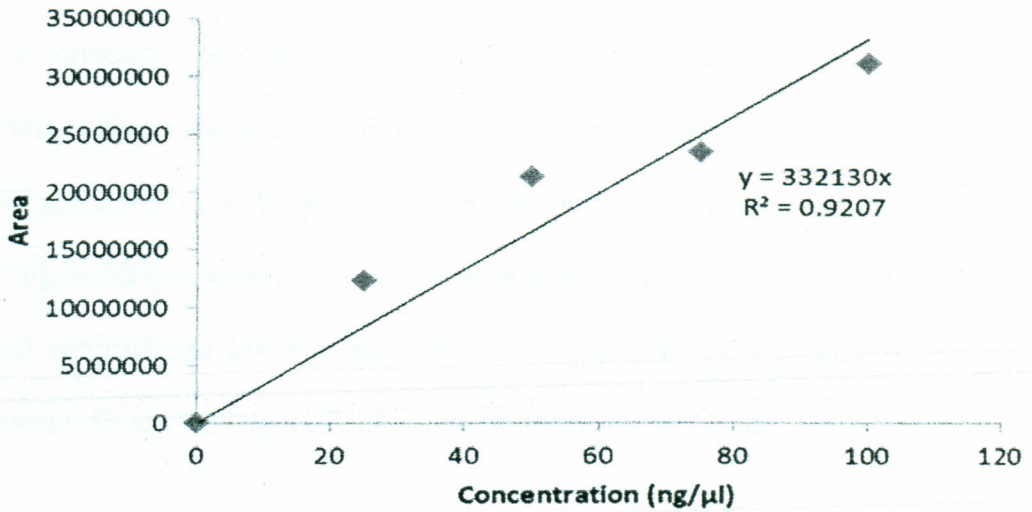


**Figure 5.7:** GC-MS chromatogram of 6 pure compounds the HE system of *ipm* and dehydro-*ipm* indicated as x and y respectively

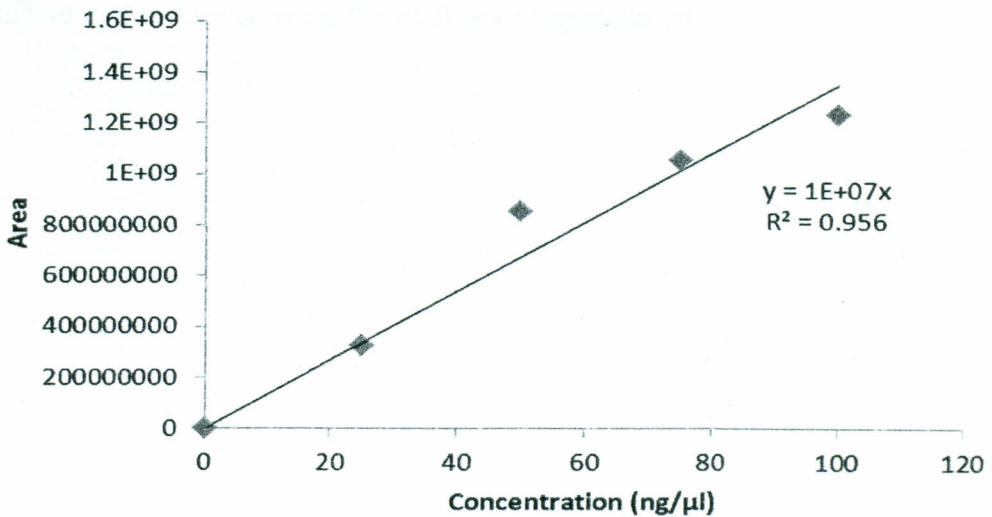


**Figure 5.8:** Representative HPLC chromatogram of six pure compounds from HE system with the *ipm* and dehydro-*ipm* indicated as 16.4 min and 11.5 min, respectively

The calibration curve was obtained using a series of five *ipm* concentrations with a regression coefficient of 92.07 % (Fig. 5.9). Similarly, a calibration curve was obtained for dehydro-*ipm* with a regression coefficient of 95.6 % (Fig. 5.10).



**Figure 5.9:** Representative calibration curve for *ipm* determined using GC-MS obtained from the pure standard



**Figure 5.10:** Representative calibration curve for dehydro-*ipm* determined using GC-MS from the pure standard

### 5.3.6 Inoculation and furanoterpenoid levels of sweetpotato extracts

In the first method of inoculation of MI-1 on sweetpotato samples resulted to infection over whole samples and different cultivars had variations in infection. There were 2 experiments done (i) on sweetpotato cultivars samples inoculated with MI-1 fungus and (ii) on their corresponding controls (those with no MI-1 inoculation). For the samples from different cultivars inoculated with MI-1, the furanoterpenoid concentrations ranged between 600 and 2,900  $\mu\text{g/g}$ . Kemb had highest *ipm* with 2,914  $\mu\text{g/g}$  followed by Nyawo with 1,089  $\mu\text{g/g}$ , Naspot with 833  $\mu\text{g/g}$  and Bungoma with 676  $\mu\text{g/g}$  (Table 5.2). The uninoculated controls had lower concentrations compared to MI-1 inoculated samples ranging between 56 and 105  $\mu\text{g/g}$  of either *ipm* or dehydro-*ipm* (Table 5.2).

Generally, 2 furanoterpenoids were observed namely *ipm* and dehydro-*ipm*. The highest concentrations were observed with *ipm* as compared to those of dehydro-*ipm*. Dehydro-*ipm* was present in all the samples including controls (Table 5.2). There were significant ( $p < 0.05$ ) differences of *ipm* between the cultivars (Appendix 10).

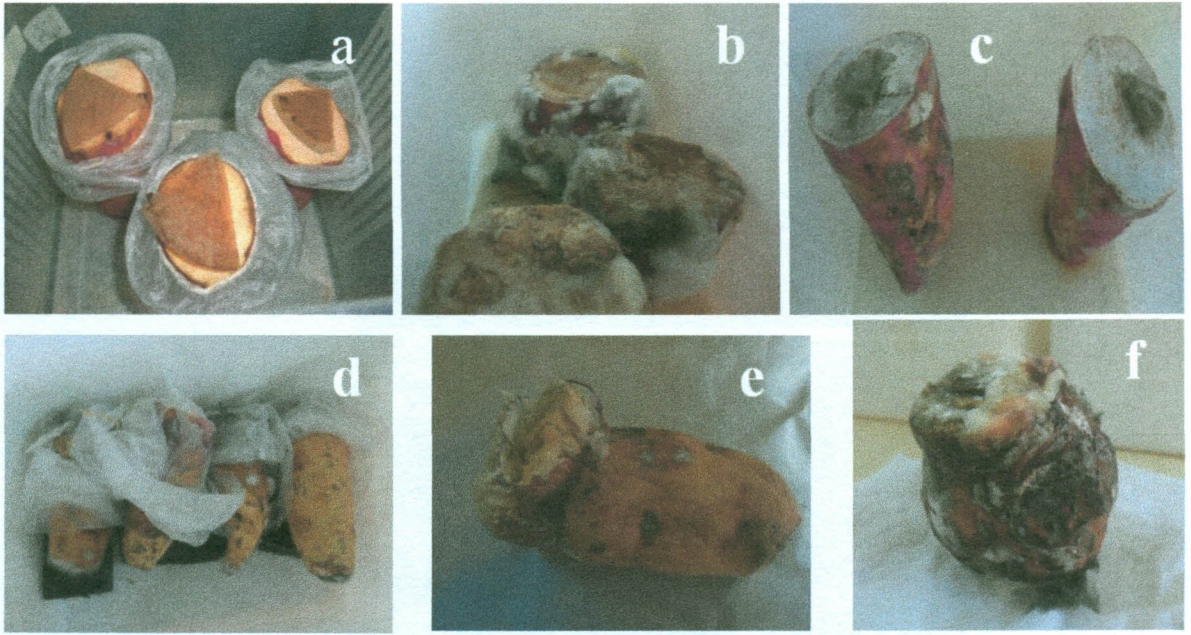
**Table 5.2:** Furanoterpenoid concentrations in 4 cultivars inoculated with MI-1

| Cultivar samples inoculated with MI-1 and respective controls with no inoculation | Phytolaxin present | Mean concentration levels ( $\mu\text{g/g}$ ) $\pm$ s.e. |
|---|--------------------|--|
| Kemb samples  | ipm                | 1476.2 $\pm$ 278.7 a                                     |
| Kemb samples  | dehydro-ipm        | 2914.2 $\pm$ 420.8 a                                     |
| Control   | ipm                | 95.9 $\pm$ 8.2 c   |
| Control   | dehydro-ipm        | 56.2 $\pm$ 9.3 c   |
| Nyawo samples   | ipm                | 1089.9 $\pm$ 269.5 ab                                    |
| Nyawo samples   | dehydro-ipm        | 1459.7 $\pm$ 339.9 b                                     |
| Control   | ipm                | 96.0 $\pm$ 8.2 c   |
| Control   | dehydro-ipm        | 61.8 $\pm$ 9.3 c   |
| Naspot samples  | ipm                | 833.7 $\pm$ 245.4 ab                                     |
| Naspot samples  | dehydro-ipm        | 1153.2 $\pm$ 223.9 b                                     |
| Control   | ipm                | 56.1 $\pm$ 8.2 c   |
| Control   | dehydro-ipm        | 61.8 $\pm$ 9.3 c   |
| Bungoma samples   | ipm                | 676.5 $\pm$ 132.2 bc                                     |
| Bungoma samples   | dehydro-ipm        | 910.0 $\pm$ 208.7 bc                                     |
| Control   | ipm                | 105.1 $\pm$ 8.2 c  |
| Control   | dehydro-ipm        | 92.6 $\pm$ 9.3 c   |

There were 4 cultivars used in this experiments and each cultivar had 3 replicates. The controls were not inoculated with MI-1. There were significant differences ( $p < 0.05$ ) between ipm and the cultivars.

### 5.3.7 Controlled infection by MI-1 fungus

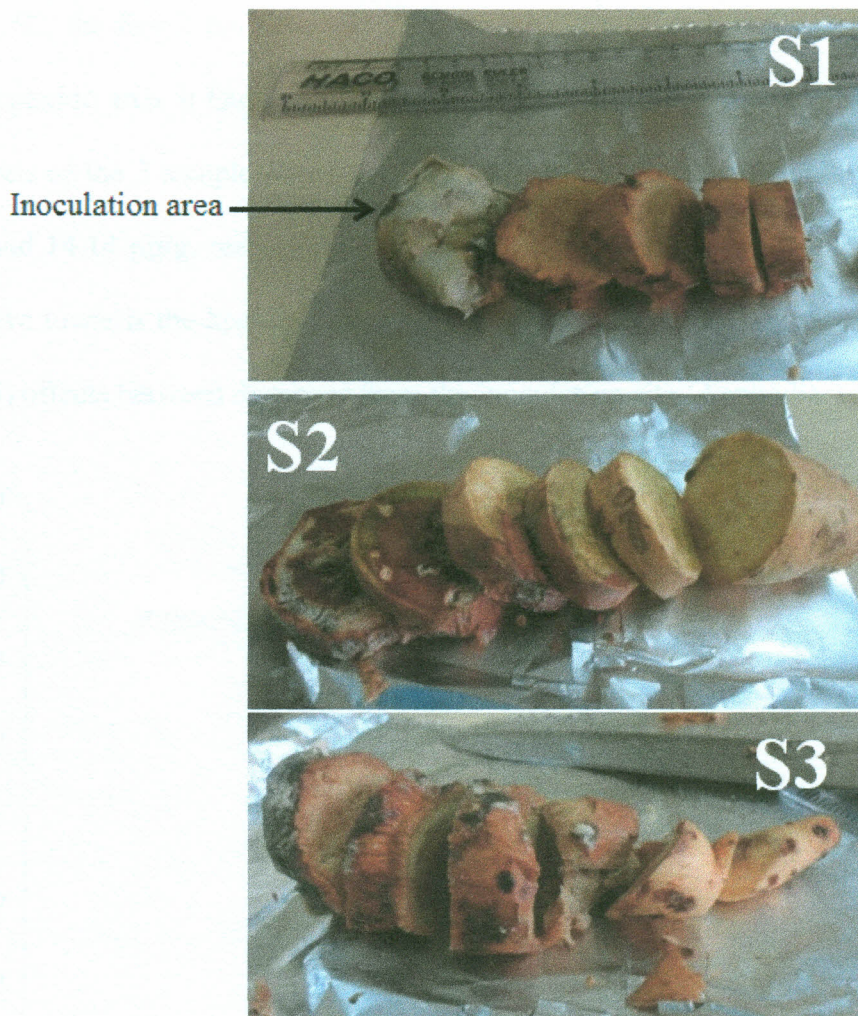
Results from the second experiment of inoculating healthy sweetpotato roots using agar plugs and suspensions of mycelia with spores on Bungoma (Plate 5.6a,b,c) and Kemb (Plate 5.6d,e,f) cultivars, showed growth of the fungus in both cases. The use of agar plugs placed to roots followed by partially covering them with polythene bags (Plate 5.6a) led to either (a) very high infection (Plate 5.6b) or (b) drying-out of the roots (Plate 5.6c). Samples inoculated with suspensions of mycelia and spores led to (a) progressive, controlled infection for those covered using a polythene bag placed round the cut and inoculated surface (Plate 5.6d) where some of the samples showed controlled infection (Plate 5.6e) and (b) others had infection throughout the root samples (Plate 5.6f).



