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Regeneration and transformation systems for improving resistance to weevils in Ugandan sweetpotato cultivars

BY

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DECLARATION

I Abel Yoas Sefasi, declare that the work presented in this dissertation is my original research and has not been submitted for any other degree award at any University.

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DEDICATION

This dissertation is dedicated to my parents.

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ABSTRACT

Productivity of sweetpotato [*Ipomoea batatas* (L.) Lam.] in Sub-Saharan Africa is significantly reduced due to damage caused by weevils, *Cylas puncticollis* Boheman and *C. brunneus* Fabricius. The improvement of weevil resistance in sweetpotato using classical breeding has been limited because no sources of resistance have been identified in the crop germplasm. Additionally, the application of genetic transformation has been hampered since different cultivars respond differently to both regeneration and transformation conditions. The main objective of the reported research was to optimise regeneration and transformation conditions for recalcitrant Uganda sweetpotato cultivars in order to pave the way for genetic transformation with weevil resistance genes.

'Whole leaf' explants of twenty Ugandan sweetpotato cultivars were screened for their embryogenic response using a common auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) (0.2 mg/L) supplemented to Murashige and Skoog (MS) basal medium. Cultivar showed a highly significant ($P < 0.001$) effect on frequency of embryogenic callus and efficiency of both shoot and root regeneration. The best responding Ugandan cultivars Bwanjule, Kyebandula, Magabali, New Kawogo and Semanda were included in a follow-up study where three different auxins were investigated; 4-fluorophenoxyacetic acid (4FA) (1 mg/L), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (1.3 mg/L) and 2,4-D. This experiment was laid in a completely randomised design. The three factors namely auxin type, plant organ and cultivar had a significant ($p \leq 0.05$) effect on the frequency of embryogenic callus. 2,4-D was found to be the best ($p \leq 0.05$) auxin for induction of embryogenic callus while cultivar Bwanjule had the highest (20.2%) embryogenic callus frequency. Although some Ugandan cultivars demonstrated high ability to regenerate roots, shoot regeneration was restricted to non-African cultivars.

Cultivars, Kyebandula and Bwanjule, were further investigated on medium supplemented with different levels of thidiazuron (0.5, 2.0 and 4.0 μM) and 0.25 μM α-Naphthalene acetic acid. The best response ($P < 0.001$) was from stem explants of cv. Kyebandula. The conversion of adventitious buds into shoots was improved significantly ($P < 0.001$) when thidiazuron (TDZ) was reduced or completely removed in subsequent stages of culture.

Stem internode pieces excised from internode position 3 from the apex were the best (70.0 %) in adventitious bud formation.

An efficient *Agrobacterium*-mediated transformation method was developed using readily accessible explants of cv. Kyebandula. Transformation efficiency was optimized by screening for transient β-glucuronidase (GUS) expression using histochemical localization of GUS activity after co-culture of primary roots, petioles, whole leaves and stem internode segments under various conditions. Concentration of *Agrobacterium* at an optical density (OD) of 1.0 at 600 nm gave significantly ($p < 0.001$) high transformation frequency for all the sweetpotato organs. The best ($p < 0.001$) transformation results were obtained when explants were co-cultured for 4 days. Kanamycin concentration of 100 mg/L significantly ($p < 0.001$) inhibited survival of non-transformed explants and callus.

When coupled with the established thidiazuron-based regeneration protocol, the optimized transformation conditions led to regeneration of plants from explants co-cultured with *Agrobacterium* strain EHA 105. This *Agrobacterium* harbours the pCIP84 plasmid construct which contains *cry7Aa1, cry3Ca1* and *nptII* genes in its transferred DNA (T-DNA). PCR for *cry7Aa1* gene gave an early indication of transformation for 10 randomly selected plants among the regenerated 18 plants. Six of these plants showed that they were transformed with *cry7Aa1* gene representing a 2.0 % transformation efficiency. Southern hybridization and Northern blot are the next steps to fully determine the transgenic nature of the plants. Taken together, the present data demonstrate that it is possible to genetically transform recalcitrant cultivars, including important African cultivars.

Key words: *β*-glucuronidase, genetic transformation, *in vitro* recalcitrance, sweetpotato, weevil resistance

PUBLICATIONS FROM THIS STUDY

Part of the work under this thesis has been prepared into papers/manuscripts as shown below:

1. **Sefasi, A**., Ghislain, M., Kiggundu, A., Ssemakula, G., Rukarwa, R. and Mukasa, S.B. (2013). Embryogenenic callus and root regeneration induced by 2,4-dichlorophenoxyacetic acid in sixteen African sweetpotato cultivars. (Manuscript).

2. **Sefasi, A.**, Kiggundu, A., Kreuze. J., Ghislain, M., Manrique, S., Ssemakula, G. and Mukasa, S. (2012). Induction of somatic embryogenesis in recalcitrant sweetpotato (*Ipomoea batatas* L.) cultivars. *African Journal of Biotechnology* **11**:16055-16064.

3. **Sefasi, A.**, Ghislain, M., Kiggundu, A., Ssemakula, G. and Mukasa, S. B. (2013). Thidiazuron improves adventitious bud and shoot regeneration in recalcitrant sweetpotato. *African Crop Science Journal* **21**: 85-95.

4. **Sefasi, A.**, Ghislain, M., Ssemakula, G., Prentice, K., Kiggundu, A., Mwanga, R. and Mukasa, S.B. (2013). Transient expression of *β*-glucuronidase in recalcitrant Ugandan sweetpotato and putative transformation with two *cry* genes. (Manuscript).

CHAPTER ONE

INTRODUCTION

1.1   Origin and distribution of sweetpotato

Sweetpotato (*Ipomoea batatas* (L.) Lam.) is cultivated as an annual crop although it is a perennial plant (Janssens, 2001). The sweetpotato genus *Ipomoea* is in the *Convolvulaceae* botanical family (Austin, 1988). Sweetpotato is not found in the wild although it is estimated to have more than 400 relatives in the wild (Woolfe, 1991). Most wild relatives of sweetpotato are diploids (2x=30) and two are tetraploids. However sweetpotato is a hexaploid, although a (wild) tetraploid cytotype (2n=4x=60) was identified in Ecuador (Bohac *et al.,* 1993).

It is widely believed that sweetpotato originated from a cross between the ancestors of *Ipomoea trifida* and another wild *Ipomoea* species in Central America or northern part of South America (Jarret *et al.,* 1984). *I. trifida* (2x) and *I. tabascana* (4x) are the most closely related species to sweetpotato (Rajapakse *et al.,* 2004; Srisuwan *et al.,* 2006). The tetraploid (4x) form has been suggested to have originated from the crossing of two diploid wild relatives *I. triloba* (A genome) and *I. trifida* (B genome), and the hexaploid (6x) through hybridization of a diploid species with an unknown tetraploid, with subsequent spontaneous chromosome doubling (Yen, 1976). Another hypothesis suggests that sweetpotato originated through autopolyploidy of *I. trifida* or an unknown species (Shiotani, 1988).

1.2   Economic importance of sweetpotato

Sweetpotato is mainly cultivated for its expanded edible roots which contain high carbohydrate and beta-carotene. In sub-Saharan Africa, the roots can be boiled, steamed, baked or fried. Sweetpotato roots are also canned or dried and made into flour, cereal and noodles (Woolfe, 1991). In some countries the roots are processed to produce a wide variety of products: alcoholic and non-alcoholic beverages, sweet desserts, snacks, a variety of convenience processed products, fast foods, multipurpose flour, starch, animal feeds and basic industrial raw material (Woolfe, 1991). The crop has high potential to

reduce hunger, malnutrition and poverty since it gives better and early yield with less input (Lim *et al.,* 2007).

Sweetpotato is considered as the most important species of the *Ipomoea* genus. The total area of sweetpotato cultivation in the world is about 8.2 million hectares, with production of 102 million tons and an average yield of 12.1 tons/ha (FAOSTAT, 2010). More than 95 % of the world area dedicated to sweetpotato is in developing countries. China grows more than 80 % of the world's sweetpotato production, followed by Uganda. Sweetpotato ranks seventh among all crops in the world in terms of production (Kays, 2005). It produces more biomass and nutrients per hectare than any other food crop. In the tropics, sweetpotato ranks fifth in terms of caloric value contribution (Reddy *et al.,* 2007). In Africa sweetpotato is the second most important root crop after cassava. Uganda is the biggest producer of sweetpotato in Africa (FAOSTAT, 2010) and third in the world at an annual production of 1.8 million tons (FAOSTAT, 2010).

Fresh sweetpotato roots provide about 50 % more calories than irish potatoes (*Solanum tuberosum* L.) (Baybutt and Molteni, 2000). In addition, sweetpotato provides fiber, iron, potassium, ascorbic acid and the amino acid lysine, which is absent in most common foods such as rice. The yellow- and orange-fleshed varieties of sweetpotatoes are high in beta-carotene, which can be converted into vitamin A in the intestines and liver (Low *et al.,* 2007). The crop is also considered a dual-purpose crop, since the storage roots are consumed fresh or cooked and the vines and leaves are eaten as a green vegetable. Sweetpotato leaves rank as one of the healthiest vegetables, because of high levels of vitamins A and C, iron, potassium, and fiber (Baybutt and Molteni, 2000). In addition, both the roots and vines can be used as feed in either fresh or dried form. They can be fed to both ruminants and non-ruminants (Woolfe, 1991).

Because of its tolerance to a wide range of agro-ecological conditions, high yield potential, and high nutritive value, sweetpotato is grown in both tropical and temperate zones (Woolfe, 1991). However the potential production for Africa is not attained as yields are very low at about 5 t/ha (Luo *et al.,* 2006). The low yields are mainly due to various challenges including high incidence of virus disease and increasing attacks by sweetpotato weevils (Fuglie, 2007).

1.3 Constraints to sweetpotato productivity

Although the inherent yield of sweetpotato is up to 80 tons per ha (Woolfe, 1991), the yields achieved by farmers in Africa is about 5 tons per hectare, while the world's average yield is 15 tons per hectare (Luo *et al.,* 2006). The yields in Asia are significantly higher, averaging 12.41 tons/ha while in South America the average yield is 10.74 tons/ha. In contrast, the average yield in the USA is 20.1 tons/ha (FAOSTAT, 2007). The low and variable yields in sub-Saharan Africa are mainly due to poor quality of the propagation material and attacks by various diseases and pests (Namanda *et al.,* 2011). In East Africa, sweetpotato featherly mottle virus (SPFMV) occurs together with sweetpotato chlorotic stunt virus (SPCSV), causing the devastating sweetpotato virus disease (SPVD) (Mukasa *et al.,* 2006; Njeru *et al.,* 2008). Sweetpotato is generally resistant to other diseases although some leaf diseases can infect it when conditions favour their development. Such diseases include leaf blight (*Alternaria* spp.), leaf spot (*Cercospora* spp.), bacterial wilt (*Pseudomonas solanacearum*), and leaf scab (*Elsinoe batatas*).

Sweetpotato productivity is also constrained by insect pests like weevils, white grubs, thrips, aphids, planthoppers, whiteflies, mites and bugs. Among these insect pests, the sweetpotato weevil which mainly feeds on the expanded roots of the plant has emerged as the most devastating pest in Africa (Fuglie, 2007). In Uganda, more than 28% of sweetpotato yield is lost due to weevil damage (Kiiza *et al.,* 2009). Losses of the entire crop due to weevil attacks in sub-Saharan Africa have been reported previously (Smit *et al.,* 2001). There are two common species of weevils attacking sweetpotato in Africa: *Cylas puncticollis* Boheman and *C. brunneus* Fabricius. The larval stage of weevil development causes the most damage by chewing its way into stems and exposed roots. The larva feeding is associated with loss in quality due to terpenoids and phenols produced by the plant in response to the weevil feeding. These compounds make the roots unpalatable for human and animal consumption. Hence, even low-level infestations can result in considerable loss in quality of roots.

Due to the rising production losses caused by weevils, the management of this pest was recently ranked as the highest priority for sweetpotato improvement in sub-Saharan Africa (Andrade *et al.,* 2009). Since most of the sweetpotato producers in Africa are small-scale,

resource-poor farmers (Bashaasha *et al.,* 1995), management of sweetpotato weevils relies on cultural control practices, with low levels of material inputs. Nevertheless most common control strategies based on integrated pest management (IPM) still face challenges in Africa due to the cryptic feeding habit of the weevil, the practice of piecemeal harvesting, the cultivation of small plots throughout the year and the lack of reliable weevil resistance among the sweetpotato cultivars. In addition, the use of sex pheromones has proved less effecitve in Uganda although the approach was shown to reduce attacks by *C. formicalius* in Cuba and other countries (Smit *et al.,* 2001). Thus, in sub-Saharan Africa (SSA) it would require a large scale approach for comprehensive IPM approaches to have an impact.

The larvae is the most destructive growth stage of the weevil. It forms tunnels while feeding within the sweetpotato stems or expanded roots. This nature of feeding inside roots renders the weevil difficult to control especially through the use of contact pesticides. The application of pesticides for control of weevils is also limited by low accessibility to chemicals by rural farmers of SSA (Stathers *et al.,* 2003). Furthermore, the use of chemicals in pest management is usually a last resort due to fears of food and environmental contamination.

Apart from poor access to insecticides, another unique feature of SSA farmers is that they usually only harvest what will be eaten or sold immediately. This practice implies that some plants always remain in the field thus rendering impractical the inclusion of field sanitation as a component of IPM. With these unique challenges in SSA, there is a greater need to develop alternative or additional technologies which would provide adequate and affordable crop protection. One of the most feasible strategies for the rural farmers in SSA is the deployment of plant resistance against weevils. Sweetpotato resistance to weevils is both economically and ecologically feasible under the conditions of cultivation in SSA. Furthermore, plant resistance has historically been considered as a critical component of IPM (Jansson and Raman, 1991).

1.4   Conventional breeding strategy to weevil control

Screening the adapted sweetpotato cultivars has revealed differences in damage caused by weevils, which may be governed by additive genetic factors. However, progress in

breeding weevil-resistant cultivars has been slow because the heritability of the trait is extremely low (Stevenson *et al.,* 2009). Some workers have reported that cultivars with high foliage weight show lower levels of weevil damage (Stathers *et al.,* 2003). High foliage causes a moist microclimate that SPW detest. Other factors such as high dry matter and starch contents have been associated with lower damage by weevils (Hahn and Leushner, 1981) although other workers found no evidence of this relationship (Anota and Odebiyi, 1984). These mixed results would suggest that continuing screening for tolerance in cultivars may generate interesting results in the future. However, stable resistance to weevil is yet to be found in the sweetpotato gene pool (Luo *et al.,* 2006; Stevenson *et al.,* 2009).

It is likely that any weevil resistance that exists is due to a combination of antibiosis, tolerance, escape and non-preference (Andrade *et al.,* 2009). Immunity to weevil infestation may not exist, but factors that adversely affect survival or development of *Cylas* spp may be at play (Stathers *et al.,* 2003). Under field conditions a great many factors could potentially affect the susceptibility of a cultivar to weevil damage, for instance maturation date, root depth, root shape, root arrangement, plant canopy and root attraction (Stathers *et al.,* 2003). Furthermore, Stevenson *et al.* (2009) suggested the possibility of both deterrent and toxic effects on weevil adults and larvae, respectively. However this group also demonstrated that the inheritance of the implicated root-latex chemicals is not undersood yet.

Apart from the lack of reliable weevil resistance in the sweetpotato germplasm, the crop poses many challenges to plant breeding due to its complexity (Xing *et al.,* 2008). It is a hexaploid (2n = 6x = 90) and has problems such as pollen sterility, cross incompatibility, poor seed germination and special physiological requirements for flowering (Dhir *et al.,* 1998). The crop is also highly heterozygous (Cervantes-Flores *et al.,* 2008).