**Plate 5.6:** Infection methods of MI-1 on Kemb and Bungoma cultivars. **(a)** Agar plugs cultured on Bungoma cultivar samples **(b)** High infection of Bungoma with no cover **(c)** Dried samples of Bungoma **(d)** Half-covered samples of Kemb to restrict infection **(e)** controlled infection of Kemb **(f)** Full infection of Kemb

### 5.3.7.1 Inoculation of sweetpotato samples

Controlled infection of Kemb cultivar by MI-1 showed progressive infections from inoculated area towards healthy tissues but Bungoma cultivar did not (Plate 5.7). Kemb cultivar showed uneven infection through the layers, some had infection spread over whole slices, others were spread half-way while others had no visible infection. The progression of MI-1 infection varied with each inoculated sample where one sample was almost fully infected in all but the last 1 cm (Plate 5.7, S1). In other samples infection was present in the first 3 cm but little or no infection in the last 2 cm (Plate 5.7, S2) and infection in the first 4 cm (Plate 5.7, S3).

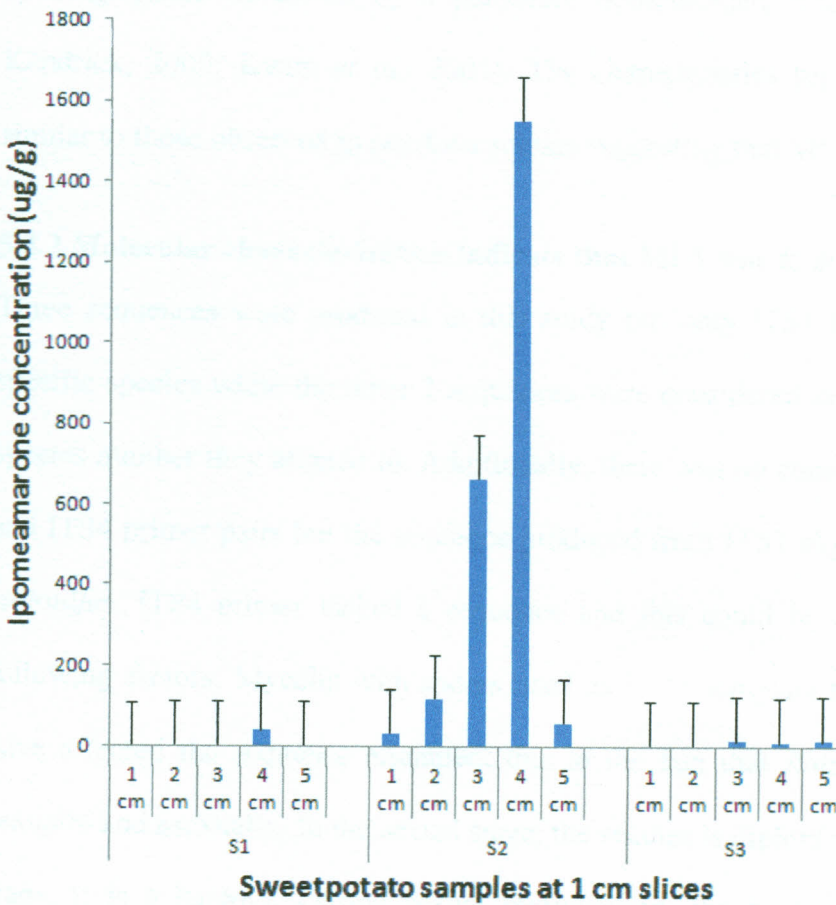


**Plate 5.7:** Infection on 1 cm slices from Kemb cultivar samples (S1, S2 and S3) by MI-1 fungus. Each of the 3 samples had five or six 1 cm slices

#### 5.3.7.2 Furanoterpenoid levels of samples from controlled inoculation method

Generally, *ipm* levels were high in slices with low noticeable infection (Fig. 5.11). Three sweetpotato samples, S1, S2 and S3 were shown to have progressive infection (in section 5.3.7.1) and different slices for each sample were analysed for *ipm* levels. Quantification of the sweetpotato slices revealed progressive increase in *ipm* levels away from site of inoculation where S2 slices had the highest concentrations followed by S3 and then S1. For the specific samples, S1 had the lowest *ipm* level of 0.35  $\mu\text{g/g}$  at 1 cm away from the inoculation site while at 4 cm, the *ipm* concentration was 40.45  $\mu\text{g/g}$ . For the second

sample, S2, the first 1 cm slice had high *ipm* level of 33  $\mu\text{g/g}$  while at 4 cm away from the inoculation area, it had 1547.14  $\mu\text{g/g}$ . The third sample, S3, generally had the lowest *ipm* levels of the 3 samples where at 1 cm, it had 4.51  $\mu\text{g/g}$  while at 3 and 5 cm, it had 15.50 and 14.14  $\mu\text{g/g}$ , respectively. There was a general progressive increase of *ipm* as you move towards the healthy tissue (Fig. 5.11). Nevertheless, there were no significant ( $p>0.05$ ) effects between distances from the inoculation site (Appendix 11).



**Figure 5.11:** Ipomeamarone concentrations at 1-cm slices of 3 sweetpotato root samples of Kemb cultivar

## 5.4 DISCUSSION

### 5.4.1 Morphological characteristics indicate that MI-1 was *R. stolonifer*

Morphological structures on MI-1 from microscopy included rhizoids, well developed stolons, sporangiophores and a dome-shaped collumella. These structures are consistent with those reported for *R. stolonifer* having white cotton-like colonies with black/brown sporangia speckles, well-developed rhizoids which are interconnected by stolons and sporangiophores followed by a persistent dome-shaped columella (Schippers, 1984; Kendrick, 2000; Kwon *et al.*, 2001). The characteristics reported in this study were similar to those observed in previous studies suggesting that MI-1 was *R. stolonifer*.

### 5.4.2 Molecular characterization indicate that MI-1 was *R. stolonifer*

Three sequences were produced in this study but only ITS1 had an alignment to one specific species while the other 2 sequences were considered ineffective due to the high species number they aligned to. Additionally, there was no consensus sequence for ITS1 and ITS4 primer pairs but the sequence produced from ITS1 aligned mainly to *Rhizopus stolonifer*. ITS4 primer lacked a sequence and this could be attributed to one of the following factors. Mycelia with spores used as DNA template for PCR reactions could have affected the sequence alignment due to the fact that *Rhizopus* species reproduce sexually and asexually. In the sexual stage, the species is diploid while during the asexual stage, it is a haploid. During sexual reproduction, the fungus has two mating types designated as ‘-’ and ‘+’ that fuse to form zygosporangium (Gauger, 1977). Since *Rhizopus stolonifer* is a diploid during the reproduction period and DNA template used in this study had both spores and mycelia, MI-1 could have been at reproduction hence the difference. There could also have been the presence of many secondary structures thereby

inhibiting the analysis as reported by Abe *et al.* (2006) for *Rhizopus* species. The variations could also be attributed to mutations which are known to highly occur in fungi. It could also have been due to splice variants present in at transcription, although they did not affect expression of the specific genes. Another factor could have been occurrence of recombination or insertions during sexual reproduction. Another concern could have been that ITS method may not have been effective in identification of MI-1 hence lack of ITS4 sequence. However, this concern was highly unlikely because species identification using ITS has been reported as reliable and good quality products have been generated that directly translated into sequencing results (Schoch *et al.*, 2012). Results from molecular characterization gave an indication that MI-1 was *R. stolonifer*.

In addition to identification of MI-1 as *R. stolonifer*, phylogenetic analysis showed MI-1 in a separate cluster, alone. MI-1 could have been clustered separately due to the difficulty in generating a consensus sequence. A study by Abe *et al.* (2006) indicated that they also had difficulty in sequencing of PCR amplicons and they attributed it to presence of many secondary structures thereby inhibiting analysis relationships of *Rhizopus* species and MI-1 could have been a different sub-species under *Rhizopus* species therefore alignment to a known sequence was not possible. Nevertheless, there was likelihood that MI-1 was *R. stolonifer* species because of the total 26 hits from BLAST, 23 were *R. stolonifer* while only 1 was *R. oryzae*.

#### **5.4.3 The methods used were effective for furanoterpenoid analysis**

Controlled fungal infection using crude but simple methods such as covering the root samples with polythene bags proved successful in this study. Secondly, mycelia and

spores used as inoculum successfully infected the sweetpotato roots under controlled infection. Similar inoculation methods have been reported to generate high incidence of infection (Jenkins, 1981). In addition to inoculation methods, general infection of fungus on sweetpotato samples has been reported (Clark *et al.*, 1981) but in contrast to this inoculation method, progressive infection by fungus has not previously been reported in sweetpotato for further furanoterpenoid analysis. In addition, controlling fungal growth on sweetpotato samples has also not been reported or is unknown hence making this study unique and important for preparation of samples for extraction of furanoterpenoids.

Methods used in isolating and identifying furanoterpenoids were successfully done using TLC, GC-MS, HPLC and NMR. Similar isolation methods have been reported for another phytoalexin 7-hydroxycoumarin in sweetpotato at a retention time of 11.5 mins by HPLC and GC-MS with peaks at  $m/z$  51, 78, 105, 134, 162 (Smith *et al.*, 2001). The major contribution made by this study on identification and isolation of *ipm* and dehydro-*ipm* was the mass to ratio peak values and the retention times, which are specific to these compounds and has not previously been done for GC-MS and HPLC. In contrast to methods used in this study, other methods have previously been used for *ipm* analysis including column, gas chromatography which used radioactivity for isolation of furanoterpenoids (Oguni and Uritani, 1974). GC-MS technique used in this study is better than simple gas chromatography because there is additional functions availed when mass spectroscopy is included such as ionisation to produce fragmentations, separation of ions by mass, measurement of mass and abundance of the metabolites (McLafferty and Turecek, 1993). In addition, there was no use of radioactivity in this study hence safer. Nevertheless, all the methods mentioned above had similar chemical structures for

dehydro-*ipm* and *ipm* (Oguni and Uritani, 1974) based on spectroscopic techniques (GC-MS and NMR) from this study.

#### **5.4.4 Variations in furanoterpenoid levels on the sweetpotato samples**

Presence of MI-1 elicited production of high *ipm* levels in the sweetpotato. These levels exceed those reported for *R. stolonifer* of 200-1,100 mg/kg but comparable to high *ipm* elicitors such as *C. fimbriata* and *Fusarium solani* with levels from 1,100-9,300 mg/kg in sweetpotato (Clark *et al.*, 1981). In addition, since *ipm* levels as low as 250-500 mg/kg are reported to cause liver damage (Pandey, 2008) production of relatively high levels of furanoterpenoids as shown herein suggests that they are of economic importance.

The MI-1 inoculated sweetpotato samples showed consistently high *ipm* levels compared to the non-inoculated controls. Similar reports have been documented of low *ipm* levels between 56-105 mg/kg for non-infested sweetpotato cultivars in United Kingdom (Coxon *et al.*, 1975). It has also been reported that mechanical injury or damaged but uninfested sweetpotato elicit low furanoterpenoid levels while the infested ones have high levels due to increased enzyme activity to convert mevalonate to isopentylpyrophosphate, correlating to furanoterpenoid production (Greisbach and Ebel, 1978; Uritani, 1999; Vidhyasekaran, 2008). The relatively low *ipm* levels in roots of the uninfested samples from the present study confirm that high furanoterpenoid levels are elicited in presence of an agent.

#### **5.4.5 Different cultivars elicit varying concentration levels of ipomeamarone**

The *ipm* levels differed even within cultivar. For instance, in Nyawo cultivar, one sample had 602 mg/kg while the other had 2,126 mg/kg. Similar observations have been reported

for *ipm* in other studies with such high variations of 11 to 2,000 mg/kg (Coxon *et al.*, 1975; Clark *et al.*, 1981). These variations have been speculated to be associated with moisture loss, root maturity and susceptibility or resistance of sweetpotato to fungal infection (Jenkins, 1981). The observations made for this study suggests that these variations are a common occurrence and since the samples were not biological replicates, such variations could be attributed moisture, root maturity and cultivar-type.

In addition to *ipm* concentration variations, the yellow-fleshed cultivars had higher levels than lighter coloured ones. Similarly, Clark and Hoy (1994) reported high resistance to fungal attack for orange-fleshed compared to white-fleshed sweetpotato. High *ipm* levels in flesh of deeper coloured cultivars suggests higher resistance than lighter coloured cultivars as shown by Nyawo, Kemb and Naspot with higher *ipm* than Bungoma of white flesh.

#### **5.4.6 Ipomeamarone concentrations on 1 cm slices**

Analysis of 1 cm slices for *ipm* revealed wide margins between different samples from 0.35-1,547 mg/kg. In addition, the *ipm* concentrations increased progressively from the site of inoculation. It has been reported that phytoalexins are produced in healthy tissues surrounding wounded parts but terminate in the necrotic tissue (Agrios, 2005). Catalano *et al.* (1977) reported that 3-10 mm elimination of diseased portions beyond infested area removes most *ipm*. In the present study, high *ipm* levels were reported 4 cm from area of infection suggesting that removal of 1 cm from infection area will not remove all *ipm*. Differences in the 2 reports could either arise due to the size of the infected region on the sweetpotato sample or the current study took into consideration the *ipm* levels for several slices not undertaken by the previous workers.

#### 5.4.7 Possible effects of high ipomeamarone levels on animals and humans

High *ipm* concentrations of 2,900 mg/kg were reported herein in this thesis. These levels far exceed those reported to cause liver damage in rats of 84-500 mg/kg (Wilson and Burka, 1979; Pandey *et al.*, 2006; Pandey, 2008). Although exact concentrations have not been documented for large animals like cattle, there are reports of respiratory complications and deaths due to presence of furanoterpenoids in sweetpotato roots fed to these animals (Figuera *et al.*, 2003; Meikle, 2008; Donovan, 2011). Symptoms in cattle have been documented to start within 24 hours after consumption of toxic roots and death follows after a few days (Figuera *et al.*, 2003).

It is documented that investment in research is a low priority in developing countries (Perry and Grace, 2009) hence lack of available information on effect of furanoterpenoids in cattle. The lack of information relating furanoterpenoids and farm animals in SSA could have contributed to respiratory infections and some deaths, although the cause may not have been known. Such deaths could have adverse effects for small-scale farmers since they practise mixed farming (growing crops and keeping cattle), which contributes 30 to 80% gross farm income (FAO, 2002).

High *ipm* levels (2,900 mg/kg) were reported in this study. It has also been documented that respiratory complications and hepatotoxicity were caused by levels as low as 250-500 mg/kg (Pandey, 2008). Although there is no report linking *ipm* to consumption by humans, earlier studies indicated cooking does not destroy *ipm* (Wilson *et al.*, 1970); others reported 80% destroyed by cooking (Cody and Haad, 1976) and baking or boiling does not eliminate *ipm* but on the contrary may increase *ipm* levels for blemished parts but if blemished parts are removed, non-traceable amounts are detected in the remaining

tissue (Catalano *et al.*, 1977). For instance, if 80 % of 2,900 mg/kg of *ipm* was eliminated by cooking, a considerable amount would still be left over in the cooked roots of about 580 mg/kg and could still cause harm to animals when fed to them. This amount is much higher than documented levels causing harm to animals of 250-500 mg/kg (Pandey, 2008). Although direct effects of consumption by humans is not documented, clinical toxicity of liver and severe body pains was reported in some patients administered with high doses of 4-ipomeanol (Rowinsky *et al.*, 1993), a probable drug against lung cancer (Zimmermann *et al.*, 2010).

## CHAPTER SIX

### CONCLUSSIONS, RECOMMENDATIONS AND FURTHER RESEARCH

#### 6.1 GENERAL CONCLUSSIONS

The regeneration protocols developed here have been optimized for several SSA sweetpotato cultivars. Low levels of 2,4-D (0.05 mg/L) are reported as efficient in calli induction for regeneration via organogenesis in sweetpotato. It was also noted that kinetin at 0.2 mg/L seemed to be the most effective concentration for production of regenerants, irrespective of cultivar. Multiple regenerants per explant were also produced in some cultivars but the media containing kinetin produced the highest numbers. These regenerants could then be used as a source of propagules. Additionally, although different hormones work better for different cultivars, results from this study suggest that media containing kinetin (0.2 mg/L) could be the most effective cytokinin when compared to thidiazuron and zeatin in step-2 media for organogenesis. In conclusion, from the 32 SSA screened, this study identified 6 cultivars with high regeneration efficiencies that could be used for transformation experiments including Imby, Luapula, Ukerewe, Mafutha, Mugande and Zambezi.

Development of a transformation protocol through organogenesis of the SSA sweetpotato cultivars was successful both for *uidA* and weevil resistance genes. Optimal kanamycin concentrations ranged between 5 and 25 mg/L for the SSA cultivars. Gene integration of WR transgene, *cry7Aa1*, showed several copy numbers but also showed gene truncation owing to the size of the gene hence future gene constructs should have smaller inserts or have enhancers to enhance transformation efficiencies such as catalase introns. If they

had some resistance to weevils, copy number would not have been a major factor since sweetpotato is generally clonally propagated and cross pollinating happens but flower abortion mostly occurs due to incompatibility and sterility issues.

Protein concentrations of ET33-34 differed for transgenic events produced in the range of 0 to 0.03  $\mu\text{g/g}$ . When the events were fed to weevils, there was no evidence of resistance. This could have been due to the root-specific promoters used to drive *cry7Aa1* driven by  $\beta$ -amylase and *ET33-34* driven by sporamin. Although both proteins are normally found in sweetpotato storage roots, the relatively low levels suggested that either these proteins were not as effective or other gene enhancers needed to be included in the gene construct to increase the transformation efficiencies. It also suggests that future gene constructs should have constitutive promoters such as CaMV-35S.

The ipomeamarone concentrations were high up to 2,900 mg/kg for one cultivar. The rationale behind this study was that if the transgenic events could have been resistant to weevils, storage roots could have had one major venue through which weevils lay eggs and larvae burrow through, closed. This in turn would reduce the secondary infection by microbes thereby reducing *ipm* concentrations as high as those reported herein. In addition, the information availed in this study was novel since there have been no previous reports or studies that have evaluated *ipm* concentrations across a microbe infested samples, through the layers. This gives guidance to those who produce or consume them to beware of *ipm* levels that are high when infected by microbes.

In conclusion, this study has shown that SSA sweetpotato cultivars can be regenerated for future improvement through organogenesis but it requires an initial screen to identify the

best performing genotypes and the optimal hormone concentrations and combinations. Secondly, genetic engineering of SSA sweetpotato cultivars is achievable although it requires generation of numerous events in order to identify the most effective events against weevils. Once transgenic events have been successfully developed, it would be easier for farmers to grow sweetpotato without fear of losing them in case of a drought. Finally, *ipm* should be considered as a potential economic problem to both the rural poor farm families and the large-scale farmers all over the world due to its effect on farm animals and possibly humans.

## **6.2 GENERAL RECOMMENDATIONS AND FUTURE RESEARCH**

For the regeneration experiments through organogenesis, it is recommended to start with kinetin as the first cytokinin, to screen for regenerating cultivars. From the regeneration experiments, it was noted that there are differences in RE for the SSA cultivars, which could suggest differences in endogenous hormone levels; further research should therefore be conducted on these cultivars to verify that the endogenous levels vary between cultivars.

There was evidence of gene integration and truncation for the weevil resistance genes from the southern blot results, which suggests that a need for shorter inserts or optimize conditions under which whole T-DNA transfers are made. In addition to this, further research by adding a catalase intron to the gene construct should be conducted since it has been documented to improve transformation efficiency in other crops.

In the case of low protein expression, there could be a problem with conformity of the protein due to post-transcription and post-translation modification and could be addressed by protein crystallization leading to a 3-D structure elucidation. In addition to this,

sequencing of the amino acids that make up these proteins from the transgenics should be conducted and compared them to pure protein sequence from bacterium. Further research should be conducted to identify factors that are most likely to affect transcription and translation followed by identifying methods that would help solve this.

For the ipomeamarone work, it is recommended that more work should be done on farmer grown sweetpotato on farms to find out the ipm levels present. In addition, farm animal research should be conducted to find out the ipm levels that affect these farm animals once ingested since such information is lacking.

## REFERENCES

- Abe, A.; Oda, Y.; Asano, K. and Sone, T. (2006) The molecular phylogeny of *Rhizopus* based on rRNA sequences. *Bioscience, Biotechnology and Biochemistry* 70 (10): 2387-2393.
- Agili, S.; Nyende, B.; Ngamau, K. and Masinde, P. (2012) Selection, yield evaluation, drought tolerance indices of orange-flesh sweetpotato (*Ipomoea batatas* Lam.) hybrid clone. *Journal of Nutrition and Food Sciences* 2: 138. doi:10.4172/2155-9600.1000138.
- Agrios, G.N. (2005) Plant Pathology. 5<sup>th</sup> edition. Elsevier Academic Press. Burlington, USA Pg. 235-236.
- Ahuja, I.; Kissen, R. and Bones, A.M. (2012) Phytoalexins in defense against pathogens. *Trends in Plant Science* 17 (2): 73-90.
- Akazawa, T. and Uritani, I. (1960) Isolation of ipomeamarone and two coumarin derivatives from sweet potato roots injured by the weevil, *Cylas formicarius elegantulos*. *Archives of Biochemistry and Biophysics* 88:150-156.
- Akuta, T.; Eguchi, A.; Okuyama, H.; Senda, T.; Inokuchi, H.; Suzuki, Y.; Nagoshi, E.; Mizuguchi, H.; Hayakawa, T.; Takeda, K.; Hasegawa, M. and Nakanishi, M. (2002) Enhancement of phage-mediated gene transfer by nuclear localization signal. *Biochemistry and Biophysical Research Communications* 297: 779-786.
- Al-Mazrooei, S.; Bhatti, M.H.; Henshaw, G.G.; Taylor, N.J. and Blakesley, D. (1997) Optimisation of somatic embryogenesis in fourteen cultivars of sweetpotato (*Ipomoea batata* (L.) Lam). *Plant Cell Reports* 16 (10): 710-714.
- Ames, T.; Smit, N.E.J.M.; Braun, A.R.; O'Sullivan, J.N. and Skoglund, L.G. (1996) Sweetpotato: major pests, diseases, and nutritional disorders. International Potato Center (CIP). Lima, Peru. 152 p.
- Anderson, M.S. and de Vicente, M.C. (2010) Gene flow between crops and their wild relatives. The John Hopkins University Press, Baltimore, USA Pg. 483-498.
- Bajguz, A. and Piotrowska, A. (2009) Conjugates of auxin and cytokinin. *Phytochemistry* 70 (8): 957-969.
- Baker, D.A. (2000) Vascular transport of auxins and cytokinins in *Ricinus*. *Plant Growth Regulation* 32: 157-160.

- Baldwin, B.G.; Sanderson, M.J.; Porter, M.J.; Wojciechowski, M.F.; Campbell, C.S. and Donoghue, M.J. (1995) The ITS region of nuclear ribosomal DNA: A valuable source of evidence on angiosperm phylogeny. *Annals of the Missouri Botanical Garden* 82 (2): 247-277.
- Bashaasha, B.; Mwanga, R.O.M.; Ocitti pObwoya, C. and Ewell, P.T. (1995) Sweet potato in the farming and food systems of Uganda: A Farm Survey Report. December 1995. NARO and CIP.
- Beier, C. (1990) Natural pesticides and bioactive components in foods. *Reviews of Environmental Contamination and Toxicology* 113: 47-137.
- Betz, F.S.; Hammond, B.G. and Fuchs, R.L. (2000) Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. *Regulatory Toxicology and Pharmacology* 32: 156-173.
- Binka, A.; Orczyk, W. and Nadolska-Orczyk, A. (2012) The *Agrobacterium*-mediated transformation of common wheat (*Triticum aestivum* L.) and triticale (x *Triticosecale Wittmack*): role of the binary vector system and selection cassettes. *Journal of Applied Genetics* 53: 1-8.
- Birch, R.G. (1997) Plant Transformation: Problems and strategies for practical application. *Annual Review of Plant Physiology Plant Molecular Biology* 48: 297-326.
- Bjourson, A.J. and Cooper, J.E. (1992) Band-stab PCR: a simple technique for the purification of individual PCR products. *Nucleic Acids Research* 20 (17): 4675.
- Blasco, I.C. (2007) Regeneration "In vitro" via organogenesis de cinco cultivares de camote (*Ipomoea batatas* Lam): Tanzania, SPK004, Wagabolige, 199004.2 y Nanshu. Bachiller tesis. Universidad Nacional Agraria, La Molina. Facultad de Ciencias Lima, Peru, 106 p.
- Bravo, A.; Gill, S.S. and Soberón, M. (2007) Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon* 49 (4): 423-435.
- Bravo, A. and Soberon, M. (2010) *Bacillus thuringiensis*: Mechanisms and use. In eds. Gilbert, L.I. and Gill, S.S. *Insect Control*. Elsevier B.V. Academic Press, London, UK. Pg. 247-259.