1.5   Genetic transformation strategy

Genetic transformation could help overcome some of the biological complications of sweetpotato and the limitations of sexual hybridization since it is fairly more accurate in transferring target genes (Yang *et al.,* 2011). More importantly, genetic transformation

allows insertion and expression of specific genes, from related or unrelated species, into the genome of a target organism (Shimada *et al.,* 2006). This advantage is extended to the use of synthesized genes that have more similarities in codon usage to genes of the plant targeted for transformation (Ghislain *et al.,* 2008). Genetic transformation is especially advantageous for clonally propagated crops like sweetpotato, as the primary transformation event is after thorough characterization of the final transformed plant (Luo *et al.,* 2006). In addition, genetic transformation is most useful for introduction of traits like weevil resistance which have not been identified in the sweetpotato germplasm (Kreuze *et al.,* 2008). The need for increased efforts to exploit genetic transformation is more justified now after the discovery of three different Bt toxins (Cry7Aa1, cryET33/cryET34, Cry3Ca1) that have shown effectiveness in controlling sweetpotato weevils, *Cylas puncticollis* and *C. brunneus* (Ekobu *et al.,* 2010).

1.6   Problem statement

Genetic transformation of sweetpotato is underdeveloped as compared to other crops due to the difficulties in *de novo* plant regeneration from transformed explants or single cells (Okada *et al.,* 2001; Santa-Maria *et al.,* 2009; Song *et al.,* 2004). The crop is considered to be recalcitrant to both regeneration and transformation (Aloufa, 2002; González *et al.,* 2008; Yu *et al.,* 2007), especially African cultivars (Luo *et al.,* 2006; Moar *et al.,* 2007). The crop has high genetic variability which in turn causes different cultivars to have significant differences in their responses to *in vitro* regeneration (Islam *et al.,* 2002; Wang *et al.,* 1998). As a result, most groups recommend investigating more than one cultivar when developing regeneration and transformation protocols for cultivars that have not been regenerated before (Al-Mazrooei *et al.,* 1997; Dessai *et al.,* 1995; Santa-Maria *et al.,* 2009).

1.7   Justification

The protocols of regeneration that have shown potential for application in genetic transformation of sweetpotato are somatic embryogenesis (Anwar *et al.,* 2010; Kreuze *et al.,* 2008; Song *et al.,* 2004) and organogenesis (Gong *et al.,* 2005; Gosukonda *et al.,* 1995a; Luo *et al.,* 2006; Santa-Maria *et al.,* 2009). Both methods have advantages and disadvantages and it is best to attempt both approaches in experimenting with novel

cultivars. Organogenesis has shown to be easier to manipulate *in vitro* but most of the regenerated plants after transformation are non-transformed escapes surviving on selection medium (Luo *et al.,* 2006). On the contrary, an important advantage with somatic embryogenesis is that most of the regenerated plants surviving on selection media are transformed (Song *et al.,* 2004). However, somatic embryogenesis remains a difficult process to control for the regeneration of sweetpotato (Song *et al.,* 2004; Yang *et al.,* 2011; Yu *et al.,* 2007). Most somatic embryogenesis protocols are cultivar dependent, have low regeneration frequencies, and require long periods of culture and frequent media changes (Anwar *et al.,* 2010; Kreuze *et al.,* 2008; Yu *et al.,* 2007).

Apart from the large variations in regeneration efficiency among sweetpotato cultivars, the transformation with *Agrobacterium tumefaciens*is is highly complex (Valentine, 2003; Xing *et al.,* 2008). However, despite its biological complexity, *Agrobacterium*-mediated genetic transformation remains more popular than particle bombardment because it does not involve sophisticated equipment, and it usually results in intact and single copy transgene integrations (Gustavo *et al.,* 1998; Yang *et al.,* 2011). Due to the biological complexity of genetic transformation with *Agrobacterium* species, the optimisation of factors known to influence *de novo* regeneration need to be coupled with establishment of optimum conditions for genetic transformation (Valentine, 2003; Xing *et al.,* 2008; Yu *et al.,* 2007). The present study was therefore undertaken with the aim of developing *in vitro* regeneration and transformation systems for some popular African sweetpotato cultivars that have not been reported in previous studies.

1.8   Objectives of the study

The main objective of this study was to develop a protocol for regeneration and genetic transformation that could enable transformation of selected Ugandan sweetpotato cultivars with weevil-resistance genes.

The specific objectives were:

1. To determine the ability of twenty Ugandan sweetpotato cultivars to form embryogenic callus
2. To develop a protocol for producing high frequency of embryogenic callus for application in regeneration of popular Ugandan sweetpotato cultivars

3. To study the effect of TDZ on morphogenesis of African sweetpotato cultivars with the aim of inducing adventitious shoots.

4. To develop an *Agrobacterium*-mediated transformation system based on transient expression of *β*-glucuronidase (GUS) as a reporter gene.

The hypotheses of the study were:

1. Sweetpotato somatic mebryogenesis and organogenesis depends on genotype, plant organ and concentration of growth hormone used in tissue culture

2. Genetic transformation is affected by plant genotype, condition of plant organ used as explants and duration of co-cultivation after infection with *Agrobacterium*

3. The transformation frequency of sweetpotato explants can be demonstrated with transient expression of *β*-glucuronidase (GUS)

CHAPTER TWO

LITERATURE REVIEW

2.1   General introduction

Apart from the dip-flower method for *Arabidopsis thaliana*, genetic transformation of plants requires an efficient method to regenerate shoots from *in vitro* tissue culture (Luo *et al.*, 2006; Tovar *et al.*, 2009). Infiltration of *Agrobacterium* into parts of whole plants has been proposed as a means of transforming higher plants without the need for a regeneration step but this has not been experimentally proven to be effective (Mahfouz and Li, 2011). In addition, such a method would be very difficult to apply in vegetatively propagated crops like sweetpotato where plants with both transformed and non-transformed sections would be a common result (Gong *et al.*, 2005). Therefore the ability to regenerate a whole plant from isolated plant cells or tissues which have been genetically transformed remains a major component of most plant transformation systems, and can often prove to be the most challenging and critical aspects of a plant transformation protocol (Sivparsad and Gubba, 2012). In this regard, the availability of a method for regenerating plants from cultivars that have not been reported to be amenable to regeneration is a preliquisite (Triqui *et al.*, 2007; Yang *et al.*, 2011).

2.2   *De novo* plant regeneration

Every cell in an organism contains all the genetic information which was present in the first single cell; the zygote. Additionally, single plant cells have been shown to be totipotent; possessing the capacity to form a complete plant identical to the mother plant (Slater *et al.*, 2008). In this case if the original cell is genetically transformed then the resulting plant after regeneration will carry the transgene in all its cells (Gong *et al.,* 2005; Kreuze *et al.,* 2008). The process of plant regeneration consists of many aspects that are induced by perception of plant growth regulators. In general, three phases of organogenesis are recognizable, on the basis of temporal requirement for a specific balance of plant growth regulators in the control of organogenesis. In the first phase, cells in the explants acquire 'competence' which is defined as the ability (not capacity) to respond to hormonal signals of organ induction. The process of acquisition of organogenic competence is referred to as 'dedifferentiation'. The competent cells in cultured explants are canalized

and determined in the second phase, for specific organ formation under the influence of plant growth regulators. Then the morphogenesis proceeds independently of the exogenously supplied plant growth regulators during the third phase (George *et al.,* 2008).

Generally, the regeneration of plants after transformation can be achieved through either organogenesis or embryogenesis, but not both (Carman, 1990). While the response of sweetpotato cultivars to the organogenesis protocols is better than to embryogenesis, the higher efficiency of selection of transformed plants (often 100 %) obtained during embryogenesis may be the underlying factor why this regeneration method is more popular (Luo *et al.,* 2006; Song *et al.,* 2004). A critical disadvantage of embryogenesis protocols, however, is that they are generally lengthy, often requiring eight months or more from beginning of the experiment before transgenic plants are obtained. However the fact that sweetpotato is a clonally propagated plant also determines preference towards somatic embryogenesis which ensures that plants originate from single cells and thus avoids the presence of chimeric vines (Gong *et al.*, 2005).

2.2.1   Shoot organogenesis in sweetpotato

Organogenesis is the production of organs, either directly from an explant or from a callus culture without the production of somatic embryos. Organogenesis relies on the inherent plasticity of plant tissues, and is regulated by altering the components of the medium (Slater *et al.*, 2008). It is usual to induce shoot formation by increasing the cytokinin to auxin ratio of the culture medium. Organogenic shoots can be induced directly from explants or indirectly from an intervening callus. These shoots can then be rooted relatively simply. The first success with transforming sweetpotato was obtained using organogenesis protocol without antibiotic selection (Dodds *et al.,* 1991b). The plants were obtained by adventitious shoot (organogenesis) formation from hairy roots after infection with *Agrobacterium rhizogenes*.

Direct shoot organogenesis in sweetpotato explants has been demonstrated in many studies (Gong *et al.,* 2005; Gosukonda *et al.,* 1995a; Santa-Maria *et al.,* 2009). Regeneration efficiencies ranged from 2 to 93% depending on the cultivar. Cytokinin in the first-stage medium is not critical and Dessai *et al.* (1995) used a modified protocol with only 2,4-D in the first medium. They achieved 10–83% regeneration efficiency with 15 out of the 25

tested cultivars. The optimal type of auxin and cytokinin used in each stage of the protocol may vary between cultivars. Treatment with the auxin 4-fluorophenoxyacetic acid (4-FA) for 5 to 6 days followed by the cytokinin zeatin gave satisfactory results for regeneration of cultivar Jewel (Luo *et al.,* 2006).

Using a two-stage (auxin-cytokinin) protocol, Luo *et al.* (2006) found that leaf-disks formed only non-regenerative compact callus. They also tested stem internode pieces, which regenerated shoots with a high efficiency but all were non-transgenic. Whole leaves (including petiole) produced 57 regenerants out of the 160 explants tested and 15 regenerants were confirmed to be transgenic. In their experiments in which the most efficient hormone combination (4FA-zeatin) was used, the transformation efficiency was 10–20% and plantlets were obtained within 6–10 weeks after inoculation with *Agrobacterium*. Low selection efficiency with 60–85% of regenerants representing escapes was however also observed. The presence of escapes on selection medium is a major disadvantage of organogenesis protocols (Gong *et al.,* 2005). However, despite this disadvantage, even in the highly responsive sweetpotato genotypes, the shorter time required to regenerate plants as compared to somatic embryogenesis protocols makes organogenesis a more attractive regeneration method (Luo *et al.,* 2006; Santa-Maria *et al.,* 2009).

2.2.2 Somatic embryogenesis in sweetpotato

During the process of evolution, many plants have evolved different methods of asexual embyogenesis, including somatic embryogenesis, to overcome various environmental and genetic factors that prevent fertilization. Somatic embryogenesis is a process whereby somatic cells differentiate into somatic embryos (George *et al.,* 2008). Somatic embryos resemble zygotic embryos morphologically. They are bipolar and bear typical embryonic organs. The embryo-like structures from somatic cells can develop into whole plants in a way analogous to zygotic embryos. *S*omatic embryos can be developed either from callus (indirect) or directly from the explant without any intermediate callus stage (George *et al.,* 2008). Direct somatic embryogenesis has better applicability in the improvement of crops since plant regeneration from callus cultures is often associated with somaclonal variations (Chee *et al.,* 1989; Greco *et al.,* 1984). However, direct somatic embryogenesis is not common (George *et al.,* 2008).

The callus formed is either embryogenic or non-embryogenic (Anwar *et al.,* 2010). Embryogenic callus is composed of pro-embryogenic masses (PEMs) which have potential to form somatic embryos. Indirect somatic embryogenesis is better understood with the process of secondary embryogenesis. Secondary embryogenesis is termed continuous, recurrent or accessory, when the first formed somatic embryo fails to develop into a plant but instead gives rise to successive cycles of embryos, secondary, tertiary and so on (George *et al.,* 2008). This can be an advantage if many plants are required, but is also difficult to halt and convert the somatic embryos into plants. Abscisic acid has been deployed in sweetpotato embryogenesis to halt this process, and thus indirectly leading to regeneration of plants (Anwar *et al.,* 2010; Song *et al.,* 2004). Because of the difficulty in strictly separating direct and indirect somatic embryogenesis, most discussions focus on what would be classified as indirect somatic embryogenesis, where callus is first formed.

The process of somatic embryogenesis is often initiated in media containing high levels of auxins, but embryos usually do not develop further until the auxin concentration is reduced. It has been proposed that auxin induces an embryogenic determination in a proportion of the cells in callus or suspension cultures but at the same time causes these induced cells to cease further development into embryos (Lakshmanan *et al.,* 1997). It was suggested that pre-embryogenic cells and their development into embryos is only resumed at lower auxin concentrations (George *et al.,* 2008).

For the embryogenic initiation step, it has also been proposed that PGRs and stress play a central role in mediating the signal transduction cascade leading to the reprogramming of gene expression. This results in a series of cell divisions that induce either unorganised callus growth or polarised growth leading to somatic embryogenesis (George *et al.,* 2008). The initiation of the embryogenic pathway is restricted only to certain responsive cells which have the potential to activate those genes involved in the generation of embryogenic cells. Alternatively it may be due to the presence of different auxin receptors, one responsible for cell division as such, and another responsible for the asymmetric division that generates an embryogenic cell (George *et al.,* 2008). Two mechanisms appear to be important for *in vitro* formation of embryogenic cells; asymmetric cell division and control of cell elongation (De Jong *et al.,* 1993). Asymmetric cell division is promoted by PGRs

that alter cell polarity by interfering with pH gradient or the electrical field around cells (Smith and Krikorian, 1990).

Somatic embryogenesis is the preferred method in sweetpotato regeneration since plants originate from single cells and this avoids the occurance of chimeras for a crop that is vegetatively propagated (Gong *et al.,* 2005; Kreuze *et al.,* 2008; Song *et al.,* 2004). Since this method is through callus induction it remains cultivar-specific in sweetpotato. Most reports show that callus induction is possible in many cultivars of sweetpotato over a wide number of auxins and plant organs (Al-Mazrooei *et al.,* 1997; Anwar *et al.,* 2010; Otani and Shimada, 1996). However, evidence for the induction of somatic embryogenic tissues at high frequencies is restricted to a few cultivars, while most are found to be recalcitrant or to respond poorly (Cavalcante Alves *et al.,* 1994; Desamero *et al.,* 1994; Zheng *et al.,* 1996).

Regeneration of sweetpotato plants from somatic embryos in semi-solid and liquid media after induction of embryogenic tissues from apical meristems was first reported for cv. 'White Star' (Liu and Cantliffe, 1984). Okada (2001) regenerated only 19 plants from 900 callus and only four of these plants showed to be transformed. In most instances, the callus that is initiated is usually either embryogenic or non-embryogenic (Song *et al.,* 2004). The two types of calli differ in terms of colour and texture (Anwar *et al.,* 2010). One callus type (type I) is white in colour and friable in appearance, while the other (Type II) is firm and green or yellow in color. Only type I calli were capable of somatic embryo formation on medium that induces somatic embryo formation (Anwar *et al.,* 2010). The type I calli were capable of inducing horn-shaped somatic embryos within 3 weeks on embryo initiation medium.

The transformation of explants directly or their embryogenic callus is sometimes preferred over embryogenic suspension cultures due to the ready availability of explant material and easy generation and maintenance of material for routine transformation experiments (Song *et al.,* 2004). Apart from difficulties to predict the type of callus induced, the establishment of the embryogenic suspension cultures has shown to be technically demanding and reproducibility between laboratories has been low (Zhai and Liu, 2003). Al-Mazrooei (1997) demonstrated that longer induction periods were required for subsequent

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maturation, and large numbers of mature embryos could only be regenerated from tissues which had been cultured for eight weeks on the first-stage medium. Likewise, the ability of the mature embryos to convert to plantlets (defined as the formation of shoot and root tissues) was found to depend on the duration of exposure to the medium. This was observed mostly in cv. Papota and TIB 10, in which only embryos regenerated from tissue cultured for eight weeks were able to convert into plants. The regeneration of of cv. Huachano through somatic embryogenesis took between 5 to 12 months in the hands of Kreuze *et al.* (2008).

An increasing number of the recently developed somatic embryogenesis protocols for sweetpotato transformation are based on embryogenic suspension cultures (Xing *et al.,* 2007; Yang *et al.,* 2011; Yu *et al.,* 2007). In these protocols, embryogenic clusters of callus is crushed and resuspended in liquid medium. Then the transformation is carried out on the cell clusters in the liquid medium. The transformed cell clusters are later transferred to solid medium for plant regeneration. Although these protocols have been reported to give relatively high transformation frequencies (Sihachakr *et al.,* 1997), they are time consuming and difficult to establish (Anwar *et al.,* 2010; González *et al.,* 2008).

Apart from somatic embryogenesis protocols being costly, the long durations of exposure to medium can lead to somaclonal variation (Song *et al.,* 2004). In general, the use of 2,4-D and/or a prolonged callus phase are responsible for inducing genetic as well as epigenetic variation. Song *et al.* (2004) reported some abnormalities (5–15%) among sweetpotato plants regenerated through somatic embryogenesis; some plants had reduced apical dominance or had an altered leaf and/or a reduced internode length, and failed to produce roots and recover a normal phenotype. However, the long duration of exposure and frequent medium changes was indicated as one of the main weaknesses of the somatic embryogenesis although 15 commercial cultivars from China were successfully regenerated (Liu *et al.,* 2001). Newell *et al.* (1995) also found regeneration through somatic embryogenesis to be complex. This group noted that orange-coloured, embryogenic callus was first discernible as early as eight weeks after inoculation of leaf discs. However, shoots were only regenerated after several more months of culture, during which time numerous media were evaluated for embryo germination potential.

2.2.3   Factors that affect regeneration efficiency

2.2.3.1 Plant genotype

Genotype has been shown to be a major limiting factor in induction of plant regeneration in sweetpotato. Many cultivars give low or no embryogenic responses at all (Cavalcante Alves *et al.,* 1994; Desamero *et al.,* 1994). Triqui *et al.* (2007) found that three out of six sweetpotato cultivars were completely recalcitrant to regeneration even after experimenting with them on medium supplemented with three different types of auxins. In addition different laboratories report different results for the same cultivar indicating that regeneration of sweetpotato is difficult to reproduce (Moran *et al.,* 1998). Inconsistencies in regeneration responses within the same cultivar may be due to a variation in the developmental and physiological stage of *in vitro* plants, affecting the cultural behaviour of explants (Jones *et al.,* 2007; Triqui *et al.,* 2007).

2.2.3.2 Type of plant organ

Apart from the overall genotype of the plant, the plant organ used as explants also has an effect on regeneration efficiency (Dodds *et al.,* 1992). Although growth regulators may help to induce regeneration, cells in some parts of the plant appear to be partially pre-determined to a particular morphogenetic pathway so that it takes only a slight change in environment to induce the tissues of some organs to form an adventitious meristem or somatic embryo instead of progressing to become a differentiated cell within the mother intact plant. This is clearly seen in cells that are embyogenically committed. There is a high probability that somatic embryogenesis can be initiated from such tissues when they are explanted, either directly, or indirectly after some initial cell proliferation.

In sweetpotato, somatic embryogenesis can be initiated from anther-derived callus (Tsay and Tseng, 1979), leaf, storage root discs (Newell *et al.,* 1995), shoot tip, stem internodes (Song *et al.,* 2004), root explants (Liu and Cantliffe, 1984) and lateral buds (Cavalcante Alves *et al.,* 1994; Jarret *et al.,* 1984). When using different types of sweetpotato explants, various groups obtained different results. Song *et al.* (2004) found stem explants of cultivar Beniazuma better than both leaf discs and petioles for both transformation efficiency and regeneration through somatic embryogenesis. However, when experimenting with transformation through organogenesis Luo *et al.* (2006) found that the shoots and roots

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regenerated from all tested sixty stem internodes were not transgenic. These authors suggested that the strong regeneration capacity of this tissue seems to compete with the regeneration from the rare transformed cells (Luo *et al.,* 2006).

2.2.3.3 Age and size of explant

Apart from genotype and type of plant organ, the age and size of the explants has also been implicated in influencing regeneration efficiency. Triqui *et al.* (2006) found that embryogenic response in sweetpotato highly depended on the size of lateral buds incubated. Only buds with a size of 0.5–1 mm were suitable for embryogenic induction. Similar observations were made by Al-Mazrooei *et al.* (1997) when experimenting with axillary buds. They found that buds greater than 1 mm in length had a tendency to form non-embryogenic green callus, while those under 0.5 mm failed to grow or develop in any manner. The age of explants is also an important factor. Dessai *et al.* (1995) observed that sweetpotato leaves from the fourth position downward (older) generally responded poorly. The differential effect of the age of the explant on proliferative and regenerative abilities in other crops namely barley, wheat and tritordeum, is well known (Barcelo *et al.,* 1991)*.* Differences in regenerative abilities of leaves of varying ages may be the result of differences in internal auxin, cytokinin and/or abscisic acid (ABA) levels. Also the high metabolic activity in young developing leaves may contribute to organogenesis (Ritchie and Hodges, 1993).

2.2.3.4 Plant growth regulators

The main signaling systems in plants are hormone-dependent since there is no nervous system present. There are five main classes of hormones or their synthetic analogues used as plant growth regulators. These are auxins, cytokinins, gibberellins, abscisic acid and ethylene. Generalisations about plant growth regulators and their use in plant cell culture media have been developed from initial observations made in the 1950s (Skoog and Miller, 1957). There is, however, some considerable difficulty in predicting the effects of plant growth regulators in different species, cultivars and even plants of the same cultivar grown under different conditions.

Apart from variations caused by the plant species, any given plant growth regulator may affect the biosynthesis or metabolism of another, thus affecting endogeneous levels. The

issue is further complicated by the fact that environmental factors – light, water status, wounding, pathogens – may modify responses and indeed affect the levels of plant growth regulators. The reason for this appears to be that growth regulators and environmental factors share many components in their transduction chains. These transduction chains interact to produce an integrated response (George *et al.,* 2008). Unsurprisingly therefore, it is difficult to predict how any hormone (or growth regulator or inhibitor) will affect any given plant system.

Auxins. One of the important findings of early physiological analysis on organogenesis *in vitro* was the identification of a predominant role of auxin as a chemical determinant in plant development (Skoog and Miller, 1957). Auxins play an important role in many aspects of growth and differentiation including cell enlargement, cell division, vascular differentiation, apical dominance, and root formation. Both exogenous and endogenous auxins are closely involved in the process of somatic embryogenesis (Michalczuk *et al.,* 1992). Usually, the bulk of auxin molecules present in plants are in conjugated forms. Hormone conjugation reactions are mostly for regulation of endogeneous hormone levels. The synthetic auxins, 2,4-D and IAA are often converted, after uptake into plant tissue, to conjugates, mainly glucosyl esters. This reversible conjugation may regulate levels of free active substances.

In tissue culture, depending on other hormones present in the medium, changes in auxin concentrations may change the type of growth; for instance, the stimulation of root formation may switch to callus induction. Applied auxins seem to be capable of fundamentally altering the genetically programmed physiology of whole plant tissues, which had previously determined their differentiated state. Cells, which respond to auxin, revert to a dedifferentiated state and begin to divide. It has been reported that auxins cause DNA to become more methylated than usual and that this might be necessary for the re-programming of differentiated cells (George *et al.,* 2008).

Exposure to synthetic auxins such as 2,4-D stimulates the accumulation of endogenous IAA (indoleacetic acid), which in turn is considered to be important to the competence of carrot cells to undergo somatic embryogenesis (Michalczuk *et al.,* 1992). The apparent response to changes in levels of 2,4-D decreased progressively in the continued presence of

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this auxin analog. This implied that 2,4-D was important for proliferation of embryogenis callus, but long duration of exposure reduces regeneration. Following this observation, some workers have adopted two-stage auxin-cytokinin regeneration protocols that briefly (3 to 5 days) expose sweetpotato explants to 2,4-D followed by transfer to other growth regulators (Dessai *et al.,* 1995; Kreuze *et al.,* 2008; Santa-Maria *et al.,* 2009). Sihachakr *et al* (1997) only obtained sweetpotato regenerants through somatic embryogenesis when they transferred white callus from auxin to zeatin-containing medium.

In many respects, each tissue culture system is unique, and the effects of different concentrations of auxins and other hormones must be tested for each case individually and only to some extent can the results be transferred to other cultures. Al-Mazrooei (1997) tested five different auxins for induction of embryogenic callus and found 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) to be the most effective. The use of 2,4,5-T induced the production of embryogenic tissues in seven cultivars which responded poorly or not at all to 2,4-D. The auxins 1-naphthaleneacetic acid (NAA), picloram and dicamba (at 10–200 μM) were also investigated but did not show good results (Al-Mazrooei *et al.,* 1997). Triqui *et al* (2007) also reported that the auxin 2,4,5-T was best for sweetpotato embryogenesis from lateral buds, while the auxin 2,4-D produced best results when combined with the cytokinin BAP or kinetin. This group also found that the regeneration based on 2,4,5-T showed significant interaction between the auxin and cultivar implying that the effect of auxin was dependent on type of cultivar. In any case only very low levels of 2,4-D (5 to 10 μM) were helpful. Other researchers have successfully regenerated sweetpotato through somatic embryogenesis after supplementing the auxin 4-fluorochloroacetic acid (4-FA) to culture medium (Anwar *et al.,* 2010; Muramoto *et al.,* 2012; Song *et al.,* 2004). Taken together, these results confirm that it is important to test many auxin types and concentration ranges when novel genotypes are being tested, given that responses among them can be quite different (Santa-Maria *et al.,* 2009)

Cytokinins. Cytokinins play multiple roles in the control of plant development; however, the mode of their action at the molecular level is uncertain. Some are present in t-RNA molecules, but it is not yet clear whether incorporation into t-RNA is necessary before typical cytokinin effects can become apparent. In some circumstances, cytokinins activate

RNA synthesis, stimulate protein synthesis and the activities of some enzymes (George *et al.*, 2008). The action of cytokinins is light-dependent, they inhibit shoot development in conditions without light. Together with auxins, cytokinins take part in the regulation of the cell cycle in plant cells. They probably induce D-type cyclin CycD3 and thus stimulate the cell cycle progression from the G1 to the S phase, and possibly also G2/M transition via induction of expression of the genes *CDC*2 for histon-H1-kinase and stimulation of its dephosphorylation by cdc25 (George *et al.,* 2008). The commonly used cytokinins in sweetpotato regeneration are Zeatin, kinetin and BAP (Kreuze *et al.,* 2008; Pido *et al.,* 1995; Santa-Maria *et al.,* 2009).

Auxin-cytokinin interactions. Regeneration *in vitro* depends on the application of exogenous phytohormones, in particular auxin and cytokinin, and also on the ability of the tissue to respond to these phytohormone changes during culture. Since the classic studies of Skoog and Miller (1957), it has been known that cytokinins interact with auxin and that the relative ratio of these two hormones determines the type of organs regenerated *in vitro*. The auxin:cytokinin ratio in culture media represents a signal that regulates subsequent cell phenotype and also in the onset and maintainance of the process of cell division (George *et al.,* 2008). Subsequent studies on whole plants and excised tissues revealed that cytokinins are negative regulators of both lateral and adventitious root development and that they can reverse the inductive effect of auxin (Konieczny *et al.,* 2009).

Skoog and Miller (1957) found that shoot formation could be induced predictably from tobacco callus using relatively low levels of auxin and high levels of cytokinin. A balance between auxin and cytokinin growth regulators is most often required for the formation of adventitious shoot and root meristems (Slater *et al.*, 2008). The requisite concentration of each type of regulant differs greatly according to the kind of plant being cultured, the cultural conditions and the compounds used. Interactions between the two classes of regulants are often complex, and more than one combination of substances is likely to produce optimum results (George *et al.,* 2008).

In recent years, interest has grown for the application of thidiazuron (TDZ) in both adventitious and somatic embryogenesis regeneration of plants (Sriskandarajah and Lundquist, 2009). Originally, TDZ, a synthetic phenylurea-type plant growth regulator,

was considered as a cytokinin inducing responses similar to those caused by natural cytokinins (Guo *et al.,* 2011). However, TDZ is able to induce both cytokinin and auxin morphogenic responses (Jones *et al.,* 2007).

Gibberellins. Gibberellins are involved in a wide range of developmental responses. These include promotion of elongation in stems and grass leaves, due in part to activation of intercalary meristem. They promote cell division and elongation (Harberd *et al.,* 1998). In sweetpotato regeneration through embryogenesis, gibberellins are usually applied to induce elongation of somatic embryos (Anwar *et al.,* 2010; Kreuze *et al.,* 2008; Song *et al.,* 2004). Auxin and gibberellin responsive genes play a role in modification of the cell wall (Tanimoto, 2005). More than 100 members of this group of plant hormones are now known. They all share the gibbane ring structures and are either dicarboxylic (C_{20}) or monocarboxylic (C_{19}), they have all been assigned 'gibberellin numbers' (GA_x) and are usually referred to by these rather than by conventional chemical nomenclature.

The timing of application of GA_3 in culture is very important and it is recommended that GA_3 be applied only when somatic embryos are already mature or when shoots are already evident. When GA_3 is added to plant tissue culture media, it often diminishes or prevents the initiation of adventitious roots, shoots or somatic embryos. Thus, the prior treatment of callus or explants (George *et al.,* 2008) with GA_3, or the addition of GA_3 to the medium together with auxin and cytokinin at concentrations which normally promote morphogenesis, is usually inhibitory (George *et al.,* 2008).

Abscisic acid. Abscisic acid (ABA) is another naturally-occuring growth substance. It has many roles in plants, such as the regulation of stomatal closure, control of water and ion uptake by roots, and of leaf abscission and senescence. Hence, like other hormones, ABA has multifaceted effects *in vitro* (George *et al.,* 2008). In tissue culture, ABA sometimes promotes morphogenesis or growth. More specifically, ABA has been shown to control the expression of genes specific to embryo development and maturation. At another level, some of the side effects of ABA lie in the hormone antagonizing or modifying the effect of other hormones, notably cytokinins and gibberellins, but also auxins (George *et al.,* 2008). Although the mechanism of ABA action in embryo development has not been well

elucidated, this compound has been used widely in maturation and synchronization of sweetpotato somatic embryos (Anwar *et al.,* 2010; Song *et al.,* 2004; Triqui *et al.,* 2007).

2.3   Genetic transformation of crop plants

Genetic transformation is the stable integration of a gene or genes from one organism into the genome of another, often a different species (Bohorova *et al.,* 1999). This technique is employed for both basic and applied research (Birch, 1997). Areas of particular interest in basic research using genetic transformation include the functional analysis of gene regulatory elements, the study of protein function and the molecular biology of viruses and hormonally regulated genes (Haines *et al.,* 2003; Kim and Botella, 2004; Nakagawa *et al.,* 2003). The applied use of genetic transformation is in crop improvement through transfer of one or a few genes, thereby delivering desirable traits by themselves rather than in the company of other unwanted genes (Kasukabe *et al.,* 2006; Kreuze *et al.,* 2008; Shimada *et al.,* 2006). This allows breeders to retain the gains made through thousands of years of breeding when they develop new varieties (Slater *et al.*, 2008).

2.3.1   Direct gene transfer

The first step in genetic transformation is DNA uptake by the target host plant cells (Bohorova *et al.,* 1999). This step can be achieved through either direct or indirect gene transfer methods (Ogawa and Mii, 2007). Examples of direct gene transfer methods are microinjection, macroinjection, electroporation (Fromm *et al.,* 1986; He *et al.,* 2001), silicon carbide fibers, access by pollen tube pathway, dry embryo incubation and biolistic gene transfer. Biolistic gene trasfer (particle bombardment) is the most popular among the direct gene transfer methods (Valentine, 2003). It involves introduction of DNA into intact cells and tissues through the use of high-velocity microprojectiles (Valentine, 2003). The process has allowed scientists to transform many important crop species that have been difficult to transform using other methods (Bohorova *et al.,* 1999). Microprojectile bombardment is able to breach cell walls and cell membranes, the principle barriers to DNA delivery. In addition, biolistic particle gun yields more transformants than other technologies.