- Brian, P.W. (1959) Effects of Gibberellins on Plant Growth and Development. *Biological Reviews* 34 (1): 37-77.
- Burgos, L. and Albuquerque, N. (2003) Ethylene inhibitors and low kanamycin concentrations improve adventitious regeneration from apricot leaves. *Plant Cell Reports* 21: 1167-1174.
- Callis, J.; Fromm, M. and Walbot, V. (1987) Introns increase gene expression in cultured maize cells. *Genes and Development* 1: 1183-1200.
- Cao, J.; Tang, J.D.; Strizhov, N.; Shelton, A.M. and Earle, E.D. (1999) Transgenic broccoli with high levels of *Bacillus thuringiensis* Cry1C protein control diamondback moth larvae resistant to Cry1A or Cry1C. *Molecular Breeding* 5: 131-141.
- Catalano, E.A.; Hasling, V.C.; Dupung, H.P. and Constantine, R.J. (1977) Ipomeamarone in blemished and diseased sweetpotato. *Journal of Agriculture and Food Chemistry* 25: 94-96.
- Chrispeels, M.J. and Sadava, D.E. (2003) Plants, genes and Crop Biotechnology. Jones and Bartlett Publishers, MA, USA. 198 p.
- Chung, S.M.; Vaidya, M. and Tzfira, T. (2005) *Agrobacterium* is not alone: Gene transfer to plants by viruses and other bacteria. *Trends in Plant Science* 11 (1): 1-4.
- Clark, C.A. and Hoy, M.W. (1994) Identification of resistance in sweetpotato to *Rhizopus* soft rot using two inoculation methods. *Plant Disease* 78 (11): 1078-1082.
- Clark, C.A.; Lawrence, A. and Martin, F.A. (1981). Accumulation of furanoterpenoids in sweetpotato tissue following inoculation with different pathogens. *Phytopathology* 71: 708-711.
- Cody, M. and Haard, N.F. (1976) Influence of cooking on toxic stress metabolites in sweet potato root. *Journal of Food Science* 41 (2): 469-470.
- Coleacp (2011) Guide to good crop protection practices for sweet potato (*Ipomea batatas*) in ACP countries. Pg. 12-22. [www.coleacp.org/pip](http://www.coleacp.org/pip)
- Collier, R.; Fuchs, B.; Walter, N.; Lutke, W.K. and Taylor, C.G. (2005). Ex-vitro composite plants: an inexpensive, rapid method for root biology. *Plant Journal* 43: 449-457.

- Coxon, D.T.; Curtis, R.F. and Howard, B. (1975) Ipomeamarone, a toxic furanoterpenoid sweet potatoes (*Ipomeoa batatas*) in the United kingdom. *Food and Cosmetics Toxicology* 13: 87-90.
- Crickmore, N.; Nicholls, C.; Earp, D.J.; Hodgan, C. and Ellar, D.J. (1990) The construction of *Bacillus thuringiensis* strains expressing novel entomocidal  $\delta$ -endotoxins combinations. *Biochemistry Journal* 270: 133-136.
- Davies, P.J. (2004) Plant hormones: Biosynthesis, signal transduction, action. Springer. Pg. 750. [http://books.google.co.ke/books?id=E8LXjxt5H4C&dq=auxins&source=gbs\\_navlinks\\_s](http://books.google.co.ke/books?id=E8LXjxt5H4C&dq=auxins&source=gbs_navlinks_s)
- Day, C.D.; Lee, E.; Kobayashi, J.; Holappa, L.D.; Albert, H.; and Ow, D.W. (2000) Transgene integration into the same chromosome location can produce alleles that express at a predictable level, or alleles that are differentially silenced. *Genes and Development* 14: 2869-2880.
- Diaz, J.; Schmeidiche, P. and Austin, D.F. (1996). Polygon of crossability between eleven species of *Ipomoea*: section Batatas (Convolvulaceae). *Euphytica* 88: 189-200.
- Donovan, T.W. (2011) 200 dead cows mystery solved in Wisconsin: Toxic moldy potatoes to blame. The Huffington Post. <http://www.huffingtonpost.com> 29th January, 2011 05:30 PM ET.
- Du, C.; Martin, P.A.W. and Nickerson, K.W. (1994) Comparison of disulfide contents and solubility at alkaline pH of insecticidal and non-insecticidal *Bacillus thuringiensis* protein crystals. *Applied and Environmental Microbiology* 60 (10): 3847-3853.
- Dunlap, J.R. and Robacker, K.M. (1988) Nutrient salts promote light-Induced degradation of indole-3-acetic acid in tissue culture media. *Plant Physiology* 88: 379-382.
- Ebregt, E.; Struik, P.C.; Odongo, B. and P.E. Abidin, P.E. (2007) Piecemeal versus one-time harvesting of sweet potato in north-eastern Uganda with special reference to pest damage. *Wageningen Journal of Life Sciences* 55 (1): 75-92.
- Eickbush, T.H. and Eickbush, D.G. (2007) Finely orchestrated movements: Evolution of the ribosomal RNA genes. *Genetics* 175: 477-485.

- Ekobu, M.; Solera, M.; Kyamanywa, S.; Mwangi, R.O.M.; Odongo, B.; Ghislain, M. and Moar, W.J. (2010) Toxicity of 7 *Bacillus thuringiensis* Cry proteins against *Cylas puncticollis* and *C. brunneus* (Coleoptera: Brentidae) using a novel artificial diet. *Journal of Economic Entomology* 103 (4): 1493-1502.
- El Far, M.M.M.; El Mangoury, K. and Elazab, H.E.M. (2009) Novel plant regeneration for Egyptian sweetpotato (*Ipomoea Batatas* (L.) Lam.) Abees cultivar via indirect organogenesis stimulated by initiation medium and cytokinin effects. *Australian Journal of Basic and Applied Sciences* 3 (2): 543-551.
- FAO (1998) Storage and processing of roots and tubers in the tropics.
- FAO (2002) Cattle and small ruminant production systems in sub-Saharan Africa. A systematic review by Otte, M.J. and Chilonda, P. Rome. 36 p.
- FAOSTAT (2008-2013) <http://faostat.fao.org/>
- Felsenstein J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39: 783-791.
- Figuera, R.A.; Rozza, D.B.; Piazer, J.V.; Copetti, M.V.; Irigoyen, L.F. and Barros, C.S.L. (2003) Interstitial pneumonia in cattle fed mouldy sweetpotatoes (*Ipomoea batatas*) *Pesquisa Veterinaria Brasileira* 23 (4): 1-5.
- Firon, N.; LaBonte, D.; Villordon, A.; Kfir, Y.; Solis, J.; Lapis, E.; Perlman, T.S.; Doron-Faigenboim, A.; Hetzroni, A.; Althan, L. and Nadir, L.A. (2013) Transcriptional profiling of sweetpotato (*Ipomoea batatas*) roots indicates down-regulation of lignin biosynthesis and up-regulation of starch biosynthesis at an early stage of storage root formation. *Biomed Central Genomics* 14: 460 doi:10.1186/1471-2164-14-460.
- Fite, T.; Getu, E. and Sori, W. (2014) Economic importance of sweetpotato weevil, *Cylas puncticollis* B. (Coleoptera: Curculionidae) and its management in Eastern Oromiya, Ethiopia. *Asian Journal of Biological Sciences* 7: 198-207.
- Ford, T.L.; Cooley, J.T. and Christou, P. (1994). Current status for gene transfer into rice utilising variety-independent delivery systems. In eds. Zeigler, R.S.; Leong, S.A. and Teng, P.S. Rice blast disease. CABI, Wallingford Pg. 203.

- Gahan, P.B. and George, E.F. (2008) Adventitious regeneration. In eds. George, E.F.; Hall, M.A. and de Klerk, G.J. *Plant Propagation by Tissue Culture 3rd Edition Volume 1. The Background*. Springer, Dordrecht, The Netherlands. Pg. 355-401.
- Gaspar, T.; Kevers, C.; Faivre-Rampant, O.; Vecoeur, M.C.; Penel, C.; Greppin, H. and Dommes, J. (2003) Changing concepts in plant hormone action. *In Vitro Cellular and Developmental Biology-Plant* 39: 85-106.
- Gauger, W.L. (1977) Meiotic gene segregation in *Rhizopus stolonifer*. *Journal of General Microbiology* 101: 211-217.
- Gbigbi, M.T. (2011) Economic efficiency of smallholder sweet potato producers in delta state, Nigeria: a case study of Ughelli south local government area. *Research Journal of Agriculture and Biological Sciences* 7 (2): 163-168.
- Gelvin, S.B. (2003) *Agrobacterium*-mediated plant transformation: the biology behind the "Gene-Jockeying" tool. *Microbiology and Molecular Biology Reviews* 67 (1): 16-37.
- George, F.; Hall, M.A. and de Klerk. (2008) *Plant propagation by Tissue Culture 3<sup>rd</sup> edition*. Vol. 1: The Background. Springer, Netherlands.
- Ghislain, M.; Lagnaoui, A. and Walker, T. (2003) Fulfilling the promise of Bt potato in developing countries. *Journal of New seeds* 5 (2/3): 93-113.
- Ghuman, B.S. and Lal, R. (1983) Growth and plant-water relations of sweetpotato (*Ipomoea batatas*) as affected by soil moisture regimes. *Plant and soil* 70: 95-106.
- Gibson, R.W.; Mpembe, I.; Alicai, T.; Carey, E.E.; Mwanga, R.O.M.; Seal, S.E. and Vettene, H.J. (1998) Symptoms, aetiology and serological analysis of sweetpotato virus disease in Uganda. *Plant Pathology* 47: 95-102.
- Gonzalez, R.G.; Sánchez, D.S.; Campos, J.M.; Vázquez, E.P.; Guerra, Z.Z.; Quesada, A.L.; Valdivia, R.M. and González, M.G. (1999) Plant regeneration from leaf and stem explants from two sweet potato (*Ipomoea batatas* L. Lam.) cultivars. *Biotechnologia Aplicada* 16 (1): 11-14.
- Gonzalez, R.G; Sanchez, D.S; Guerra, Z.Z; Campos, J.M.; Quesada, A.L.; Valdivia, R.M.; Arencibia, A.D.; Bravo, K.Q. and Caligari, P.D.S. (2008) Efficient regeneration and *Agrobacterium tumefaciens* mediated transformation of

recalcitrant sweet potato (*Ipomoea batatas* L.) cultivars. *Asia-Pacific Journal of Molecular Biology and Biotechnology* 16 (2): 25-33.

Gosukonda, R.M.; Porobo-Dessai, A.; Blay, E.; Prakash, C.S. and Peterson, C.M. (1995) Thidiazuron-induced adventitious shoot regeneration of sweetpotato (*Ipomoea batatas*). *In Vitro Cellular and Developmental Biology* 31: 65-71.

Gosukonda, R.M.; Prakash, C.S. and Porobo-Dessai, A. (1995b) Shoot regeneration in vitro from diverse genotypes of sweetpotato and multiple shoot production per explant. *Hortscience* 30 (5): 1074-1077.

Greisbach, H. and Ebel, J. (1978) Phytoalexins, chemical defence substances of higher plants? *Angewandte Chemie International Edition in English* 17: 635-647.

Grüneberg, W.; Mwanga, R.; Andrade, M. and Dapaah, H. (2009) Unleashing the potential of sweetpotato in Sub-Saharan Africa: Current challenges and way forward. Challenge Theme Paper 1: Sweetpotato Breeding. CIP. Social Sciences Working Paper 2009-1.

Gutierrez, D.L.; Fuentes, S. and Salazar, L.F. (2003) Sweetpotato virus disease (SPVD): Distribution, incidence and effect on sweetpotato yield in Peru. *Plant Disease* 87: 297-302.

Harriet, M.; Talwana, H.L.; Mwanga, R.O.M. and Stevenson, P.C. (2012) Sweetpotato weevil (*Cylas* spp.) resistance in African sweetpotato germplasm. *International Journal of Pest Management* 58 (1): 73-81.