Genetic transformation of sweetpotato through particle bombardment has been reported previously (Prakash and Veradarajan, 1992; Yi *et al.,* 2007). However, the exact

mechanism for gene integration using particle bombardment is still unknown (Bohorova *et al.,* 1999). In plants, direct gene transfer leads to non-homologous integration into the chromosome, characterized by multiple copies and some degree of re-arrangement. Additionally, the occurance of chimeric plants is a common feature of biolistic gene transfer method (Bohorova *et al.,* 1999). Importantly, the presence of extraneous DNA in plants transformed through this method has remained a major challenge. In addition, the sophisticated nature of the gene gun used in this method has lead to the low adoption of this technique by most laboratories (Birch, 1997; Yang *et al.,* 2011).

2.3.2 *Agrobacterium*-mediated transformation of crop plants

The exploitation of *Agrobacterium* species to transfer genes has become popular since this soil bacterium has natural capacity to transfer its DNA (referred to as T-DNA). Usually *Agrobacterium* transformation leads to clean transgene integration events (intact and single copy DNA integrations) than particle bombardment (Yang *et al.,* 2011). The T-DNA targeted for transfer into plants is first inserted on a large circular plasmid, within the *Agrobacterium*, where it is delimited by 25 base-pair imperfect repeats, known as the left (LB) and right border (RB) sequences. In order to effectively exploit the natural ability of this bacterium, scientists have replaced the natural tumour- or rhizogenesis-inducing (Ti or Ri) T-DNA sequences with gene sequences for plant improvement (Labra *et al.,* 2001). With this change the bacterium transfers the genes for plant improvement without causing tumours or rhizogenesis in the target plant.

Because the gene for plant improvement has to first be transferred to the Ti or Ri plasmid of *Agrobacterium*, the transformation of plants with *Agrobacterium* is considered to be an indirect gene transfer method (Zambrynski *et al.,* 1983). *Agrobacterium*-mediated transformation of sweetpotato has been reported for *Agrobacterium rhizogenes* (Dodds *et al.,* 1992; Otani *et al.,* 1993) and *Agrobacterium tumefaciens* (Kreuze *et al.,* 2008; Lowe *et al.,* 1994; Zang *et al.,* 2009). In general, the results on sweetpotato genetic transformation have shown low efficiency and a high genotype dependent response (Luo *et al.,* 2006; Otani *et al.,* 2003; Song *et al.,* 2004).

One of the first reports of sweetpotato transformation was through *Agrobacterium rhizogens* (Otani *et al.,* 1993). Leaf discs of 5 cultivars were transformed and hairy roots

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were induced from them. Plants were subsequently regenerated from the hairy roots. However the Ri-T-DNA seems to have caused problems in the regenerated plants. The plants had wrinkled leaves, altered shape of flowers, reduced apical dominances, shortened internodes, small storage roots and abundant, frequently branching roots that showed reduced geotropism (Otani *et al.,* 1993). Following these disappointing observations, most scientists started experimenting with *Agrobacterium tumefaciens* for gene transfer.

Transformation of sweetpotato using *Agrobacterium tumefaciens* has been attempted by many groups (Cipriani *et al.,* 2001; Dodds *et al.,* 1992; Sheng-Jun *et al.,* 2004; Shimada *et al.,* 2006). Despite wide use of *Agrobacterium* in genetic transformation, sweetpotato has shown a high genotype dependent response with a low transformation and regeneration efficiency in most of these studies (Luo *et al.,* 2006; Sheng-Jun *et al.,* 2004; Song *et al.,* 2004). A critical challenge is that regeneration system in non-transformed sweetpotatoes can not be directly applied to transformed system coupled with *Agrobacterium* (Garcia *et al.,* 2000; Gosukonda *et al.,* 1995a; Otani *et al.,* 1998). When regeneration is coupled to transformation, the numbers of regenerated plants drop dramatically, presumably due to some modification caused by *Agrobacterium* infection step (Song *et al.,* 2004; Xing *et al.,* 2008). Furthermore, even when Okada *et al.* (2001) used electroporation they were only able to transform one cultivar although regeneration without transformation showed success with two cultivars.

Among the biological steps of transformation, the optimization of *Agrobacterium tumefaciens*-plant interaction is probably the most important aspect to be considered. It includes the integrity of the bacterial strain, its correct manipulation and the study of reaction in wounded plant tissue, which may develop in a necrotic process in the wounded tissue or affect the interaction and release of inducers or repressors of *Agrobacterium* virulence system. The type of explant is also an important fact and it must be suitable for regeneration allowing the recovery of whole transgenic plants (Song *et al.,* 2004). The establishment of a method for the efficient regeneration of one particular species is crucial for its transformation (Liu, 2011).

Differences in virulence and effective host range of commonly used *Agrobacterium* strains have been reported. Various strains of *Agrobacterium* have been used in sweetpotato

transformation, including EHA105 (Luo *et al.,* 2006; Song *et al.,* 2004), EHA101 (Otani *et al.,* 2003), C58C1 (Otani *et al.,* 1998) and LBA4404 (Newell *et al.,* 1995). Hypervirulent strains of *A. tumefaciens* such as EHA101 and EHA105 can significantly improve transformation efficiency as compared to strains such as LBA4404. The super virulent Ti plasmid pTiBo542 (Hood *et al.,* 1986) in the *Agrobacterium* strain EH105 is responsible for their high infection potential. Strain EHA105 has been reported to be very efficient for the genetic transformation of different sweetpotato cultivars (Gama *et al.,* 1996; Otani *et al.,* 1998; Song *et al.,* 2004; Yu *et al.,* 2007). Apart from the strain of *Agrobacterium*, many other factors have been experimented on in order to improve transformation efficiency. These include the effect of selective antibiotics, concentration of acetosyringone, duration of co-cultivation with *Agrobacterium*, *Agrobacterium* concentration, light/dark during co-culture, inclusion of wounded cell extracts, wounding of explants, infiltration (Bidney *et al.,* 1992) and agitation during *Agrobacterium* infection (González *et al.,* 2008; Otani *et al.,* 1998; Song *et al.,* 2004; Xing *et al.,* 2008).

2.3.3 Genes that have been used for genetic transformation of sweetpotato

The capacity to introduce and express diverse foreign genes in plants, first described for tobacco in 1984 (Paszkowski *et al.,* 1984), has been extended to over 120 species in at least 35 families (Birch, 1997). Genetic transformation has proved a practical tool for the improvement of sweetpotato although few plants were regenerated and only a few cultivars were included in most of the previous transformation studies (Yang *et al.,* 2011). Sweetpotato transformation has been reported for introducing important agronomic traits, such as herbicide resistance (Choi *et al.,* 2007; Yu *et al.,* 2007), viral resistance (Kreuze *et al.,* 2008; Okada *et al.,* 2001) and insect resistances related to the CryIIIA delta-endotoxin, cowpea trypsin inhibitor and snowdrop lectin (Moran *et al.,* 1998; Newell *et al.,* 1995). By the expression of heterogenous proteins, for example, NtFAD3 (Wakita *et al.,* 2001) and mouse adiponectin (Berberich *et al.,* 2005), the value addition to sweetpotato was also achieved. Transgenic sweetpotato expressing snowdrop lectin (Newell *et al.,* 1995), delta-endotoxin (Moran *et al.,* 1998), sweetpotato feathery mottle virus (SPFMV-S) coat protein (Okada *et al.,* 2001), tobacco microsomal ω-3 fatty acid desaturase (*NtFAD*3) (Wakita *et al.,* 2001) and *bar* gene (Yi *et al.,* 2007), among others, have been produced.

2.3.4 Selection marker genes deployed in transformation of sweetpotato

Regardless of the chosen strategy, transformation is a random and low-efficiency process. Only a small proportion of target cells typically receive the DNA during the transformation process, and only a small number of these plant cells survive the treatment and stably integrate the introduced DNA (Birch and Franks, 1991). It is therefore essential to efficiently detect or select for transformed cells among a large excess of untransformed cells (Birch, 1997). Since most foreign genes introduced into plants do not confer a phenotype that can be used conveniently for the identification or selective propagation of the transformed cells, marker genes must be introduced along with the transgene of interest to provide such a phenotype.

The selectable marker gene encodes a product that allows the transformed cell to survive and grow under conditions/media that kill (negative selection) or restrict (positive selection) the growth of nontransformed cells. There are several selectable marker genes available that can be used in plant systems including antibiotics (*nptII*, hygromycin phosphotransferase (*hph*) (Otani *et al.,* 1998), gentamycin, chloramphenicol, streptomycin, tetratcycline), herbicides (*bar* (Shou *et al.,* 2004) and glyphosate) and other non-toxic genes such as the phosphomannose isomerase gene. The gene for neomycin phosphotransferease (*nptII*) which confers tolerance to kanamycin driven by CaMV 35S constitutive promoter, is widely used in sweetpotato transformation and expression vectors (Kreuze *et al.,* 2008; Luo *et al.,* 2006; Xing *et al.,* 2008). This gene from the *E. coli* transposon Tn5 encodes an enzyme called neomycin phosphotransferase type II (*nptII*). *NptII* is one of several enzymes that detoxify the kanamycin family of amino glycoside antibiotics by phosphorylating them (Bevan, 1984).

Some groups have used the *hph* gene after observing that the *nptII* was a weak selection agent (Okada *et al.,* 2001; Song *et al.,* 2004; Yu *et al.,* 2007). Moran *et al* (1998) failed to completely eliminate untransformed escapes on medium containing 100 mg/L Kanamycin. Newell (1995) also encountered some escapes on medium supplemented with 50 mg/L kanamycin. They also observed that both the antibiotics for suppression of *Agrobacterium* and the selection agent kanamyicn have a negative effect on transformation efficiency. Okada and colleagues (2001) also reported that kanamycin is not a good selection agent and implicated this antibiotic in the low sweetpotato transformation efficiency they

obtained. Later, Song *et al.* (2004) reported that kanamycin alone was too weak as a selection agent while hygromycin was too strong to be used in the early stages of culture.

Following this observation, Song and Yamaguchi (2006) only used kanamycin (50 mg/L) in the early stages of culture followed by hygromycin (30 mg/L) in later stage. However, this regeneration system has been thought to be complicated as it requires the use of binary vectors that bear two selectable markers (Luo *et al.*, 2006). This group (Song *et al.*, 2004) later demonstrated that the exclusive use of kanamycin required higher (100 mg/L) doses to ensure selection of transformed callus of cv. Beniazuma (Song and Yamaguchi, 2006). However, Xing *et al* (2008) reported that 10 mg/L Kanamycin was sufficient for selection of transgenic plants of cultivar Xu55-2 regenerated through cell suspensions. From these results it is not surprising that most groups optimise kanamycin concentrations based on the existing conditions and have used different concentrations of kanamyicin to improve transformation efficiency (Shin *et al.*, 2007; Song *et al.*, 2004; Xing *et al.*, 2008).

2.3.5 Reporter genes deployed to assay transgenic sweetpotato

Reporter genes are often used as a quick indication of whether a certain gene has been taken up by or expressed in the cell or organism. The choice of a suitable reporter gene is important in both transient and stable expression assay systems (Jefferson *et al.*, 1987). Reporter genes can be used as an assay using colorimetric, fluorimetric, luminometric or radiometric approaches. The most popular systems for monitoring genetic activity in eukaryotic cells include chloramphenicol acetyl transferase (CAT), β-galactosidase, β-glucuronidase (*gus*), firefly luciferase (*luc*), growth hormone, alkaline phosphatase (AP), green fluorescent protein (*gfp*) and renilla luciferase (Lawton *et al.*, 2000). Although the *gfp* has been used in some cases (Lawton *et al.*, 2000), the *gus* gene remains the most common reporter for sweetpotato transformation (Song *et al.*, 2004; Yang *et al.*, 2011; Yu *et al.*, 2007). The histochemical procedure used to demonstrate GUS activity in transformed plant tissue is very powerful even for resolving differences in gene expression between individual cell and cell types within tissues (Jefferson *et al.*, 1987).

2.3.6 Transgene expression

One attraction of genetic transformation for cultivar improvement is the theoretical potential for very precise genetic change (Kung and Wu, 1992). In the longer term, a more

important goal than increased transformation efficiency is the development of transformation methods and constructs tailored for predictable transgene expression, without collateral genetic damage (Birch, 1997). Transgene expression depends basically on the type of genetic transformation used, the copy number of the transgene, number of rearranged and truncated transgene copies, the chromosomal position where the transgene got integrated, and the methylation-prone sequences the gene construct has. Levels and patterns of expression generally vary to some extent, even between independent single copy transformants (Birch, 1997). This can be due to the influence of different sequences flanking the integration sites (Van-der-Hoeven *et al.,* 1994).

Transgene introgression into chromosomes in areas that are meant to be methylated and silenced will result in transgene silencing (positional effect). The instability of transgene expression, commonly called "gene silencing" is frequently observed in transgenic plants. Loss of expression does not correlate with the loss of the transgene but rather with its inactivation. Newly integrated DNA may be recognized by plants as foreign because its insertion disrupts normal chromatin structure or alternatively, sequence characteristics of the integrated DNA could distinguish it from that of the surrounding integration site. Inactivation could also be a consequence of RNA-directed *de novo* methylation of genomic transgene sequences.

Features that are considered in preparation of gene constructs include: appropriate transcriptional promoters and enhancers (Benfey *et al.,* 1990); introns (Maas *et al.,* 1991); transcriptional terminators and 3' enhancers (Richardson, 1993); polyadenylation signals (Birch, 1997); untranslated 5' leader and 3' trailer sequences (De Loose *et al.,* 1995); codon usage (Birch, 1997); optimal sequence context around transcription and translation start sites, including absence of spurious start codons (Birch, 1997); transit sequences for appropriate subcellular compartmentation and stability of the gene product (Hicks *et al.,* 1995); absence of sequences such as cryptic introns (Birch, 1997) or polyadenylation signals resulting in inappropriate RNA processing (Birch, 1997); and absence of sequences resulting in undesired glycosylation or lipid anchor sites (Birch, 1997).

Lim *et al.* (2007) indicated that the promoter is one of the most important factors for the expression of genes. Plant promoters used in biotechnology are divided into three

categories based on gene expression pattern: constitutive (almost everywhere, every time); spatiotemporal (tissue-specific and/or stage-specific); and inducible (regulated by some specific signal). Cauliflower mosaic virus (CaMV) 35S is the promoter of choice in more than 80% of GM plants (Hernandez-Garcia *et al.,* 2009; Hull *et al.,* 2000), because it drives constitutive high levels of transgene expression (Lim *et al.,* 2007; Venter, 2007). However, despite the great success of the CaMV 35S promoter, there is a scientific interest in discovering new promoters with differentiated and specialised functions (Lu *et al.,* 2008). Moreover, the stability and expression pattern of foreign genes driven by the 35S promoter has been tested and sometimes questioned (Bakhsh *et al.,* 2009).

The major challenge with the 35S promoter is that it is regulated constitutively and has thus caused pant development problems in some cases. A high level of activity of an introduced transgene may cause too much disruption and lead to a negative overall effect e.g. severe growth retardation under normal growing conditions (Kasuga *et al.,* 1999; Lim *et al.,* 2007). Therefore the use of inducible genes is sometimes preferable. Lim *et al.* (2007) successfully achieved strong tolerance to oxidative and chilling stress in transgenic plants when they used an oxidative stress-inducible SWPA2 promoter to drive CuZnSOD and APX expression in chloroplasts using a chloroplast-targeted signal peptide. It is also necessary to sometimes use tissue-specific promoters like *gSPOA1* promoter: promoter from the *gSPOA1* (sporamin producing) gene from sweetpotato and *ß-amy* promoter: promoter from the *ß-amy* (*ß*-amylase producing) gene from sweetpotato (Ghislain *et al.,* 2008). A previous study demonstrated that the sporamin promoter can effectively direct high-level recombinant protein expression in potato tubers (Hong *et al.,* 2008).

Wakita *et al.* (2001) found that the expression of the ω-3 fatty acid desaturase gene from tobacco (*NtFAD3*) was higher in roots and root tubers for plants transformed with El2Ω promoter than in both transgenic roots with CaMV and non-transgenic roots, although for these plant organs the expression driven by CaMV 35S was higher than non-transgenics (Wakita *et al.,* 2001). Apart from revealing the importance of considering the target tissue and promoter, this work also reveals the importance of understanding the gene product. While the CaMV 35S promoter driving the *hpt* gene was sufficient for the production of hygromycin-resistant transgenic plants, the expression level of the *NtFAD3* gene under the

control of this promoter was not high enough to significantly change the composition of the targeted fatty acids in the transgenic lines (Wakita *et al.,* 2001).

Interestingly the same group (Otani *et al.,* 2003) later found that the expression level of *bar* gene under the control of the El2Ω promoter was lower than that under the control of CaMV 35S promoter. This group concluded that for the bar gene the CaMV 35S promoter may be suitable than the improved El2Ω promoter (Otani *et al.,* 2003). This work also showed that the different transgenic events from the CaMV 35S-*bar* had no significant differences in the expression of the *bar* gene while the expression among events of the El2Ω-bar system were variable.

2.3.7 Management of insects through expression of *Bt* cry proteins

Bacillus thuringiensis (*Bt*) is a gram-positive bacterium, which produces proteinaceous crystalline (*Cry*) inclusion bodies during sporulation (Hoftey and Whiteley, 1989). The *Bt* δ–endotoxins are known to constitute a family of related proteins for which 140 genes have been described (Crickmore *et al.,* 1998), with specificities for Lepidoptera, Coleoptera and Diptera. The crystalline protoxins are inactive, until they are solubilized by the insect midgut proteases at high pH (Gill *et al.,* 1992; Milne and Kaplan, 1993). Brush border membrane vesicles (BBMV) have been identified as the primary binding site for several insect species (Lee *et al.,* 1992). The active toxins bind to the specific receptors located on the apical brush border membrane of the columnar cells of the midgut (Knowles, 1994). There may be many toxin binding protein receptors, and some have been identified as 12 to 180 kDa glycoproteins (Garczynski *et al.,* 1991; Knowles, 1994). After binding to the receptor, the toxin inserts irreversibly into the plasma membrane of the cell leading to lesion formation. There is a positive correlation between toxin activity and ability to bind BBMV (Gill *et al.,* 1992), and the toxicity is correlated with receptor number rather than receptor affinity (van Rie *et al.,* 1989). Differences in the extent of solubilisation of different toxins may explain the differences in the toxicity of various proteins (Meenakshisundaram and Gujar, 1998). Decreased solubility could be one potential mechanism for insect resistance to *Bt* proteins (McGaughey and Whalon, 1992). In cotton bollworm (*Helicoverpa zea*), Cry IIA is less soluble than Cry 1A(c) and fails to bind to a saturable binding component in the midgut brush border membrane (English *et al.,* 1994).

A common feature of *Bt* toxins is that their toxicity is specific to some insect orders thus rendering the technology safe to the environment. Another critical advantage of Bt toxins is that they exhibit an acute toxicity with immediate effect on the target insect. Seven proteins from *Bacillus thuringiensis* were previously tested for activity against the two African sweetpotato weevil species, *Cylas puncticollis* and *C. brunneus* (Tovar *et al.,* 2009). Three of them (*Cry7Aa1, CryET33/CryET34, Cry3Ca1*) were effective against both African weevils with an LC_{50} below 1 ppm (Moar *et al.,* 2007). Therefore, *cry7Aa1, cry3Ca1, and cryET33/cryET34* gene constructs were developed taking into account sweetpotato optimized codon usage, promoters with storage root expression and wound response, and two selectable marker genes (Kreuze *et al.,* 2009). The sporamin promoter and 3' regions were attached to both cryET33/cryET34 and cry3Ca1 coding sequences while the gene cry7Aa1 was attached to the ß-amylase promoter and 3' region (Tovar *et al.,* 2009). The selection of these genes was also based on their low sequence identity, which is important for the potential of cross-resistance (Tovar *et al.,* 2009). A total of 10 gene constructs have been developed with some constructs having combinations of two weevil resistant (WR) genes as an insect-resistance management component (Tovar *et al.,* 2009).

2.3.8 Other biotechnological approaches for insect management

At present the genetic transformation of plants with *Bacillus thuringiensis (Bt)* genes ranks among the successful strategies for improving tolerance to certain insects (Sanahuja *et al.,* 2011). The use of *Bt* crops has already led to effective pest control and reduced use of insecticides resulting in higher crop yields (Agbios, 2009; Mutuc *et al.,* 2011). However, details about the mechanism of action by *Bt cry* proteins, such as post-binding effects and receptor specificities, are not yet clear (Gomez *et al.,* 2002; Zhang *et al.,* 2005). On the other hand, other biotechnological approaches have also shown potential to control insects relying on protease inhibitors (Atkinson *et al.,* 2001; Zhang *et al.,* 2000), spider and scorpion venom toxins (Pham Trung *et al.,* 2006), plant lectins, ribosome inactivating proteins, secondary plant metabolites, small RNA viruses (Hilder and Boulter, 1999) and RNA-mediated interference (RNAi) (Ibrahim *et al.,* 2010).

The early work on sweetpotato transformation for pest management focused on proteins that decrease the digestibility of sweetpotato for insects (Newell *et al.,* 1995). Newell *et al.*

(1995) transformed sweetpotato with a cowpea (*Vigna unguiculata*) trypsin inhibitor (CTI) and the mannose binding snowdrop lectin (Zhang *et al.,* 2000). Later, Cipriani *et al.* (1999, 2001) transformed sweetpotatoes with a soybean (*Glycine max*) Kunitz-type trypsin inhibitor (SKTI) and a rice (*Oryza sativa*) cystein proteinase inhibitor (OCI). The results of feeding tests involving the transformed sweetpotato in this work and weevils suggested that trypsin inhibitors could be useful for control of weevils (Zhang *et al.,* 2000).

RNA-mediated interfering (RNAi) based on post-transcriptional gene silencing (PTGS) has recently come as a strategy to compliment *Bt* technology (Ibrahim *et al.,* 2010; Klink *et al.,* 2009; Urwin *et al.,* 2002). RNAi has successfully been used to improve nutrition and virus disease resistance in sweetpotato and other crops (Kreuze *et al.,* 2008; Shimada *et al.,* 2006; Swain and Powell, 2001). For insect management, some reports have already shown the potential application of anti-feeding strategies based on proteinase gene silencing by RNAi (Urwin *et al.,* 2002).

CHAPTER THREE

EMBRYOGENIC CALLUS AND ROOT REGENERATION INDUCED BY 2,4-DICHLOROPHENOXYACETIC ACID IN SIXTEEN AFRICAN SWEETPOTATO CULTIVARS

3.1 Introduction

Previous reports show that there is a correlation between the quality and quantity of embryogenic callus and subsequent plant regeneration in sweetpotato (Al-Mazrooei *et al.,* 1997). The auxin 2,4-D has been used in most somatic embryogenesis protocols involving different cultivars of sweetpotato (Kim *et al.,* 2012; Liu *et al.,* 2001; Sihachakr *et al.,* 1997; Yang *et al.,* 2011). Low embryogenic frequencies and complete recalcitrance have been reported for many important and adapted sweetpotato cultivars (Al-Mazrooei *et al.,* 1997; Liu *et al.,* 2001; Zang *et al.,* 2009), particularly for African cultivars (Luo *et al.,* 2006). The non-African cultivars that have been transformed so far, including Jewel (Luo *et al.,* 2006), Huachano (Kreuze *et al.,* 2008), White Star (Gama *et al.,* 1996), Kokei 14 (Otani *et al.,* 1998), Yulmi (Choi *et al.,* 2007) and Lizixiang (Yu *et al.,* 2007), can only be used for research purposes in Africa. These are not farmer-preferred cultivars due to several drawbacks, such as low dry matter and vulnerability to diseases and pests (Yang *et al.,* 2011). An investigation was conducted to determine the ability of twenty Ugandan cultivars to form embryogenic callus on medium supplemented with 2,4-D.

3.2 Materials and Methods

3.2.1 Plant material and culture conditions

Twenty farmer-preferred Ugandan sweetpotato cultivars and one USA cultivar, Jonathan, were used in this study. The Ugandan cultivars (Table 3.1) were selected based on preferred attributes among consumers, including high dry matter content (Mwanga *et al.,* 2001; Mwanga *et al.,* 2007). The non-African cultivar, Jonathan, was previously reported to regenerate through somatic embryogenesis (Cipriani *et al.,* 1999). The cultivars were grown in a screenhouse at Makerere University Agricultural Research Institute, Kabanyolo (MUARIK). Vines from the screenhouse grown plants were grafted on *Ipomoea setosa,* an indicator plant for virus infection (Figure 3.1 B). Cuttings from those sweetpotato plants

corresponding to scions whose indicator plants were asymptomatic were used in establishment of virus-free *in vitro* cultures.
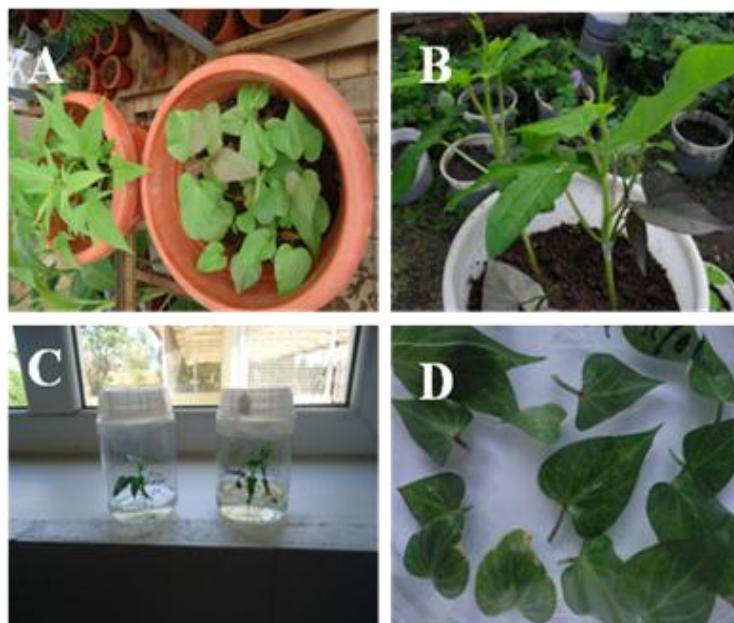
Figure 3.1: Preparation of plant material for somatic embryogenesis experiments.
  A, Sweetpotato cultivars growing in screenhouse ready for bio-indexing. **B**, Bio-indexing of sweetpotato (scion) by grafting on Ipomoea setosa (rootstock) indicator plants. **C**, *In vtro* cultures growing from virus-free and decontaminated plants. **D**, "Whole leaves' used for callus induction on medium supplemented with 2,4-D.

Cuttings containing five to eight nodes were taken from the sreenhouse to the laboratory. The cuttings were washed for fifteen minutes with flowing tap water and submerged in 70% ethanol for two minutes. Thereafter, the vines were immersed for twenty minutes in 39% (v/v) commercial JIK containing 3.85% sodium hypochlorite (NaOCl) and 0.03% Tween 20®. After surface sterilization, the vines were transferred to sterile water and rinsed three times. They were then each cut into one or two nodes and established on sweetpotato propagation medium.

Table 3.1: Some attributes of Ugandan cultivars selected for somatic embryogenesis investigation.

Cultivar	Flesh color	SPVD resistance	Maturity (Days)	Dry matter (%)	Year of release and specific location of collection/release in Uganda
Kakamega	Intermediate orange	M	135	33	2004 in Uganda. Kenya landrace
NASPOT 1	Cream	M	135	32	1999 (bred clone)
New Kawogo	Cream	R	140	32	1995 (Landrace), Germplasm (1988)
Bwanjule	White/Cream	M	135	30	Landrace/ 1995, Germplasm (1988)
Araka	Cream/white	F	105	32	Landrace: Germplasm 2005
NASPOT 8	Pale orange	M	120	32	2007 (bred clone)
Dimbuka-Bukulula	Cream	S	128	32	2001, Germplasm from Masaka
Silk omupya	White	R	100	34	Germplasm from Pallisa
Kyebandula	Cream	M	120	32	Landrace: Germplasm -Mbale
Munyeera	Cream	R	165	33	Landrace: Germplasm from Mpigi
Ssemanda	Cream/white	R	120	33	Landrace: Germplasm from Mpigi
Kisakyamaria	Cream	S	120	32	Landrace: Germplasm from Mbarara
Luwero Silk	Cream	S	120	34	Landrace: Germplasm -Luwero
Namusonga	Cream	F	120	34	Landrace: Germplasm from Pallisa
Magabali	Cream	R	165	33	Landrace: Germplasm-Kabale
Kigaire	Cream	M	120	32	Landrace: Landrace Soroti/Serere
Nyidoyamulalo	Cream	R	120	32	Landrace: Germplasm-Kamuli
Luwero 2 (Uknown)	Cream/white	S	120	33	Landrace: Germplasm -Luwero
Luwero (Unknown)	White	M	165	30	Landrace: Germplasm -Luwero
Jamada	Cream	M	150	32	Landrace: Germplasm -Luwero

Note: all cultivars are adapted to Uganda with potential for adoption in other areas of East Africa. SPVD sweetpotato virus disease resistance, M = Moderate resistance, R = resistant, S= Susceptible, F=Fair, Dry matter content (%) = per cent of fresh weight

The propagation medium was composed of MS (Murashige and Skoog, 1962) salts premix (4.3 g/L), sucrose (30 g/L), myo-inositol (0.1 g/L), 5 ml/L sweetpotato vitamin stock comprised of 40 g/L ascorbic acid, 20 g/L L-arginine, 4 g/L putrescine HCl, 0.01 mg/L gibberellic acid (GA$_3$) and 0.4 g/L calcium pantothenate (Kreuze *et al.,* 2008). All reagents used in this study were sourced from Sigma-Aldrich. The medium was adjusted to pH 5.8 before adding 3 g/L phytagel and autoclaved at 121 °C for 15 minutes at 15 kPa.

3.2.2   Callus induction

Four-week-old whole leaves (petiole with lamina) from the *in vitro*-grown plants were placed on callus induction medium (CIM) under dark conditions at 28 ℃. The cut end of the petiole was slightly pushed into medium. CIM was made of 4.3 g/L MS premix (without vitamins), 0.2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 1 ml/L Vitamins stock (0.5 mg/ml nicotinic acid, 0.1 mg/ml thiamine and 0.5 mg/ml pyridoxine), 100 mg/L myo-inositol, 30 g/L sucrose and 7 g/L agar. The cultures were transferred to fresh media every two weeks. A total of ten to fifteen explants were used per cultivar in each petri dish, and this was replicated three times and arranged in a completely randomized design. After twelve weeks the callus formed was transferred to embryo initiation medium (EIM) under a photoperiod of 16 h, regardless of it being embryogenic or non-embryogenic. EIM was made as CIM but 2,4-D was replaced with 1 mg/L abscisic acid (ABA). After four weeks on EIM, the callus was transferred to plant regeneration medium devoid of plant growth regulators. Fully developed plants were transferred to soil in a screenhouse where they developed normally.

Data on number of explants forming callus and those forming embryogenic callus was collected after twelve weeks. Data on shoot and root regeneration was collected every week since some callus formed roots within three weeks of culture initiation. Cultures were discarded after twenty-four weeks since at this stage most calli lost their embryogenic capacity and could not regenerate shoots. Frequency data was transformed using the arcsine square root before analysis to stabilise the variance. The tables presented show non-transformed data expressed as percentages. Statistical analysis for the effect of cultivar on total callus proliferation and formation of embryogenic callus was performed using general linear model of analysis of variance (ANOVA) at 5% level of significance. Mean values were compared using the least significance difference (LSD) method at the 5% level.