Hassanein, A., Loridon, K. And Dorion, N. (2007) Comparison of two transformation method efficiency Using *Pelargonium* × *Hortorum* 'Panaché Sud' protoplasts and leaf discs as explants. *Acta Horticulturae* 743: 127-132.

Heeres, P.; Schippers-Rozenboom, M.; Jacobsen, E. and Richard Visser, G.F. (2002) Transformation of a large number of potato varieties: genotype-dependent variation in efficiency and somaclonal variability. *Euphytica* 124 (1): 13-22.

Hicks, G.S. (1994) Shoot induction and organogenesis in-vitro: A developmental perspective. *In Vitro Cellular and Developmental Biology-Plant* 30P (1): 10-15.

Hofte, H. and Whiteley, H.R. (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiological Reviews* 53 (2): 242-255.

- Hoque, M.E. and Mansfield, S.W. (2004) Effect of genotype and explant age on callus induction and subsequent plant regeneration from root-derived callus of Indica rice genotypes. *Plant Cell, Tissue and Organ Culture* 78 (3): 217-223.
- Huaman, Z. (1999) Botany, origin, evolution and biodiversity of the sweetpotato. Germplasm training manual, CIP. Section 1.2 Taxonomy and Biodiversity. 26 p.
- Humara, J.M.; Lopez, M. and Ordas, R.J. (1999) Transient expression of the *uidA* gene in *Pinus pinea* cotyledons: A study of heterologous promoter sequences. *Plant Cell, Tissue and Organ Culture* 56: 69-78.
- Ichihashi, Y.; Kawade, K.; Usami, T.; Horiguchi, G.; Takahashi, T. and Tsukaya, H. (2011) Key proliferative activity in the junction between the leaf blade and leaf petiole of *Arabidopsis*. *Plant Physiology* 157 (3): 1151-1162.
- James, C. (2009) Global status of commercialized Biotech/ GM crops. International Service for the Acquisition of Agri-Biotech Applications, Brief 41.
- Jefferson, R.A.; Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions:  $\beta$ -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal* 6 (13): 3901 -3907.
- Jenkins, P.D. (1981) Differences in susceptibility of sweetpotato (*Ipomoea batatas*) to infection by storage fungi in Bangladesh. *Phytopathology* 102: 247-256.
- Jiang, T.; Xing, B. and Rao, J. (2008) Recent developments of biological reporter technology for detecting gene expression. *Biotechnology and Genetic Engineering Reviews* 25: 41-76.
- Jimenez, V.M. (2001) Regulation of in-vitro somatic embryogenesis with emphasis on the role of endogenous hormones. *The Revista Brasileira de Fisiologia Vegetal* 13 (2): 196-223.
- Joersbo, M. and Okkels, F.T. (1996) A novel principle for selection of transgenic plant cells: positive selection. *Plant Cell Reports* 16: 219-221.
- Kapinga, R.; Ortiz, O.; Ndunguru, J.; Omiat, E. and Tumwegamire. S. (2007) Handbook of sweetpotato. Integrated crop management. Research outputs and programs for East Africa. CIP.

- Kasili, R.W. (1998) Plant regeneration via direct and indirect organogenesis in some cultivars of the sweetpotato (*Ipomoea batatas* (L.) Lam). M.Sc. Botany, Thesis, Kenyatta University.
- Kendrick, B. (2000) Eumycotan fungi. The mainstream Phyla Zygomycota and Dikaryomycota. In *The Fifth Kingdom*, 3<sup>rd</sup> ed. Mycologue Publications. Pg. 27-37.
- Kihara, M.; Okada, Y.; Kuroda, H.; Saeki, K.; Yoshigi, N. and Ito, K. (2000) Improvement of  $\beta$ -amylase thermostability in transgenic barley seeds and transgene stability in progeny. *Molecular Breeding* 6 (5): 511-517.
- Kiiza, B., Mwanga, R.O.M., Kitembo, L. and Kawooya, F. (2009) An assessment of the incidence and damage by the sweetpotato weevil (*Cylas* spp.) and virus disease in Uganda. Zero Draft Report - Phase One Survey, CIP.
- Ko, K.; Brown, S.K.; Norelli, J.L. and Aldwinckle, H.S. (1998) Alterations in *nptII* and *gus* expression following micropropagation of transgenic M.7 apple rootstock lines. *Journal of the American Society for Horticultural Science* 123 (1): 11-18.
- Kota, M.; Daniell, H.; Varma, S.; Garczynski, S.F.; Gould, F. and Moar, W.J. (1999) Overexpression of the *Bacillus thuringiensis* (*Bt*) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and *Bt*-resistant insects. *Proceedings of the National Academy of Sciences* 96: 1840-1845.
- Koyama, Y., Tsuchiya, T. and Kakeda, K. (2000) Sporophytic self-incompatibility in *Ipomoea trifida*, a close relative of sweetpotato. *Annals of Botany* 85 (supplement A): 191-196.
- Kreuze, J.F.; Klein, I.S.; Lazaro, M.U.; Cuellar, W.J.C.; Morgan, G.L.; Mejia, P.G.C.M.; Ghislain, M. and Valkonen, J.P.T. (2008) RNA silencing-mediated resistance to a crinivirus (Closteroviridae) in cultivated sweetpotato (*Ipomoea batatas* L.) and development of sweetpotato virus disease following co-infection with a potyvirus. *Molecular Plant Pathology* 9 (5): 589-598.
- Kuc, J. (2001) Concepts and direction of induced systemic resistance in plants and its application. *European Journal of Plant Pathology* 107: 7-12.

- Kumar, P.P.; Lakshmanan, P. and Thorpe, T.A. (1998) Regulation of morphogenesis in plant tissue culture by ethylene. *In Vitro Cellular and Developmental Biology-Plant* 34: 94-103.
- Kwon, J.H.; Kang, S. W.; Kim, J.S. and Park, C.S. (2001) *Rhizopus* soft rot on cherry tomato caused by *Rhizopus stolonifer* in Korea. *Mycobiology* 29 (3): 176-178.
- Li, X.; Krasnyanski, S.F. and Korban, S.S. (2002) Optimization of the *uidA* gene transfer into somatic embryos of rose via *Agrobacterium tumefaciens*. *Plant Physiology and Biochemistry* 40: 453-459.
- Liston, A.; Robinson, W.A.; Oliphant, J.M. and Alvarez-buylla, E.R. (1996) Length variation in the nuclear ribosomal DNA internal transcribed spacer region of non-flowering seed plants. *Systematic Botany* 21 (2): 109-120.
- Loebenstein, G. (2009) Origin, distribution and economic importance. In eds Loebenstein, G. and Thottappilly, G. The Sweetpotato Chap. 2 The Sweetpotato. Springer Science Pg. 9-12.
- Low, J.; Lynam, J.; Lemaga, B.; Crissman, C.; Barker, I.; Thiele, G.; Namanda, S.; Wheatley, C. and Andrade, M. (2009) Sweetpotato in Sub-Saharan Africa. In eds. Loebenstein, G. and Thottappilly, G. The Sweetpotato Chap. 16 Pg. 359-390.
- Low, J.; Kapinga, R.; Cole, D.; Loechl, C.; Lynam, J. and Andrade, M. (2009b). Challenge theme paper 3: Nutritional impact with orange-fleshed sweetpotato (OFSP). In 'Unleashing potential of sweetpotato in Sub-Saharan Africa'. CIP Social Sciences Working 2009-1 Paper Pg. 73-105.
- Luo, H.R.; Maria, M.S.; Benavides, J.; Zhang, D.P.; Zhang, Y.Z. and Ghislain, M. (2006) Rapid genetic transformation of sweetpotato (*Ipomoea batatas* (L.) Lam) via organogenesis. *African Journal of Biotechnology* 5 (20): 1851-1857.
- Macaluso, A. and Mettus, A. (1991) Efficient transformation of *Bt* requires non-methylated plasmid DNA. *Journal of Bacteriology* 173 (3): 1353-1356.
- Machakova, I.; Zazimalova, E. and George, E.F. (2008) Plant growth regulators I: Introduction; auxins, their analogues and inhibitors. In eds. George, E.F.; Hall, M.A. and de Klerk, G.J. Plant Propagation by Tissue Culture 3rd Edition Volume 1. The Background. Springer, Dordrecht, The Netherlands. Pg. 175-204.

- Maeo, K.; Tomiya, T.; Hayashi, K.; Akaike, M.; Morikami, A.; Ishiguro, S. and Nakamura, K. (2001) Sugar-responsible elements in the promoter of a gene for  $\beta$ -amylase of sweetpotato. *Plant Molecular Biology* 46 (5): 627-637.
- Maeshima, M.; Sasaki, T. and Asahi, T. (1985) Characterization of major proteins in sweet potato tuberous roots. *Phytochemistry* 24: 1899-1902.
- Manrique, S.T.; Diaz, D.; Reano, R.; Ghislain, M. and Kreuze, J. (2013) Sweetpotato plant regeneration via an improved somatic embryogenesis protocol. *Scientia Horticulturae* 161: 95-100.
- Mansaray, A.; Sundufu, A.; Yilla, K. and Fomba, S.N. (2013) Evaluation of cultural control practices in the management of sweetpotato weevil (*Cylas puncticollis* Boheman (Coleoptera: Curculionidae). *QScience Connect* 44 <http://dx.doi.org/10.5339/connect.2013.44>.
- Manyumwa, D.; Baars, R. and Kingma, K. (2012) Cassava and sweetpotato production for HIV/AIDS affected smallholder farmers: A case study of Zvishavane District, Zimbabwe. *Global Advanced Research Journal of Peace, Gender and Develop Studies* 1 (1): 12-19.
- Masumba, E.A.; Kulembeka, H.; Tollano, S.M. and Yongolo, M. (2004) Participatory evaluation of improved sweetpotato varieties in Eastern Tanzania. *African Crop Science Journal* 12 (3): 259-265.
- McLafferty, F.W. and Turecek, F. (1993) Interpretation of mass spectra. Fourth edition. University Science Books. Sausalito, California. Pg. 1-15.
- Meikle, J. (2008) Six cows killed by sweet potato poisoning. The Guardian, Tuesday 15 January <http://www.guardian.co.uk/theguardian>.
- Miller, C.O.; Skoog, F.; Von Saltza, M.H. and Strong, F.M. (1955) Kinetin, a cell division factor from deoxyribonucleic acid. *Journal of the American Chemical Society* 77 (5): 1392-1392.
- Miller, C.O. (1965) Evidence for the natural occurrence of zeatin and derivatives: compounds from maize which promote cell division. *Proceedings of the National Academy of Sciences* 54: 1052-1058.

- Ming-Xia, C.; Jian-Qiu, H.; Zhi-Ming, W.; Quan-Hong, Y.; Chang-Zhao, W. and Jia-An, L. (2005) *Agrobacterium*-mediated multiple gene transformation in rice using a single vector. *Journal of Integrated Plant Biology* 47 (2): 233-242.
- Mohr H. and Schopfer P. (1995) *Plant Physiology*. Springer-Verlag, Berlin Pg. 394.
- Moliszewska, E.B. and Wisniewski, W. (2006) Infestation of sugar beet fields by *Rhizopus arrhizus* in atypical weather conditions in the summer of 2006. *Phytopathologia Polonica* 41: 75-78.
- Mont, J.; Iwanaga, M.; Orjeda, G. and Watanabe, K. (1993) Abortion and determination of stages for embryo rescue in crosses between sweetpotato, *Ipomoea batatas* Lam. ( $2n=6x=90$ ) and its wild relative, *I. trifida* (H.B.K.) G. Don. ( $2x=2x=30$ ). *Sexual Plant Reproduction* 6: 176-182.
- Moran, R.; Garcia, R.; Lopez, A.; Zaldua, Z.; Mena, J.; Garcia, M.; Armas, R.; Somonte, D.; Rodriguez, J.; Gomez, M. and Pimentel, E. (1998) Transgenic sweet potato plants carrying the delta-endotoxin gene from *Bacillus thuringiensis* var. *tenebrionis*. *Plant Science* 139: 175-184.
- Morikami, A.; Matsunaga, R.; Tanaka, Y.; Suzuki, S.; Mano, S. and Nakamura, K. (2005) Two cis -acting regulatory elements are involved in the sucrose-inducible expression of the sporamin gene promoter from sweetpotato in transgenic tobacco. *Molecular Genetics and Genomics* 272: 690-699.
- Moshkov, I.E.; Novikova, G.V.; Hall, M.A. and George, E.F. (2008) Plant growth regulators III: Gibberellins, ethylene, abscisic acid, their analogues and inhibitors; miscellaneous Compounds. In eds. George, E.F.; Hall, M.A. and de Klerk, G.J. *Plant Propagation by Tissue Culture 3rd Edition Volume 1. The Background*. Springer, Dordrecht, The Netherlands. Pg. 227-282.
- Moss, M.O. (2008) Fungi, quality and safety issues in fresh fruits and vegetables. *Journal of Applied Microbiology* 104: 1239-1243.
- Mugo, S.N.; Mwimali, M.; Taracha, C.O.; Songa, J.M.; Gichuki, S.T.; Tende, R.; Karaya, H.; Bergvinson, D.J.; Pellegrineschi, A. and Hoisington, D.A. (2011) Testing public Bt maize events for control of stem borers in the first confined field trials in Kenya. *African Journal of Biotechnology* 10 (23).

- Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays of tobacco tissue culture. *Physiologia Plantarum* 15: 473-497.
- Murata, T. and Matsuda, Y. (2003) Histological studies of the relationship between the process from fertilisation to embryogenesis and the low seed set of sweetpotato (*Ipomoea batatas* L. Lam). *Breeding Science* 53: 41-49.
- Muyinza, H.; Talwana, H.L.; Mwangi, R.O.M. and Stevenson, P.C. (2012) Sweetpotato weevil (*Cylas* spp.) resistance in African sweetpotato germplasm. *International Journal of Pest Management* 58 (1): 73-81.
- Mwangi, R.O.M.; Odongo, B.; Niringiye, C.; Alajo, A.; Abidin, P.E.; Kapinga, R.; Tumwegamire, S.; Lemaga, B.; Nsumba, J. and Carey, E.E. (2007) Release of two orange fleshed sweetpotato cultivars 'SPK004' ('Kakamega') and 'Ejumula' in Uganda. *HortScience* 42 (7): 1728-1730.
- Mwangi, R.O.M.; Niringiye, C.; Alajo, A.; Kigozi, B.; Namukula, J. and Mpenbe, I. (2011) 'NASPOT 11', a sweetpotato cultivar bred by a participatory plant breeding approach in Uganda. *HortScience* 46 (2): 317-321.
- Naimov, S.; Dukiandjiev, S. and de Maagd R.A. (2003) A hybrid *Bacillus thuringiensis* delta-endotoxin gives resistance against a coleopteran and a lepidopteran pest in transgenic potato. *Plant Biotechnology Journal* 1 (1): 51-57.
- Nayak, P.; Basu, D.; Das, S.; Busu, A.; Ghosh, D.; Ramakrishnan, N.A.; Ghosh, M. and Sen, S.K. (1997) Transgenic elite indica rice plants expressing Cry1Ac  $\delta$ -endotoxin of *Bacillus thuringiensis* are resistant against yellow stem borer (*Scirpophaga incertulas*). *Proceedings of the National Academy of Sciences* 94: 2111-2116.
- Naylor, R.L.; Falcon, W.P.; Goodman, R.M.; Jahn, M.M.; Sengooba, T.; Tefera, H. and Nelson, R.J. (2004) Biotechnology in the developing world: a case for increased investments in orphan crops. *Food Policy* 29: 15-44.
- Ndolo, P.J.; Mcharo, T.; Carey, E.E.; Gichuki, S.T.; Ndinya, C. & Malinga, J. (2001) Participatory on-farm selection of sweetpotato varieties in Western Kenya. *African Crop Science Journal* 9 (1): 41-48.
- Nedunchezhiyan, M.; Rao, R.K.; Laxminarayana, K. and Satapathy, B.S. (2010) Effect of strip cropping involving sweetpotato (*Ipomoea batatas* L.) on soil moisture,

- conservation, weevil infestation and crop productivity. *Journal of Root Crops* 36 (1): 53-58.
- Nopo, L.; Medrano, G. and Ghislain, M. (2005) Applied biotechnology laboratory protocols: Genetic engineering of potato and sweetpotato, production, selection and testing. International Potato Centre, Lima, Peru. Pg. 2-3.
- OECD (2008) Task Force for the Safety of Novel Foods and Feeds: Draft consensus document on sweetpotato (*Ipomoea batatas*). Env/JM/Food(2007)2/Rev1.
- Oggema, J.N.; Kinyua, M.G.; Ouma, J.P. (2007) Optimum 2, 4-D concentration suitable for embryogenic callus induction in local Kenyan sweetpotato cultivars. *Asian Journal of Plant Sciences* 6 (3): 484-489.
- Oguni, I. and Uritani, I. (1974) Dehydroipomeamarone as an intermediate in the biosynthesis of ipomeamarone, a phytoalexin from sweet potato root infected with *Ceratocystis fimbriata*. *Plant Physiology* 53: 649-652.
- Ohto, M.; Nakamura-Kito, K. and Nakamura, K. (1992) Induction of expression of genes coding for sporamin and  $\beta$ -amylase by polygalacturonic acid in leaf-petiole cuttings of sweet potato. *Plant Physiology* 99 (2): 422-427.
- Okada, Y.; Saito, A.; Nishiguchi, M.; Kimura, T.; Mori, M.; Matsuda, Y. and Murata, T. (2002) Microprojectile bombardment-mediated transformation of sweetpotato (*Ipomoea batata* (L.) Lam). *Sabrao Journal of Breeding and Genetics* 34 (1): 1-8.
- Okada, Y.; Saito, A.; Nishiguchi, M.; Kimura, T.; Mori, M.; Hanada, K.; Sakai, J.; Miyazaki, C.; Matsuda, Y. and Murata, T. (2001) Virus resistance in transgenic sweetpotato (*Ipomoea batatas* L. Lam) expressing the coat protein gene of sweet potato feathery mottle virus. *Theoretical and Applied Genetics* 103: 743-751.
- Omosa, M. (1997) Current and potential demand for fresh and processed sweetpotato products in Nairobi and Kisumu, Kenya. Social Science Department, Working paper No. 1997-1. Post-harvest Management, Marketing Program CIP Lima, Peru.
- Opabode, J.T. (2006) *Agrobacterium*-mediated transformation of plants: Emerging factors that influence efficiency. *Biotechnology and Molecular Biology Reviews* 1 (1): 12-20.
- Ormachea, M.A. (2008) Estandarización de un protocolo de cultivo in vitro para la regeneración vía organogénesis de camote (*Ipomoea batatas* (L.) Lamarck, 1793)

- variedades Tanzania y Wagabolige. Tesis para licenciada en biología. Facultad de Ciencias Biológicas, Universidad Ricardo Palma.
- Pandey, G. (2008) Acute toxicity of Ipomeamarone, a phytotoxin isolated from the injured sweetpotato. *Pharmacognosy Magazine* 4 (15): 89-92.
- Pandey, G.; Srivastava D.N.; Shrivastav A.B. and Madhuri, S. (2006) Ipomeamarone induced hepatotoxicity in rats: A pathological evaluation. *Indian Journal of Veterinary Research* 15 (1): 40-45.
- Payne, R.W.; Harding, S.A.; Murray, D.A.; Soutar, D.M.; Baird, D.B.; Glaser, A.I.; Welham, S.J.; Gilmour, A.R.; Thompson, R. and Webster, R. (2011) GenStat for Windows (14<sup>th</sup> Edition). VSN International, Hemel Hempstead, Hertfordshire HP1 1ES, UK.
- Pedersen, A.G.; Baldi, P.; Chauvin, Y. and Brunak, S. (1999) The biology of eukaryotic promoter prediction-a review. *Computers and Chemistry* 23 (3-4): 191-207.
- Pernisova, M.; Klima, P.; Horak, J.; Valkova, M.; Malbeck, J.; Soucek, P.; Reichman, P.; Hoyerova, K.; Dubova, J.; Friml, J.; Zazimalova, E. and Hejatko, J. (2009) Cytokinins modulate auxin-induced organogenesis in plants via regulation of the auxin efflux. *Proceedings of the National Academy of Sciences* 106 (9): 3609-3614.
- Perry, B. and Grace, D. (2009) The impacts of livestock diseases and their control on growth and development processes that are pro-poor. *Philosophical Transactions of the Royal Society* 364: 2643-2655.
- Porobo Dessai, A.; Gosukonda, R.M.; Blay, E.; Korsi Dumenyo, C.; Medina-Bolivar, F. and Prakash, C.S. (1995) Plant Regeneration of sweetpotato (*Ipomoea batatas* L.) from leaf explants in vitro using a two-stage protocol. *Scientia Horticulturae* 62: 217-224.
- Prakash, C.S. and Varadarajan, U. (1992) Genetic transformation of sweetpotato by particle bombardment. *Plant Cell Reports* 11 (2): 53-57.
- Qaim, M. (2001) A prospective evaluation of biotechnology in semi-subsistence agriculture. *Agricultural Economics* 25: 165-175.

- Rai, M.K.; Shekhawat, N.S.; Harish; Gupta, A.K.; Phulwaria, M.; Ram, K. and Jaiswal, U. (2011) The role of abscisic acid in plant tissue culture: a review of recent progress. *Plant Cell, Tissue and Organ Culture* 106 (2): 179-190.
- Ray, P.M. (1985) Destruction of auxin. *Annual Review of Plant Physiology* 9: 81-118.
- Reddy, G.N.; Gadi, N. and Taianao, A.J. (2012) Efficient sex pheromone trapping: catching the sweetpotato weevil, *Cylas formicarius*. *Journal of Chemical Ecology* 38 (7): 846-853.
- Reddy, G.V.; Zhao, Z. and Humber, R.A. (2014) Laboratory and field efficacy of entomopathogenic fungi for the management of the sweetpotato weevil, *Cylas formicarius* (Coleoptera: Brentidae). *Journal of Invertebrate Pathology* 122: 10-15.
- Ribnicky, D.M.; Cohen, J.D.; Hu, W.S. and Cooke, T.J. (2002) An auxin surge following fertilization in carrots; A mechanism for regulating plant totipotency. *Planta* 214: 505-509.
- Riva, G.A.; Gonzalez-Cabrera, J.; Vazquez-Padron, R. and Ayra-Pardo, C. (1998) *Agrobacterium tumefaciens*: a natural tool for plant transformation. *Electronic Journal of Biotechnology* 1 (3) ISSN: 0717-3458.
- Rowinsky, E.K.; Noe, D.A.; Ettinger, D.S.; Christian, M.C.; Lubejko, B.G. Fishman, E.K.; Sartorius, S.E.; Boyd, M.R. and Donehower, R.C. (1993) Phase I and pharmacological study of the pulmonary cytotoxin 4-ipomeanol on a single dose schedule in lung cancer patients: Hepatotoxicity is dose limiting in humans. *Cancer Research* 53: 1794-1801.
- Ruzicka, K.; Simaskova, M.; Duclercq, J.; Petrasek, J.; Zazimalova, E.; Simon, S.; Friml, J.; Van Montagu, M.C.E. and Benkov, E. (2008) Cytokinin regulates root meristem activity via modulation of the polar auxin transport. *Proceedings of the National Academy of Sciences* 106 (11): 4284-4289.
- Saha, S. and Sircar, P.K. (1990) Cytokinin changes in the subtending axillary leaves of developing flower of *Cosmos sulphureus* Cav. *Botanical Bulletin of Academia Sinica* 31: 291-294.
- Salisbury, F.B. and Ross, C.W. (1986) *Plant Physiology*. CBS Publishers and Distributors Shahdara, Delhi, India.

- Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning, A laboratory Manual Vol.3*. Cold Spring Harbor Laboratory Press, New York Pg. 16.57.
- Santa-Maria, M.; Pecota, K.V.; Yencho, C.G.; Allen, G. and Sosinski, B. (2009) Rapid shoot regeneration in industrial 'high starch' sweetpotato (*Ipomoea batatas* L.) genotypes. *Plant Cell, Tissue and Organ Culture* 97: 109-117.
- Schippers, M.A.A. (1984) A revision of the genus *Rhizopus* I. The *Rhizopus stolonifer*-group and *Rhizopus oryzae*. *Studies Mycology* 25: 1-19.
- Schneider, J.A.; Lee, C.J.; Naya, Y.; Nakanishi, K.; Oba, K. and Uritani, I. (1984) The fate of phytoalexin ipomeamarone: Furanoterpenes and butenolides from *Ceratocystis fimbriata*-infected sweetpotato. *Phytochemistry* 23: 759-144.
- Schnepf, E.; Crickmore, N.; Van Rie, J.; Lereclus, D.; Baum, J.; Feitelson, J.; Zeigler, D.R. and Dean, D.H. (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiology and Molecular Biology Reviews* 62 (3): 775-806.
- Schoch, C.L.; Seifert, K.A.; Huhndorf, S.; Robert, V.; Spouge, J.L.; Levesque, C.A.; Chen, W. and Fungal Barcoding Consortium. (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. *Proceedings of the National Academy of Sciences* 109 (16); 6241-6246.
- Scott, J.B. and Chakraborty, S. (2008) Identification of 11 polymorphic simple sequence repeat loci in the phytopathogenic fungus *Fusarium pseudograminearum* as a tool for genetic studies. *Molecular Ecology Resources* 8 (3): 628-30.
- Scott, G.J.; Rosegrant, M.W. and Ringler, C. (2000) Roots and tubers for the 21<sup>st</sup> century: Trends, projections and policy options. IFPRI 2020 Brief 66.
- Seo, J. and Lee, K. (2004) Post-translational modifications and their biological functions: Proteomic analysis and systematic approaches. *Journal of Biochemistry and Molecular Biology* 37 (1): 35-44.
- Sharma, H.; Sharma, K.K.; Seetharma, N. and Ortiz, R. (2000) Prospects for using transgenic resistance to insects in crop improvement. *Electronic Journal of Biotechnology* 3 (2).
- Shen Y. (1997) Determination of furanoterpenoid toxins from sweet potato by thin-layer chromatography. *Chinese Journal of Chromatography* 15 (4): 328-330.

- Shin, Y.; Choe, G.; Shin, B.; Yi, G.; Yun, P.; Yang, K.; Lee, J.S.; Kwak, S. and Kim, K. (2007) Selection of *nptII* transgenic sweetpotato plants using G418 and paromomycin. *Journal of Plant Biology* 50 (2): 206-212.
- Sihachakr, D. and Ducreux, G. (1987) Plant regeneration from protoplast culture of sweet potato (*Ipomoea batatas* Lam.). *Plant Cell Reports* 6: 326-328.
- Sitango, K. and Doyo, N. (2004) Early maturing sweet potato varieties for high altitude highlands of Papua New Guinea. In eds. Fischer T. *et al.* New directions for a diverse planet: Proceedings for the 4<sup>th</sup> International Crop Science Congress, Brisbane, Australia, 26 September - 1 October 2004.
- Smit, N.E.J. and Van Huis, A. (1999) Biology of the African sweetpotato weevil species *Cylas puncticollis* (Boheman) and *Cylas brunneus* (Fabricius) (Coleoptera: Apionidae). *Journal of Food Technology Africa* 4 (3): 103-107.
- Smit, N.E.J.; Magenya, O. and Parker, B.L. (1994) Biology and pheromone studies with the sweetpotato weevils: *Cylas Puncticollis* (Bohe.) and *C. Brunneus* (Fabr.). *Acta Horticulturae* 380: 399-404.
- Smith, R.A.; Drummond, S.; Haines, A.; Porter, J.R. and Hock, R.S. (2001) Induction of umbelliferone in sweetpotato cell suspension culture using mercuric chloride. *Biotechnology Letters* 23: 1397-1400.
- Song, G.; Honda, H. and Yamaguchi, K. (2004) Efficient *Agrobacterium tumefaciens*-mediated transformation of sweetpotato (*Ipomoea batatas* (L.) Lam.) from stem explants using a two-step kanamycin-hygromycin selection method. *In Vitro Cellular and Developmental Biology* 40 (2): 359-365.
- Srivastava, L.M. (2002) Plant growth and development hormones and environment. Elsevier Science, USA 202 p.
- Stathers; T. E.; Rees, D.; Kabi, S.; Mbilinyi, L.; Smit, N.; Kiozya H.; Jeremiah, S.; Nyango, A. and Jeffries, D. (2003). Sweetpotato infestation by *Cylas* spp. in East Africa. I. Cultivar differences in field infestation and the role of plant factors. *International Journal of Pest Management* 49 (2): 131-140.
- Steimel, J.; Engelbrecht, C.J.B. and Harrington, T.C. (2004) Development and characterization of microsatellite markers for the fungus *Ceratocystis fimbriata*. *Molecular Ecology Notes* 4: 215-218.

- Sugimura, T. and Tai, A. (1994) Total synthesis of (+)-ipomeamarone and (-)-ngaione via novel intramolecular hydrogen abstraction. *Tetrahedron* 50 (40): 11647-11658.
- Surak, J.G. (1978) Phytolaexins and human health. *Proceedings of the Florida State Horticultural Society* 91: 256-258.
- Suzuki, M.T.; Lereclus, D.; Olivia M.N. A. (2004) Fate of *Bacillus thuringiensis* strains in different insect larvae. *Canadian Journal of Microbiology* 50 (11): 973-975.
- Talekar, N.S. (1991) Integrated control of *Cylas formicarius*. In eds. Jansson, R.K. and Raman, K.V. Sweetpotato pest management: A global perspective. Westview Press, Inc. Colorado, USA Pg. 139-156.
- Tamura K.; Peterson D.; Peterson N.; Stecher G.; Nei M. and Kumar S. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. *Molecular Biology Evolution* doi: 10.1093/molbev/msr121.
- Tamura K.; Nei M. and Kumar S. (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences* 101: 11030-11035.
- Tang, W.; Newton, R.J. and Weidner, D.A. (2007) Genetic transformation and gene silencing mediated by multiple copies of a transgene in eastern white pine. *Journal of Experimental Botany* 58 (3): 545-554.
- Thomas, J.C. and Katterman, F.R. (1986) Cytokinin activity induced by thidiazuron. *Plant Physiology* 81: 681-683.
- Thottapilly, G. (2009) Introductory remarks. In eds Loebenstein, G. and Thottapilly, G. The Sweetpotato Chap. 2 The Sweetpotato. Springer Science Pg. 3-8.
- Tohidfar, M.; Zare, N.; Jouzani, G.S. and Eftekhari, S.M. (2013) *Agrobacterium*-mediated transformation of alfalfa (*Medicago sativa*) using a synthetic *cry3a* gene to enhance resistance against alfalfa weevil. *Plant Cell, Tissue and Organ Culture* 113: 227-235.
- Torrey, J.G. (1976) Root hormones and plant growth. *Annual Review of Plant Physiology* 27: 435-459.
- Treatise, A. (1972) Plant Physiology. New York. Academic Press Pg. 4-272.

- Tripathi, L.; Mwaka, H.; Tripathi, J.N. and Tushemereirwe, W.K. (2010) Expression of sweet pepper *Hrap* gene in banana enhances resistance to *Xanthomonas campestris* pv. *musacearum*. *Molecular Plant Pathology* 11 (6): 721-731.
- Uritani, I. (1999) Biochemistry on postharvest metabolism and deterioration of some tropical tuberous crops. *Botanical Bulletin Academia Sinica* 40: 177-183.
- Vancanneyt, G.; Schmidt, R.; O'Connor-Sanchez, A; Willmitzer, L and Rocha-Sosa, M. (1990) Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. *Molecular Genetics and Genomics* 220 (2): 245-50.
- Vidhyasekaran, P. (2008). Molecular biology and host defence mechanisms. 2<sup>nd</sup> ed. Taylor and Francis Group, Boca Raton, FL. USA Pg. 427.
- Villordon, A.; LaBonte, D.; Solis, J. and Firon, N. (2012) Characterization of lateral root development at the onset of storage root initiation in 'Beauregard' sweetpotato adventitious roots. *HortScience* 47 (7): 961-968.
- Von Arnold. (2008) Somatic embryogenesis. In eds. George, E.F.; Hall, M.A. and de Klerk, G.J. *Plant Propagation by Tissue Culture 3<sup>rd</sup> Edition Volume 1. The Background*. Springer, Dordrecht, The Netherlands. Pg. 335-354.
- Wang, S.; Lan, Y.; Cheng, S.; Chen, Y. and Yeh, K. (2002) Wound-response regulation of the sweetpotato sporamin gene promoter region. *Plant Molecular Biology* 48 (3): 223-231.
- White, T.J.; Bruns, T.; Lee, S. and Taylor, J.W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In, *PCR Protocols: A Guide to methods and applications*, eds. Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White. Academic Press, Inc., New York Pg. 315-322.
- Wilson, B.J. and Burka, L.T. (1979) Toxicology of novel sesquiterpenoids from the stressed sweetpotato (*Ipomoea batatas* L. Lam). *Food Cosmetics and Toxicology* 17: 353-355.
- Wilson, B. J.; Yang, D. T. C. and Boyd, M. R. (1970) Toxicity of mould-damaged sweet potatoes. (*Ipomoea batatas*). *Nature* 227 (5257): 521-522.

- Xing, Y.; Yang, Q.; Ji, Q.; Luo, Y.; Zhang, Y.; Gu, K. and Wang, D. (2007) Optimization of *Agrobacterium*-mediated transformation parameters for sweetpotato embryogenic callus using  $\beta$ -glucuronidase (GUS) as a reporter. *African Journal of Biotechnology* 6 (22): 2578-2584.
- Yang, J.; Bi, H.; Fan, W.; Zhang, M.; Wang, H.; Zhang, P. (2011) Efficient embryogenic suspension culturing and rapid transformation of a range of elite genotypes of sweet potato (*Ipomoea batatas* L. Lam.). *Plant Science* 181: 701-711.
- Yanggen, D. and S. Nagujja. (2006) The use of orange-fleshed sweetpotato to combat vitamin A deficiency in Uganda. A study of varietal preferences, extension strategies and post-harvest utilization. CIP, Lima, Peru. 80 p.
- Yasuda, K. and Kojima. M. (1986) The role of stress metabolites in establishing host-parasite specificity between sweetpotato and *Ceratocystis fimbriata* black rot fungus. *Agricultural and Biological Chemistry Journal* 50 (7): 1839-1846.
- Yildirim, M.; Bahar, B.; Genc, I.; Hatipoglu, R. and Atis. (2008) Relations among anther culture ability and some field based characters in wheat. *American-Eurasian Journal of Agricultural and Environmental Science* 4 (1): 30-33.
- Yip, W and Yang, S.F. (1986) Effect of thidiazuron, a cytokinin-active urea derivative, in cytokinin-dependent ethylene production systems. *Plant Physiology* 80: 515-519.
- Yoo, S.Y.; Bomblies, K.; Yoo, S.K.; Yang, J.W.; Choi, M.S.; Lee, J.S.; Weigel, D. and Ahn, J.H. (2005) The 35S promoter used in a selectable marker gene of a plant transformation vector affects the expression of the transgene. *Planta: An International Journal of Plant Biology*. Springer-Verlag. 10.1007/s00425-004-1466-4.
- Yookongkaew, N; Srivatanakul, M. and Narangajavana, J. (2007) Development of genotype-independent regeneration system for transformation of rice (*Oryza sativa* ssp. indica). *Journal of Plant Research* 120: 237-245.
- Yu, T.A.; Yeh, S.D. and Yang, J.S. (2003) Comparison of the effects of kanamycin and geneticin on regeneration of papaya from root tissue. *Plant Cell, Tissue and Organ Culture* 74: 169-178.

- Zang, N.; Zhaia, H.; Gaoa, S.; Chena, W.; Hec, S. and Liu, Q. (2009) Efficient production of transgenic plants using the bargene for herbicide resistance in sweetpotato. *Scientia Horticulturae* 122 (4): 649-653.
- Zalewski, W.; Orczyk, W.; Gasparis, S. and Nadolska-Orczyk, A. (2012) *HvCKX2* gene silencing by biolistic or *Agrobacterium*-mediated transformation in barley leads to different phenotypes. *Biomedical Central Plant Biology* 12 (206): 1-12.
- Zhang, L.; Wang, Q. Liu, Q. and Wang, Q. (2009) Sweetpotato in China. In eds. Loebenstein, G. and Thottappilly, G., *The Sweetpotato* Chap. 15 Pg. 325-358.
- Zimmermann, M.C.; Tilghman, S.L.; Boue, S.M.; Salvo, V.A.; Elliott, S.; Williams, K.Y.; Skripnikova, E.V.; Ashe, H.; Payton-Stewart, F.; Vanhoy-Rhodes, L.; Fonseca, J.P.; Corbitt, C.; Collins-Burow, B.M.; Howell, M.H.; Lacey, M.; Shih, B.Y.; Carter-Wientjes, C.; Cleveland, T.E.; McLachlan, J.A.; Wiese, T.E.; Beckman, B.S. and Burow. M.E. (2010) Glyceollin I, a Novel anti-estrogenic phytoalexin isolated from activated soy. *Journal of Pharmacology and Experimental Therapeutics* 332 (1): 35-45.