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3.3   Results

3.3.1   Frequency of embryogenic callus

Explants placed on callus induction medium responded by producing callus within three days. The callus was not only on the wounded tip of the petiole but also on the intact leaf lamina (Figure 3.2 B). Two types of callus were induced; embryogenic and non-embryogenic. The embryogenic callus was bright yellow or orange in colour and compact in appearance while non-embryogenic callus was white and friable and proliferated earlier and more rapidly than embryogenic callus. The embryogenic callus was induced in sixteen of the twenty investigated African cultivars cultured on media with 0.2 mg/L 2,4-D. Cultivar type had a highly significant ($P < 0.001$) effect on embryogenic callus induced (Table 3.2). The frequency of embryogenic callus ranged from zero to 25% depending on cultivar (Table 3.2). Somatic embryos started appearing on embryogenic callus within six weeks of initiation of cultures. However, the somatic embryos were more apparent after transfer of cultures from 2,4-D to ABA-containing medium.
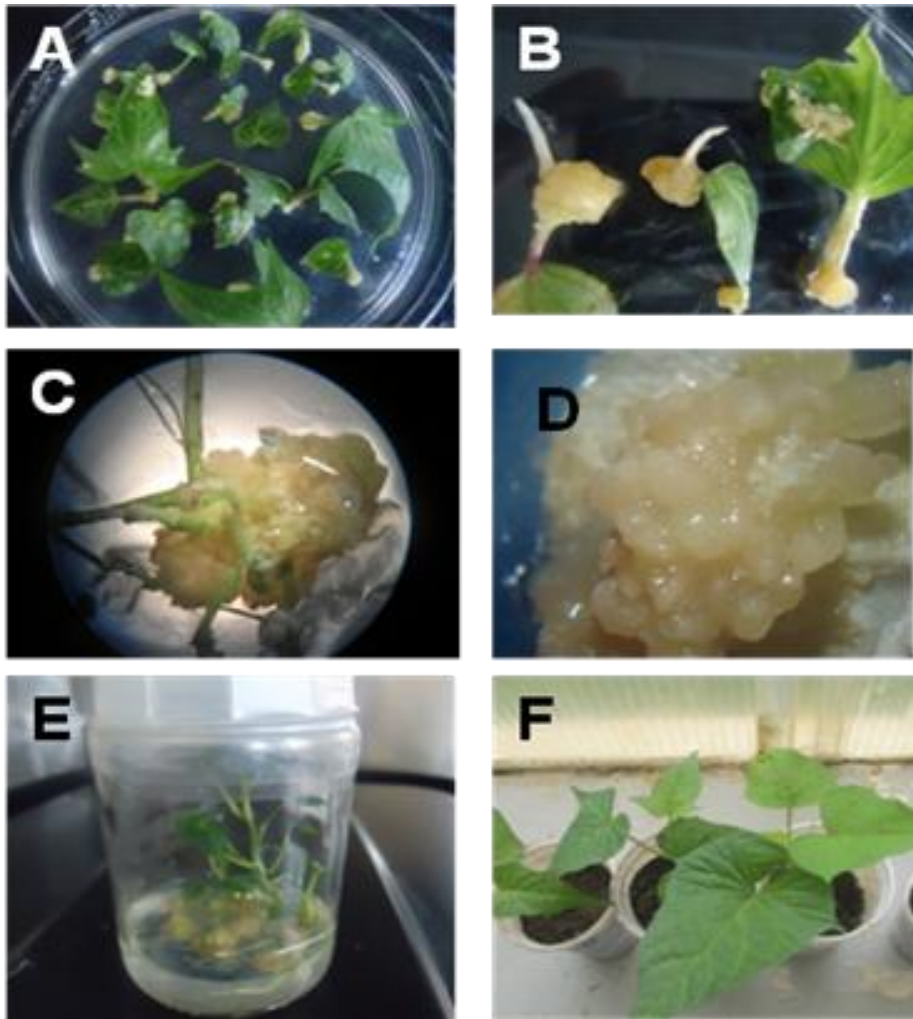
Figure 3.2: Induction of embryogenic callus in African sweetpotato cultivars and regeneration in cv. Jonathan.

A. Induction of callus from the base of petioles attached to leaves. **B.** Root regeneration from callus before development of somatic embryos. **C.** Regeneration of multiple roots from callus after 5 weeks on callus induction medium. **D.** Development of somatic embryos after placement of embryogenic callus on ABA-supplimented medium. **E.** Regeneration of shoots from callus of cv. Jonathan after transfer to plant regeneration medium. **F.** Survival of plants after transfer from *in vitro* cultures to soil in screenhouse.

Somatic embryos of cv. Jonathan developed into shoots after three weeks on ABA-containing media. Cultivar type also had a highly significant (P < 0.001) effect on the ability to regenerate shoots (Table 3.2). Regeneration of roots was achieved from callus of five African cultivars. Contrary to the regeneration of shoots which occurred through

somatic embryogenesis, the regeneration of roots appeared to be through organogenesis as they were formed directly from callus without somatic embryos. In addition, the roots were regenerated earlier than shoots, sometimes as early as three weeks after culture initiation on 2,4-D-containing media. The emergence of new roots was sustained throughout the culture period. The type of cultivar showed a highly significant ($P < 0.001$) effect on the ability to regenerate roots (Table 3.2).

Table 3.2: Embryogenic callus proliferation and regeneration from 'whole leaf' explants of 21 sweetpotato cultivars

Cultivar	Explants tested	Explants inducing callus		Explants inducing embryogenic callus		Explants regenerating roots		Explants regenerating shoots	
	No.	No.	%	No.	%	No.	%	No.	%
Naspot 1	13	10±1.5	75.0±10.4	0±0.0	0.0 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0a
Dimbuka	14	12.3±0.7	90.2±4.8	1.3±0.7	9.8±4.8bcde	0.0 ± 0.0	0.0 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0a
Semanda	14	12.3±1.8	86.0±10.9	0.3±0.3	2.3±2.2ab	0.0 ± 0.0	0.0 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0a
New Kawogo	13	12±1.5	90.0±3.8	3.0±0.6	22.5±6.7ef	1.0 ± 0.6	7.5±3.9b	0.0 ± 0.0	0.0 ± 0.0a
Bwanjule	14	12±1.0	85.7±4.3	1.3±0.9	9.5±6.8bcde	0.0 ± 0.0	0.0 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0a
Namusoga	15	11.3±0.3	77.3±2.0	0±0.0	0.0±0.0a	0.0 ± 0.0	0.0 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0a
Jamada	14	10.7±1.5	78.0±5.8	0.3±0.3	2.4±2.8ab	0.0 ± 0.0	0.0 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0a
Magabali	15	12.7±1.3	86.4±10.2	3.7±0.3	25±1.7f	2.0 ± 0.6	13.6±3.7cd	0.0 ± 0.0	0.0 ± 0.0a
Kyebandula	14	12.3±0.3	88.1±2.1	2.7±0.3	19.0±3.0def	1.3±0.9	9.5 ± 5.9bc	0.0 ± 0.0	0.0 ± 0.0a
Jonathan	14	11±0.6	78.6±7.4	2.3±0.3	16.7±1.8cdef	3.0±0.6	21.4 ± 3.9d	1.3 ± 0.9	9.5 ± 6.8b
Kakamega	15	11.3±2,2	77.3±13.5	0.0 ± 0.0	0.0±0.0a	0.0 ± 0.0	0.0 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0a
Araka red	14	10.7±1.3	78±10.8	0.7±0.7	4.9±4.8ab	0.0 ± 0.0	0.0 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0a
Naspot 8	14	12.0±0.6	83.7±2.0	0.0±0.0	0.0±0.0a	0.0 ± 0.0	0.0 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0a
Munyeela	14	11.0±1.0	80.5±7.7	0.7±0.7	4.9±4.4ab	0.0 ± 0.0	0.0 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0a
Kisakyamaria	13	9.7±0.9	72.5±3.0	0.0 ±0.0	0.0±0.0a	0.0 ± 0.0	0.0 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0a
Silk omupya	14	9.7±1.7	70.7±9.7	1.3±0.7	9.8±5.4bcdef	0.0 ± 0.0	0.0 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0a
Luwero Silk	14	10.3±0.7	72.1±4.2	0.0 ±0.0	0.0±0.0a	0.0 ± 0.0	0.0 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0a
Kigaire	14	9.7±0.7	70.7±5.7	0.7±0.3	4.9±2.5abc	0.0 ± 0.0	0.0 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0a
Nyidoyamulalo	14	10.7±0.7	74.4±5.7	0.3±0.3	2.3±2.4ab	0.0 ± 0.0	0.0 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0a
Luwero 2 (uknown)	15	13.3±0.7	90.9±2.5	1.3±0.9	9.1±5.9abcd	1.0 ± 0.6	6.8 ± 4.1b	0.0 ± 0.0	0.0 ± 0.0a
Luwero (uknown)	14	10.0±1.0	69.8±3.9	1.0±0.6	7±4.5abcd	0.0 ± 0.0	0.0 ± 0.0a	0.0 ± 0.0	0.0 ± 0.0a
F-Test			ns		P<0.001		p< 0.001		p< 0.001

Data represent means ± standard errors for three replicates (10 to 15 explants each). Values followed by the same letter in each column are not significantly

(ns) different at the P ≤ 0.05 level (LSD test).

4.3   Discussion

Sweetpotato has for long been considered recalcitrant to somatic embryogenesis (Yang *et al.,* 2011). Most of the reported protocols are cultivar specific, have low regeneration frequencies which are also difficult to reproduce (Santa-Maria *et al.,* 2009; Yang *et al.,* 2011; Yu *et al.,* 2007). In the present study, the auxin 2,4-D was used for callus induction and embryogenic callus was achieved in sixteen of the investigated twenty African cultivars. These cultivars have not been reported to respond to somatic embryogenesis in previous publications. Luo *et al.* (2006) reported that African cultivars were difficult to regenerate, but did not name the cultivars tested or their geographical location.

The induction of somatic embryogenesis with 2,4-D appears to be preferable in view of existing literature (Yang *et al.,* 2011). Liu *et al.* (2001) induced embryogenic callus from shoot apices of fifteen cultivars from China and Japan on medium supplemented with 9.05 mM 2,4-D. The embryogenic response of these cultivars was low and extremely variable ranging from 6.7 % to 85.2 % (Liu *et al.,* 2001). Similarly, Sihachakr *et al.* (1997) found large differences and low embryogenic callus frequencies among ten sweetpotato cultivars exposed to auxin (10 μM 2,4-D) treatment. Frequencies ranged from zero to 17% response when using lateral buds as explants (Sihachakr *et al.,* 1997). The frequency of embryogenic callus in the current study ranged from no response at all to 25% depending on cultivar (Table 3.2). In contrast to somatic embryogenesis protocols, most auxin-cytokinin shoot organogenesis protocols have replaced 2,4-D with other auxins in order to avoid the callus stage and achieve rapid regeneration (Gong *et al.,* 2005; Luo *et al.,* 2006; Santa-Maria *et al.,* 2009).

Although somatic embryogenesis is a complicated process to manage than shoot organogenesis (Luo *et al.,* 2006; Newell *et al.,* 1995), the regeneration of sweetpotato through somatic embryogenesis is still a preferred option than shoot organogenesis (Yu *et al.,* 2007). This is mainly because somatic embryogenesis leads to high efficiency of selection of transgenic plants on medium and avoids the regeneration of chimeric plants after genetic transformation (Song *et al.,* 2004; Yang *et al.,* 2011). Recently there have been some reports of applying somatic embryogenesis using cell suspensions (Yu *et al.,* 2007; Zang *et al.,* 2009). However, other reports suggest that the use of liquid cultures is extremely complicated and depends on availability of cell suspension cultures (Song *et al.,*

2004; Zhai and Liu, 2003). By opting for solid medium in this study, it was possible to deploy explants which are easy to access and are readily available as noted in previous reports (Cipriani *et al.,* 1999; Kreuze *et al.,* 2008; Song *et al.,* 2004). The use of solid medium for somatic embryogeneis has allowed many researchers to use various plant organs as explants e.g. leaf discs (Newell *et al.,* 1995), stem internodes (Song *et al.,* 2004), root explants (Liu and Cantliffe, 1984), root discs (Newell *et al.,* 1995) and lateral buds (Cavalcante Alves *et al.,* 1994).

The regeneration of roots in this work could be useful for the initiation of root cultures, which could serve as a source of tissue for regeneration of plants or hairy root cultures (Dodds *et al.,* 1991a; Jones *et al.,* 2007). However, it remains difficult to explain this type of morphogenesis which was evident as early as three weeks after culture initiation on 2,4-D-supplemented medium. It is likely that the explants of the cultivars had high levels of endogenous auxins. If this is the case then the exogeneous auxin 2,4-D only accelerated the root regeneration process (Becerra *et al.,* 2004; George *et al.,* 2008). Many factors have been implicated in the unpredictable responses in culture, including variations in the developmental and physiological stage of *in vitro* plants (Triqui *et al.,* 2007).

Previous reports indicate that ABA is required for induction, maturation and synchronization of somatic embryos leading to elongation into shoots (Anwar *et al.,* 2010; Song *et al.,* 2004; Triqui *et al.,* 2007). However, in the current study, two Ugandan cultivars, Magabali and Kyebandula that induced more embryogenic callus than the USA cultivar, Jonathan, did not regenerate shoots. The failure of ABA to induce somatic embryos or promote development of preformed somatic embryos in the sixteen African cultivars that successfully induced embryogenic callus could be attributed to cultivar differences in the response to ABA or the sustained effect of 2,4-D as reported previously (Becerra *et al.,* 2004; George *et al.,* 2008; Santa-Maria *et al.,* 2009).

In total, sixteen African sweetpotato cultivars were able to induce embryogenic callus on medium supplemented with the potent auxin 2,4-D (0.2 mg/L). The embryogenic callus induced in this study could be useful for the initiation of embryogenic cell suspensions which are not readily available for transformation of most sweetpotato cultivars (Yang *et al.,* 2011).

CHAPTER FOUR

INDUCTION OF SOMATIC EMBRYOGENESIS IN RECALCITRANT SWEETPOTATO CULTIVARS

4.1 Introduction

The best responding cultivars in a preliminary somatic embryogenesis study (Section 3.2) were investigated in experiments that included 2 other auxins. The experiments were arranged in a completely randomized design and the factors investigated included cultivar type, type of plant organ supplying explants and type of auxin (Section 3.3). Somatic embryogenesis and plant regeneration at a high frequency has been previously reported in a few cultivars (Al-Mazrooei *et al.*, 1997). Efforts to extend this into a wide range of genotypes has shown that most of them are recalcitrant or respond at low frequencies (Al-Mazrooei *et al.,* 1997; Anwar *et al.,* 2010; Luo *et al.,* 2006). Apart from genotype, the medium composition and type of plant organ providing the explants have been shown to influence regeneration *in vitro* (Anwar *et al.,* 2010; Song *et al.,* 2004). Thus, there is need to develop an efficient system of somatic embryogenesis and plant regeneration for recalcitrant African cultivars. The main aim of this study was to develop a protocol for producing high frequency of embryogenic callus which is useful in regeneration of popular African sweetpotato cultivars which, to our knowledge, have not been regenerated previously. The improvement of regeneration systems for some non-African cultivars and regeneration of roots in African cultivars also reported.

4.2 Materials and Methods

4.2.1 Plant material

Popular Ugandan sweetpotato cultivars namely; Bwanjule, Kyebandula, Magabali, New Kawogo and Semanda were used in this study. Three non-African varieties namely; Huachano from Peru, and Jonathan and Jewel from the U.S.A were included. The three non-African varieties were previously reported to regenerate through somatic embryogenesis or shoot organogenesis (Cipriani *et al.,* 1999; Kreuze *et al.,* 2008; Luo *et al.,* 2006). The selected cultivars were grown in a screenhouse at MUARIK and used to supply vines for initiation of *in vitro* cultures.

4.2.2 Callus induction

In vitro cultures were prepared as described in section 3.2. Three to four weeks old petioles, leaf discs and whole leaves (petiole with lamina) from the *in vitro*-grown Ugandan cultivars were tested on callus induction media. The callus induction media was made of 4.3 g/L MS premix (without vitamins), 1 ml/L Vitamins stock (0.5 mg/ml nicotinic acid, 0.1 mg/ml thiamine and 0.5 mg/ml pyridoxine), 100 mg/L myo-inositol, 30 g/L sucrose and 7 g/L agar. The media was divided into three equal parts and a different type of auxin was added to each part. The three different auxins tested in this study were 5.88 μM 4-fluorophenoxyacetic acid (4FA), 5.1 μM 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and 1.23 μM 2,4-dichlorophenoxyacetic acid (2,4-D). These concentrations were derived from previous studies on sweetpotato somatic embryogenesis (Anwar *et al.,* 2010; Cipriani *et al.,* 2001; Song *et al.,* 2004; Triqui *et al.,* 2007). All auxins were filter-sterilized and added to autoclaved media that was cooled to about 60°C. The cultures were transferred to fresh media every 2 weeks. Data on number of explants forming callus and those forming embryogenic callus was collected after 12 weeks. Embryogenic callus was identified as being compact and light green or bright yellow while non-embryogenic calli was white and friable and formed earlier and more rapidly than embryogenic callus (Cipriani *et al.,* 1999).

4.2.3 Plant regeneration

After observing that 2,4-D induced high frequency embryogenic callus than 4-FA and 2,4,5-T, the callus induction protocol of Cipriani *et al.* (2001) was adopted. The embryogenic callus from three most promising Ugandan cultivars namely New Kawogo, Bwanjule and Magabali and non-African cultivars Jewel, Jonathan and Huachano were used to investigate effect of various medium in inducing plant regeneration from embryogenic callus. Young whole leaves were placed on semisolid medium (0.43 g/L MS salts, 20 g/L D-glucose, 7 g/L agar, 0.5 g/L 2-N-morpholino-ethanesulfonic acid (MES), 0.1 ml vitamins stock, 0.1 mg/L naphthalene acetic acid (NAA), 1 mg/L benzylaminopurine (BAP), 2 mg/L gibberellic acid (GA$_3$), adjusted to pH 5.5) and incubated at 28ºC for 2 days. The explants were then transferred to F15 medium (4.3 g/L MS salts, 0.05 mg/L 2,4-D, 0.2 mg/L zeatin riboside, 0.1 g/L myo-inositol, 30 g/L sucrose, 1 ml/L vitamin stock and 3.0 g/L phytagel) for 3 days, followed by transfer to F9 medium

(Table 4.1). The explants were transferred to fresh F9 media once in every 2 weeks for callus induction (Kreuze *et al.,* 2008). Instead of transferring all the resulting embryogenic callus from F9 to G24D medium as reported by Cirpiani *et al.* (2001), the callus were separated and placed on four types of media; F25, F9, plant growth regulator free (PGR-free) and G24D medium (Table 4.1). These adjustments were made at this stage following preliminary observations of root emergence before shoot regeneration on G24D medium. Emerging shoots on the various media were taken to fresh F9 medium followed by multiplication on sweetpotato propagation medium and transfer to a screenhouse at the National Agricultural Research Laboratories (NARL) at Kawanda, Uganda.

Table 4.1: Plant regeneration media and amounts and types of growth regulators added

Type of regeneration media*	Plant growth regulator added to media (mg/L)			
	Zeatin riboside	2,4-D	GA$_3$	Abscisic acid
F9	0.2	-	-	-
G24D	-	0.05	0.1	-
F25	-	-	-	1
PGR-free	-	-	-	-

* All media listed contain 4.3 g/L MS salts, 0.1 g/L myoinositol, 30 g/L sucrose, 1ml vitamin stock and pH adjusted to 5.8 before adding 3.0 g/L phytagel. Filter-sterilized PGRs were added to all media after autoclaving and cooling to 60ºC.

4.2.4 Statistical analysis

All cultures were incubated in the growth chamber at 28ºC with a photoperiod of 16 h. A total of 10 explants were used per treatment in each petri dish, and this was replicated 3 times and arranged in a completely randomized design with cultivar, type of plant organ and type of plant growth regulator (PGR) as factors. Statistical analysis for the effect of the three factors on total callus proliferation and formation of embryogenic callus was performed using general linear model of analysis of variance (ANOVA) at 5% level of significance. Mean values were compared using the least significance difference (LSD) method at the 5% level of significance. Two-way Chi-Square was used to establish the contribution of PGR to plant regeneration from embryogenic callus.

4.3   Results

4.3.1   Conditions affecting proliferation of embryogenic callus

All cultivars, plant organs and auxins investigated in this study induced both embryogenic and non-embryogenic callus (Figure 4.1). Callus proliferation was noticed within 5 days in all experiments. Data on number of explants forming callus and those forming embryogenic callus was collected after 12 weeks. The frequency of total callus (embryogenic and non-embryogenic callus) was not significantly different ($p \leq 0.05$) among all cultivars. In general, cultivar Bwanjule had the highest frequency (51.3%) of callus while Semanda had the lowest (40.3%) frequency (Figure 4.2). However, both the plant growth regulator (PGR) and plant organ were found to have a significant effect on total callus induced when investigated within cultivars. PGR had a very high significant ($p \leq 0.05$, Table 4.3) effect on callus induction frequency (Table 4.2).
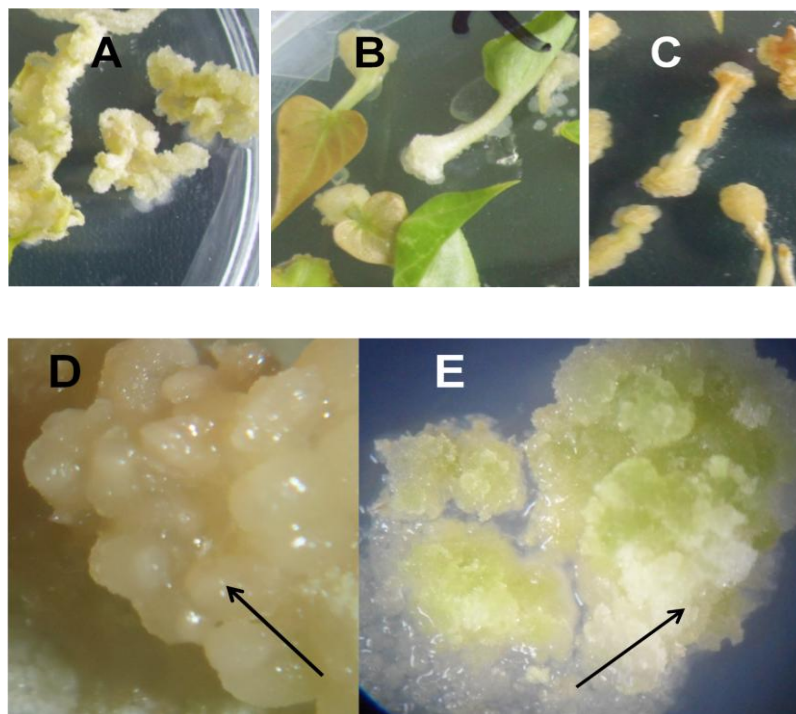
Figure 4.1: Callus formation from different types of explants.

  A, Leaf disc explants. **B,** Whole leaf explants. **C,** Petiole explants. **D,** Compact bright yellow embryogenic sections of callus (arrow). **E,** White and friable non-embryogenic sections of callus (arrow).

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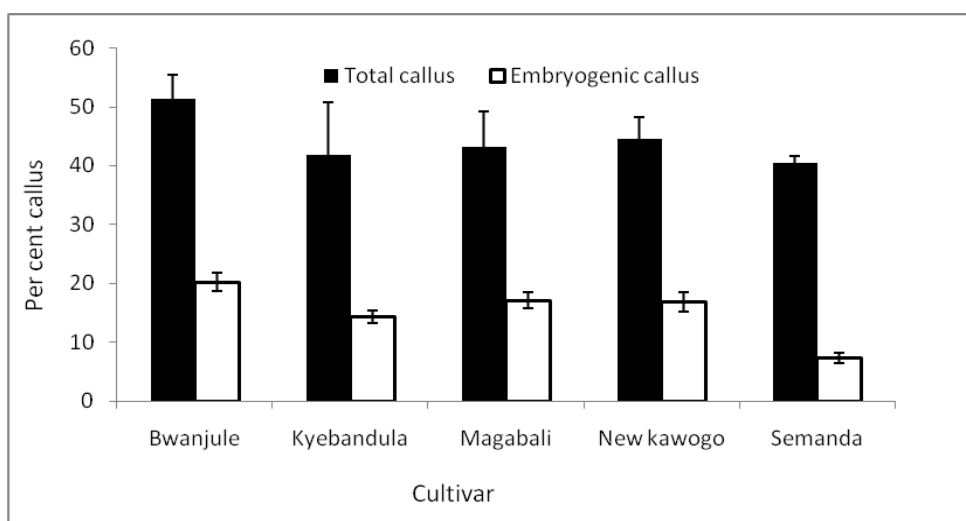
Figure 4.2: Effect of cultivar on callus induction and formation of embryogenic callus. Values are means of 10 observations (explants per petri dish replicated three times). Error bars were computed at $\alpha = 5\%$.

Table 4.2: Mean sum of squares and F- probability for frequency of callus and embryogenic callus generated under different conditions.

Source of variation	df	Callus (Frequency)		Embryogenic callus (Frequency)	
		MS	F- prob.	MS	F- prob.
Cultivar	4	0.12497	0.274	1.5055	0.002
PGR	2	3.11263	< 0.001	6.7132	< 0.001
Plant organ	2	0.3251	0.038	0.1482	0.628
Cultivar x PGR	8	0.10124	0.4	0.1714	0.823
Cultivar x Plant organ	8	0.14285	0.171	0.2796	0.535
PGR x Plant organ	4	0.19015	0.103	2.1552	< 0.001
Cultivar x PGR x Plant organ	16	0.17737	0.036	0.4934	0.097
Residual	90	0.09576		0.3168	

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The auxin type had a significant ($p \leq 0.05$) effect on the frequency of embryogenic callus. The auxin found to be best for induction of both total callus and embryogenic callus for all the 5 Ugandan genotypes investigated in this study was 2,4-D (Figure 4.3). Mean comparisons (LSD, $p < 0.05$) for total callus induction showed that petioles and whole leaves were not significantly different although the callus produced from petioles of all cultivars was higher than for both whole leaf and leaf disc (Figure 4.4). Similar results were found when means of different plant organs were investigated for their contribution to proliferation of embryogenic callus. On the other hand, the cultivar deployed did not have a significant effect on the frequency of callus proliferated although a significant ($p \leq 0.05$) effect was observed for the production of embryogenic callus.
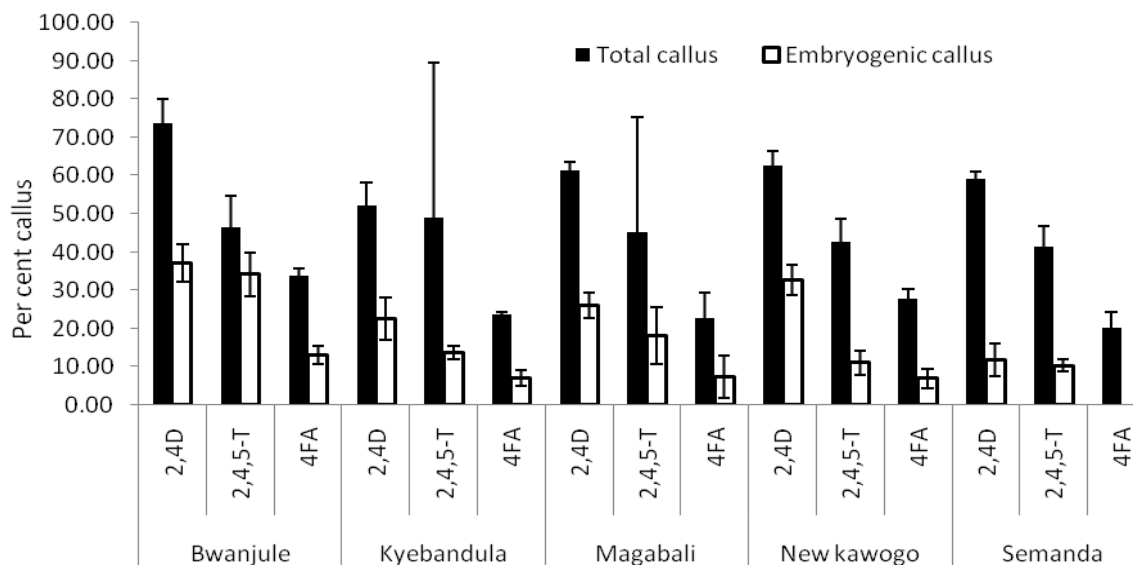
Figure 4.3: Effect of type of auxin on induction of callus and formation of embryogenic callus.

Values are means of 10 observations (explants per petri dish replicetd three times). Error bars were computed at $\alpha = 5\%$.

Figure 4.4: Effect of type of plant organ on induction of embryogenic callus.

Values are means of 10 observations (explants per petri dish replicetd three times).

Error bars were computed at α = 5%.

4.3.2 Plant regeneration from embryogenic callus

Regeneration of plants from embryogenic callus was achieved within 5 months in three non-Ugandan cultivars (Figure 4.5). Chi-square analysis showed that PGR had a significant ($p \leq 0.05$) effect on plant regeneration. Cultivar Jonathan regenerated on all the four types of media F25, F9, G24D and PGR-free. The highest number of plants of this cultivar was regenerated in F9 media (Table 4.3). A similar trend was observed for cultivar Huachano. Except for F25, all the other media led to the production of roots directly from callus in cultivar Huachano and Jonathan, with G24D media leading to high production of roots (Figure 4.5). The production of roots was observed in all cultivars, except for Bwanjule, and high frequency of roots was produced on G24D media for all cultivars, except for cultivar Jewel (Table 4.3).

Table 4.3: Effect of type of medium on regeneration from whole leaf-derived embryogenic callus

Sweetpotato variety*	Type of medium	Number of callus clusters that produced shoots	Number of callus clusters that produced roots
Huachano	F25	0	0
	F9	4	2
	PGR-free	1	2
	G24D	1	4
Jonathan	F25	3	0
	F9	7	5
	PGR-free	2	2
	G24D	1	6
Jewel	F25	2	0
	F9	3	0
	PGR-free	1	2
	G24D	1	0
New Kawogo	F25	0	0
	F9	0	2
	PGR-free	0	0
	G24D	0	3
Bwanjule	F25	0	0
	F9	0	0
	PGR-free	0	0
	G24D	0	0
Magabali	F25	0	0
	F9	0	3
	PGR-free	0	1
	G24D	0	6

* = A total of 10 embryogenic callus masses were tested for each type of medium and sweetpotato cultivar.
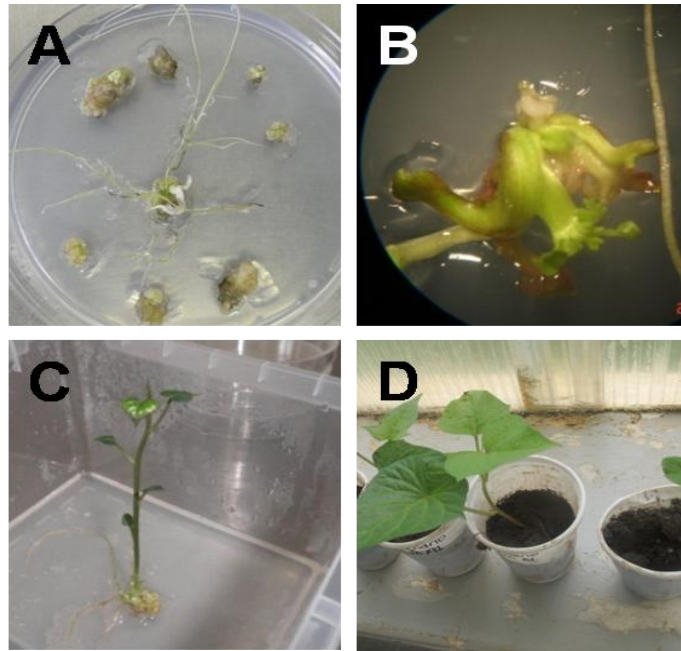
Figure 4.5: Regeneration of sweetpotato from embryogenic callus.