## APPENDICES:

## Appendix 1: Regression analysis of different explant types

Response variate: Regenerants  
 Binomial totals: Total  
 Distribution: Binomial  
 Link function: Logit  
 Fitted terms: Explant

## Summary of analysis

| Source     | d.f. | deviance | mean<br>deviance | deviance<br>ratio | approx<br>F pr. |
|------------|------|----------|------------------|-------------------|-----------------|
| Regression | 3    | 4.726    | 1.5754           | 1.96              | 0.174           |
| Residual   | 12   | 9.653    | 0.8044           |                   |                 |
| Total      | 15   | 14.379   | 0.9586           |                   |                 |
| Change     | -4   | -17.033  | 4.2583           | 5.29              | 0.011           |

## Summary statistics for regenerants

Mean = 1.938  
 Median = 1.5  
 Standard deviation = 1.124  
 Standard error of mean = 0.281  
 Variance = 1.262  
 Standard Error of Variance = 0.305  
 Coefficient of variation = 57.99

**Appendix 2:** Regression analysis of selected 7 SSA cultivars in regeneration on 0.2 mg/l kinetin

Response variate: Reg  
 Binomial totals: Total  
 Distribution: Binomial  
 Link function: Logit  
 Fitted terms: Cultivar

**Summary of analysis**

| Source     | d.f. | deviance | mean deviance | deviance ratio | approx F pr. |
|------------|------|----------|---------------|----------------|--------------|
| Regression | 6    | 67.21    | 11.202        | 7.14           | <.001        |
| Residual   | 48   | 75.35    | 1.570         |                |              |
| Total      | 54   | 142.56   | 2.640         |                |              |
| Change     | -7   | -75.44   | 10.777        | 6.87           | <.001        |

**Accumulated analysis of deviance**

| Change          | d.f. | deviance | mean deviance | deviance ratio | approx F pr. |
|-----------------|------|----------|---------------|----------------|--------------|
| - Constant      | -1   | -2.029   | 2.029         | 1.25           | 0.266        |
| + Cultivar      | 6    | 30.584   | 5.097         | 3.15           | 0.008        |
| + Conc          | 2    | 37.476   | 18.738        | 11.57          | <.001        |
| + Cultivar.Conc | 5    | 16.247   | 3.249         | 2.01           | 0.086        |
| Residual        | 82   | 132.781  | 1.619         |                |              |
| Total           | 94   | 215.059  | 2.288         |                |              |

**Accumulated analysis of deviance**

| Change             | d.f. | deviance | mean deviance | deviance ratio | approx chi pr |
|--------------------|------|----------|---------------|----------------|---------------|
| - Constant         | -1   | -2.029   | 2.029         | 2.03           | 0.154         |
| + Cultivar.Country | 6    | 30.584   | 5.097         | 5.10           | <.001         |
| Residual           | 89   | 186.504  | 2.096         |                |               |
| Total              | 94   | 215.059  | 2.288         |                |               |

**Summary statistics for RE (%)**

Mean = 52.98  
 Median = 50  
 Standard deviation = 27.93  
 Standard error of mean = 2.865  
 Standard Error of Variance = 77.10  
 Coefficient of variation = 52.71

**Appendix 3: Accumulated analysis of deviance of calli color and cultivar**

Response variate: RE  
 Distribution: Poisson  
 Link function: Log  
 Fitted terms: Constant, Calli color

## Summary of analysis

| Source     | d.f. | deviance | deviance | ratio | F pr. |
|------------|------|----------|----------|-------|-------|
| Regression | 13   | 88.9     | 6.8397   | 10.54 | <.001 |
| Residual   | 245  | 159      | 0.649    |       |       |
| Total      | 258  | 247.9    | 0.9609   |       |       |

**Appendix 4:** Regression analysis for kinetin 0.2 mg/l for all cultivars

Response variate: RE  
 Binomial totals: Total  
 Distribution: Binomial  
 Link function: Logit  
 Fitted terms: Cultivar, kinetin

## Summary of analysis

| Source     | d.f. | deviance | mean<br>deviance | deviance<br>ratio | approx<br>F pr. |
|------------|------|----------|------------------|-------------------|-----------------|
| Regression | 21   | 141.5    | 6.736            | 5.59              | <.001           |
| Residual   | 91   | 109.6    | 1.204            |                   |                 |
| Total      | 112  | 251.1    | 2.242            |                   |                 |
| Change     | 0    | 0.0      | *                |                   |                 |

## Accumulated analysis of variance

| Change             | d.f. | s.s.      | m.s.     | v.r.   | F pr. |
|--------------------|------|-----------|----------|--------|-------|
| - Constant         | -1   | -234712.4 | 234712.4 | 630.82 | <.001 |
| + Cultivar.Country | 22   | 289752.3  | 13170.6  | 35.40  | <.001 |
| Residual           | 91   | 33858.8   | 372.1    |        |       |
| Total              | 112  | 88898.7   | 793.7    |        |       |

## Summary statistics for RE

Mean = 45.58  
 Median = 33.33  
 Standard deviation = 28.17  
 Standard error of mean = 2.650  
 Variance = 793.7  
 Standard Error of Variance = 78.14  
 Coefficient of variation = 61.82

**Appendix 5:** Regression analysis of zeatin for 0.2 mg/l for all cultivars

Response variate: RE  
 Binomial totals: Total  
 Distribution: Binomial  
 Link function: Logit  
 Fitted terms: Cultivar, zeatin

## Summary of analysis

| Source     | d.f. | mean<br>deviance | deviance<br>deviance | approx<br>ratio | F pr. |
|------------|------|------------------|----------------------|-----------------|-------|
| Regression | 20   | 81.23            | 4.0615               | 4.48            | <.001 |
| Residual   | 90   | 81.56            | 0.9063               |                 |       |
| Total      | 110  | 162.79           | 1.4799               |                 |       |
| Change     | -1   | 0.00             | 0.0000               | 0.00            | 1.000 |

Dispersion parameter is estimated to be 0.906 from the residual deviance.

## Accumulated analysis of variance

| Change             | d.f. | s.s.      | m.s.     | v.r.   | F pr. |
|--------------------|------|-----------|----------|--------|-------|
| - Constant         | -1   | -209792.6 | 209792.6 | 371.91 | <.001 |
| + Cultivar.Country | 19   | 300077.2  | 15793.5  | 28.00  | <.001 |
| Residual           | 56   | 31589.5   | 564.1    |        |       |
| Total              | 74   | 121874.1  | 1646.9   |        |       |

## Summary statistics for RE (%)

Mean = 52.89  
 Median = 33.33  
 Standard deviation = 40.58  
 Standard error of mean = 4.686  
 Variance = 1647  
 Standard Error of Variance = 287.3  
 Coefficient of variation = 76.73

**Appendix 6:** Regression analysis of thidiazuron for 0.2 mg/l for all cultivars

Response variate: RE  
 Binomial totals: Total  
 Distribution: Binomial  
 Link function: Logit  
 Fitted terms: Cultivar, thidiazuron

## Summary of analysis

| Source     | d.f. | deviance | mean<br>deviance | deviance<br>ratio | approx<br>F pr. |
|------------|------|----------|------------------|-------------------|-----------------|
| Regression | 18   | 71.64    | 3.9802           | 5.39              | <.001           |
| Residual   | 56   | 41.36    | 0.7386           |                   |                 |
| Total      | 74   | 113.00   | 1.5271           |                   |                 |
| Change     | 0    | 0.00     | *                |                   |                 |

**Appendix 7: ANOVA of variation between acetosyringone concentrations****Regression analysis**

Response variate: GUS\_stained  
 Binomial totals: Total  
 Distribution: Binomial  
 Link function: Logit  
 Fitted terms: Acetosyringone concentration

**Summary of analysis**

| Source     | d.f. | mean deviance | deviance | approx | F pr. |
|------------|------|---------------|----------|--------|-------|
| Regression | 3    | 63.93         | 15.983   | 13.11  | <.001 |
| Residual   | 45   | 54.86         | 1.219    |        |       |
| Total      | 49   | 118.79        | 2.424    |        |       |
| Change     | -5   | -76.01        | 15.203   | 12.47  | <.001 |

**Regression analysis of acetosyringone levels without 0 mg/l**

Response variate: GUS\_stain  
 Binomial totals: Total  
 Distribution: Binomial  
 Link function: Logit  
 Fitted terms: no acetosyringone

**Summary of analysis**

| Source     | d.f. | deviance | mean deviance | deviance ratio | approx F pr. |
|------------|------|----------|---------------|----------------|--------------|
| Regression | 2    | 2.89     | 1.446         | 1.17           | 0.327        |
| Residual   | 27   | 33.52    | 1.241         |                |              |
| Total      | 29   | 36.41    | 1.256         |                |              |

**Summary statistics for the acetosyringone concentrations (%)**

Mean = 82.22

Median = 83.33

Standard deviation = 16.34

Standard error of mean = 2.983

Standard Error of Variance = 82.87

Coefficient of variation = 19.87

**Appendix 8: ANOVA for kanamycin levels and 6 SSA cultivars**

| Change                  | d.f. | s.s.     | m.s.    | v.r.   | F pr. |
|-------------------------|------|----------|---------|--------|-------|
| Constant                | -1   | -29299.5 | 29299.5 | 151.32 | <.001 |
| Cultivar                | 5    | 36546.6  | 7309.3  | 37.75  | <.001 |
| Kanamycin concentration | 3    | 5863.1   | 1954.4  | 10.09  | 0.001 |
| Residual                | 12   | 2323.6   | 193.6   |        |       |
| Total                   | 19   | 15433.7  | 812.3   |        |       |

**Summary statistics for kanamycin concentration (%)**

|                              |       |
|------------------------------|-------|
| Mean =                       | 46.65 |
| Median =                     | 33.33 |
| Lower quartile =             | 16.67 |
| Upper quartile =             | 66.67 |
| Standard deviation =         | 30.85 |
| Standard error of mean =     | 2.409 |
| Standard Error of Variance = | 72.99 |
| Coefficient of variation =   | 66.13 |

**Appendix 9:** Accumulated analysis of variance of feeding holes between samples

| Change    | d.f. | s.s.      | m.s.     | v.r.   | F pr. |
|-----------|------|-----------|----------|--------|-------|
| -Constant | -1   | -100489.0 | 100489.0 | 305.90 | <.001 |
| +Sample   | 3    | 102627.0  | 34209.0  | 104.14 | <.001 |
| +Reps     | 2    | 254.0     | 127.0    | 0.39   | 0.702 |
| Residual  | 4    | 1314.0    | 328.5    |        |       |
| Total     | 8    | 3706.0    | 463.3    |        |       |

**Appendix 10:** Analysis of an unbalanced design using GenStat regression for ipm**Variate:** Ipm concentration

Analysis of variance

|          | d.f. | s.s.      | m.s.     | v.r. | F pr. |
|----------|------|-----------|----------|------|-------|
| Change   |      |           |          |      |       |
| Cultiva  | 4    | 10661411. | 2665353. | 9.87 | <.001 |
| Residual | 36   | 9721948.  | 270054.  |      |       |
| Total    | 40   | 20383358. |          |      |       |

**Appendix 11: ANOVA for *ipm* in slices from controlled inoculation**

Analysis of an unbalanced design using GenStat regression

**Variate:** Concentration

Accumulated analysis of variance

| Change        | d.f. | s.s.     | m.s.    | v.r. | F pr. |
|---------------|------|----------|---------|------|-------|
| + Sample      | 2    | 749426.  | 374713. | 2.75 | 0.124 |
| + Slices_1_cm | 4    | 589916.  | 147479. | 1.08 | 0.427 |
| Residual      | 8    | 1091223. | 136403. |      |       |
| Total         | 14   | 2430566. | 173612. |      |       |

**LIST OF PUBLICATIONS ACCRUING FROM THIS THESIS**

1. Ghislain, M.; Tovar, J.; Prentice, K.; Ormachea, M.; Rivera, C.; Manrique, S.; Kreuze, J.; Rukarwa, R.; Sefasi, A.; Mukasa, S.; Ssemakula, G.; **Wamalwa, N.L.** and Machuka, J. 2013. Weevil Resistant Sweetpotato through Biotechnology. *Acta Hort. (ISHS)* 974: 91-98
2. **Wamalwa, N.L.**; Cheseto, X.; Torto, B.; Ouna, E.; Maniania J.N.; Machuka, J.; Kaplan, F. and Ghislain, M. 2014. Evidence of high furanoterpenoid levels in sweetpotato storage roots. *J. Agri.l Food Chem.* 63 (1): 335-342
3. **Wamalwa, N.L.**; Tovar, J.C.; Prentice, K.; Kreuze, J.; Machuka, J. and Ghislain, M. Organogenesis and *Agrobacterium*-mediated Transformation of Several Sub-Saharan Africa Sweetpotato Cultivars. *Under review*