> **A**, Formation of roots directly from callus. **B**, Emergence of amorphous shoots from embryogenic tissue removed from callus and placed directly on regeneration media. **C**, Growth of shoots and formation of leaves on shoots emerging from embryogenic callus. **D**, Development of regenerated plants after transfer from media to soil.

4.4   Discussion

The present study was initiated with the aim of developing a protocol for induction of embryogenic callus from recalcitrant African cultivars. These cultivars have not been reported in previous regeneration and transformation work. Particularly in important African sweetpotato cultivars, an efficient regeneration system has been very difficult to establish (Luo *et al.,* 2006). Other reports show that there is a correlation between the quality and quantity of embryogenic callus and subsequent plant regeneration in sweetpotato (Al-Mazrooei *et al.,* 1997). Therefore it is important to first establish a system for somatic embryogenesis for the recalcitrant cultivars to facilitate regeneration and enable application of genetic engineering to improve important traits (Anwar *et al.,* 2010; Moran *et al.,* 1998).

In order to establish a procedure for induction of high frequency of somatic embryogenesis that could be used in genetic transformation experiments, various plant organs and plant growth regulators that were previously reported in successful sweetpotato transformation were tested. Up to five popular Ugandan cultivars were investigated since previous reports consistently show that cultivar has a high influence on somatic embryogenesis (Al-Mazrooei *et al.,* 1997). Somatic embryogenesis was selected as a method of regeneration in this study because it results in high efficiency of selection during transformation since each whole plant originates from a single transformed cell (Sihachakr *et al.,* 1997). It is important that plant regeneration is of single cell origin, avoiding possibility of chimeras or escapes after genetic transformation (Gong *et al.,* 2005).

In this work, 2,4-D was identified as the best auxin for inducing embryogenic callus. There were significant ($p \leq 0.05$) differences in the effect of the auxins for production of embryogenic callus (Table 4.3). Various reports demonstrate the best embryogenic response from medium containing 2,4-D (Cipriani *et al.,* 2001; Sihachakr *et al.,* 1997). However other workers report that 2,4,5-T is better than 2,4-D in inducing embryogenic callus (Al-Mazrooei *et al.,* 1997; Triqui *et al.,* 2007). Successful regeneration of transgenic plants from five Japanese cultivars has also been reported when 4FA was used while there was no success with 2,4-D (Anwar *et al.,* 2010). The auxin concentrations investigated in this study were determined from those previous studies that showed successful embryogenesis over a wide number of sweetpotato cultivars (Anwar *et al.,* 2010; Cipriani *et al.,* 2001; Song *et al.,* 2004; Triqui *et al.,* 2007).

Although type of plant organ explanted did not influence the frequency of total callus, as much as plant growth regulator did, it was found to have a highly significant effect on the quality of the induced callus. In general 'petiole with lamina' explants were able to induce the highest frequency of embyogenic callus, although in some cultivars the results were comparable with 'petiole' explants. Importantly, all the shoots and roots regenerated in this work only emerged from callus of 'leaf with petiole' explants. In recent studies of induction and regeneration of adventitious shoots from sweetpotato it was found that petiole explants were the least responsive as compared to both leaf and stem explants (Gong *et al.,* 2005).

Plants were regenerated from cultivar Huachano within 5 months in the present study. This contrasts with the reported regeneration system by Kreuze *et al.* (2008) which took 12 months to regenerate this cultivar. The reduction of time to regenerate plants is of high significance since long time culture of callus-derived plants increases the chance for somaclonal variation. Other researchers have reported rapid regeneration of Jewel through organogenesis (González *et al.,* 2008; Luo *et al.,* 2006). Although these workers regenerated plants within ten weeks, they encountered the presence of escapes when polymerase chain reaction (PCR) was used to examine transformed plants regenerated through organogenesis. In contrast, workers who used somatic embryogenesis have reported 100% transformation frequency among all regenerated plants although the number of plants regenerated was low (Song *et al.,* 2004).

Although the number of plants produced in the present study is low, the protocol demonstrates reproducibility for the reported cultivars since plants were regenerated using embryogenic callus obtained from three different experiments. A reproducible protocol is more important than regenerating a high number of plants in a single experiment (Yu *et al.,* 2007). Low numbers of regenerated plants have been reported in many previous studies with sweetpotato cultivars from different geographical areas (Otani *et al.,* 2003).

The repetitive regeneration of cultivars Huachano, Jonathan and Jewel in a short time through somatic embryogenesis in this study is a critical breakthrough although some researchers regenerated these popular cultivars previously. It is difficult to reproduce regeneration results from one experiment to the next or even from one laboratory to another. Moran and co-workers (1998) reported that many protocols that had previously been reported to lead to plant regeneration did not produce good results in their hands. The inconsistencies in regeneration responses within the same cultivar may be due to a variation in the developmental and physiological stage of *in vitro* plants, affecting the cultural behaviour of explants (Jones *et al.,* 2007; Triqui *et al.,* 2007). For instance, the sweetpotato cultivar Duclos 11 which had shown ability to regenerate plants from protoplast-derived callus (Sihachakr and Ducreux, 1987) did not give any embryogenic response when lateral buds were used later (Sihachakr *et al.,* 1997).

The production of roots from callus of cultivars Huachano, Jonathan and Jewel in media supplemented with 2,4-D may be useful for the initiation of root cultures, which may serve as a source of tissue for regeneration of plants or hairy root cultures (Jones *et al.,* 2007). The regeneration of shoots was difficult following the protocol of Cipriani *et al.* (2001) who placed embryogenic callus on G24D medium. Plant regeneration was improved in this work when minor modifications were made to the protocol of Cipriani *et al.* (2001) through placement of embryogenic callus on any of the following medium: F25 (ABA), F9, G24D (2,4-D and GA_3) and PGR-free medium (Table 4.3). Both ABA and GA_3 are required after somatic embryos have been formed but have been found to have a negative effect on regeneration if applied earlier (George *et al.,* 2008). In some reports ABA has been used for synchronization and maturation of pre-formed somatic embryos of sweetpotato while GA_3 has been shown to elongate pre-formed somatic embryos (Anwar *et al.,* 2010; George *et al.,* 2008; Song and Yamaguchi, 2006).

In this study, the highest number of plants was regenerated on F9 media (Table 4.3). The production of roots was observed in all cultivars, except cultivar Bwanjule, and high frequency of roots was observed in G24D media except for cultivar Jewel (Table 4.3). Except for Bwanjule and New Kawogo, all cultivars produced roots directly from callus on medium without PGR. The reason for regeneration of roots on PGR-free medium is not straightforward although it is possible that there were high levels of endogenous auxins in the explanted organs (Becerra *et al.,* 2004; George *et al.,* 2008).

This study has identified growth regulators, plant organ type and some popular African cultivars for high production of embryogenic callus, a major constraint to regeneration through somatic embryogenesis. Additionally, by successfully reducing the time for regeneration of the non-African cultivar Huachano, the present study has overcome a major setback in regeneration through somatic embryogenesis.

**THIDIAZURON IMPROVES ADVENTITIOUS BUD AND SHOOT
REGENERATION IN RECALCITRANT SWEETPOTATO**

5.1   Introduction

The protocols of regeneration that have shown potential for application in genetic transformation of sweetpotato include somatic embryogenesis (Kreuze *et al.,* 2008; Song *et al.,* 2004) and organogenesis (Gosukonda *et al.,* 1995a; Luo *et al.,* 2006; Santa-Maria *et al.,* 2009). An important advantage with somatic embryogenesis is that it improves the efficiency of selection of transformed plants on media (Song *et al.,* 2004). However, somatic embryogenesis remains a difficult process to control for the regeneration of sweetpotato *in vitro* (Song *et al.,* 2004; Yu *et al.,* 2007). On the other hand, despite shoot organogenesis having low selection efficiency for transformed plants, the shorter time required to regenerate plants as compared to somatic embryogenesis protocols makes organogenesis a more attractive regeneration method (Luo *et al.,* 2006; Santa-Maria *et al.,* 2009). In addition, although few plants are regenerated in organogenesis as compared to somatic embryogenesis, the plants regenerated through organogenesis are usually independent transgenic events (Choi *et al.,* 2007; Luo *et al.,* 2006).

In recent years, interest has grown for the application of TDZ in both adventitious and somatic embryogenesis regeneration of plants. Originally, TDZ, a synthetic phenylurea-type plant growth regulator, was considered as a cytokinin inducing responses similar to those caused by natural cytokinins (Guo *et al.,* 2011). However, TDZ is able to induce both cytokinin and auxin morphogenic responses (Jones *et al.,* 2007). It can affect meristem induction, cause shoot development from pre-formed meristems and induce adventitious bud and shoot formation in different plant species including recalcitrant woody plants (Cuenca *et al.,* 2000). Pretreatment with TDZ can predispose a tissue to accept other inductive stimuli (Guo *et al.,* 2011). Alternately, exposure to TDZ can commit a tissue to regenerative route that is expressed even after the inductive stimulus is removed.

Thidiazuron (TDZ) has been used as a plant growth regulator to induce organogenesis, including adventitious regeneration of transformed plants in many plant species that were

thought to be recalcitrant to regeneration (Corredoira *et al.,* 2008; Cuenca *et al.,* 2000; Sriskandarajah and Lundquist, 2009). The current study was, therefore, initiated to study the effect of TDZ on morphogenesis of *I. batatas* with the aim of inducing adventitious shoots. The ultimate goal of this study was to use the regeneration protocol in genetic transformation of sweetpotato to improve its traits, particularly resistance to weevils, for which the genes have already been identified (Ekobu *et al.,* 2010).

5.2   Materials and Methods

5.2.1   Preparation of plant material and culture conditions

Two popular Ugandan sweetpotato cultivars, namely Kyebandula and Bwanjule, were used in this study. Vigorously growing vines from screenhouse plants were used to provide cuttings for establishment of *in vitro* stock cultures. The cuttings were sterilised according to the protocol of Song *et al.* (2004), except for increased immersion time in sterilising solution to from 15 to 20 minutes since a low concentration (2.0 %) of NaOCl was used. The sterile cuttings were then inserted into sweetpotato propagation (SP) medium in magenta boxes.

The medium was composed of MS (Murashige and Skoog, 1962) salts premix (4.3 g l^{-1}), sucrose (30 g l^{-1}), myo-inositol (0.1 g l^{-1}), Indole-3 acetic acid (IAA) (1.0 µM), 5 ml l^{-1} sweetpotato vitamin stock comprised of 40 g l^{-1} Ascorbic acid, 20 g l^{-1} L-arginine, 4 g l^{-1} putrescine HCl, 5.8 µM gibberellic acid (GA$_3$) and 0.4 g l^{-1} calcium pantothenate (Kreuze *et al.*, 2008). All reagents used were supplied by Sigma-Aldrich Chemie GMBH, Eschenstrasse, Taufkirchen. The medium was adjusted to pH 5.8 before adding 3 g L^{-1} phytagel, followed by autoclaving at 121 °C for 15 minutes at 15 kPa.

5.2.2   Effect of TDZ on adventitious bud induction and regeneration

This study was conducted to assess the effect of various concentrations of TDZ on induction of adventitious buds and and the conversion of adventitious buds to shoots in *I. batatas.* Bud induction medium was composed of MS basal salts, sucrose (30 g l^{-1}), myo-inositol (0.1 g l^{-1}) and sweetpotato vitamin stock (1 ml l^{-1}). Various concentrations of TDZ (0.5, 2.0 and 4.0 µM) were added to the medium after autoclaving.

Intact leaves with petiole (1.0 – 1.5 cm long) and stem internode segments (0.6 - 1.0 cm) were cut from 4-week-old *in vitro* cultures growing in SP medium in magenta boxes. The stem internode segments and leaves (with adaxial side facing up) were cultured on 25 ml of a semi-solid bud induction medium in plastic petri dishes. The base of the petiole was partially embedded into the bud induction medium, while the stem internode pieces placed horizontally on the medium were partially pressed into the medium.

The petri-dishes containing the cultures were placed in dark for 4 weeks at 25 °C to induce adventitious buds before transfer to 16 hours photoperiod under the same temperature for shoot regeneration as reported by Cuenca *et al.* (2000). The cultures were transferred onto fresh medium of the same composition as bud induction medium every 4 weeks. This duration was sufficient to ensure that the media components were not severely degraded.

5.2.3 Effect of TDZ and NAA on adventitious bud induction and regeneration

Due to low frequency of conversion of adventitious buds into shoots, the effect of the auxin, α-Naphthalene acetic acid (NAA), on conversion of adventitious buds into shoots was evaluated. In this experiment each of the three TDZ concentrations investigated earlier was added to MS medium in combination with the NAA (0.25 μM). The inclusion of NAA in the bud induction media was based on previous reports of regeneration from recalcitrant woody plant species (Corredoira *et al.*, 2008; Sriskandarajah and Lundquist, 2009).

5.2.4 Effect of age of explants on adventitious bud induction and regeneration

This experiment was carried out in order to determine whether bud regeneration frequency could be affected by internode position (age) on the mother plant. Cv. Kyebandula which exhibited high regeneration frequency was used. Five internode positions were distinguished, with node 1 being the apical-most node with unfolded leaves and node 5 the lowest from the shoot apex. The stem internodes were cut off from the mother plant as described above. These were grouped according to their position on the mother plant before culturing in petri dishes labeled according to that position. The bud induction medium was supplied with 4.0 μM TDZ combined with 0.25 μM NAA. The petri-dishes containing the cultures were sealed and cultured for four weeks in dark, followed by transfer to 16 hours photoperiod as described above.

5.2.5   Effect of duration on TDZ medium on adventitious bud formation

In this experiment, only stem internodes from positions 2, 3 and 4 of cv. Kyebandula were explanted. The internode pieces were explanted on medium containing only 4.0 μM TDZ as the only plant growth regulator (PGR). In order to assess the effect of duration of explants on medium containing TDZ, half of the explants were placed on the medium with TDZ for only 3 days; while the remaining half was allowed to stay on this medium for 7 days. After the designated period on TDZ-based medium, each group of explants was further divided into two equal groups and one group was transferred to medium containing 0.25 μM NAA while the remaining one group of the explants was transferred to PGR-free medium.

5.2.6   Experimental design and statistical analysis

All experiments were laid out in a completely randomised design. Three petri-dishes, each containing 10 explants, were used in each experiment. This gave a total of 30 explants for each experiment. These experiments were repeated three times. After 4 weeks in culture, data on total number of explants with adventitious shoot buds and number of buds per explants were recorded. The data on number of explants with adventitious shoots (with well developed leaves and rooted), number of adventitious shoots per explants, number of explants with roots and number of roots per explants were collected after 12 weeks.

The frequency of explants regenerating adventitious buds and shoots was calculated by expressing the number of explants regenerating buds or shoots as a percent of the total number of explants investigated. The frequency data were transformed using the arcsine square root before analysis to stabilise the variance. Statistical analyses were done using Analysis of variance (ANOVA) and means were compared using the least significant difference (LSD) test at the $P \leq 0.05$ level.

5.3   Results

5.3.1   Effect of TDZ on adventitious bud induction and regeneration

After three days of placement on bud induction medium, explants started showing signs of expansion. The swelling was more pronounced at the cut ends of stem explants and at the

cut base of the petioles of leaf explants (Figure 5.1). Most explants formed non-regenerative callus at the cut ends within 2 weeks of placement on the bud medium. This phenomenon was evident in all concentrations of TDZ (data not presented).
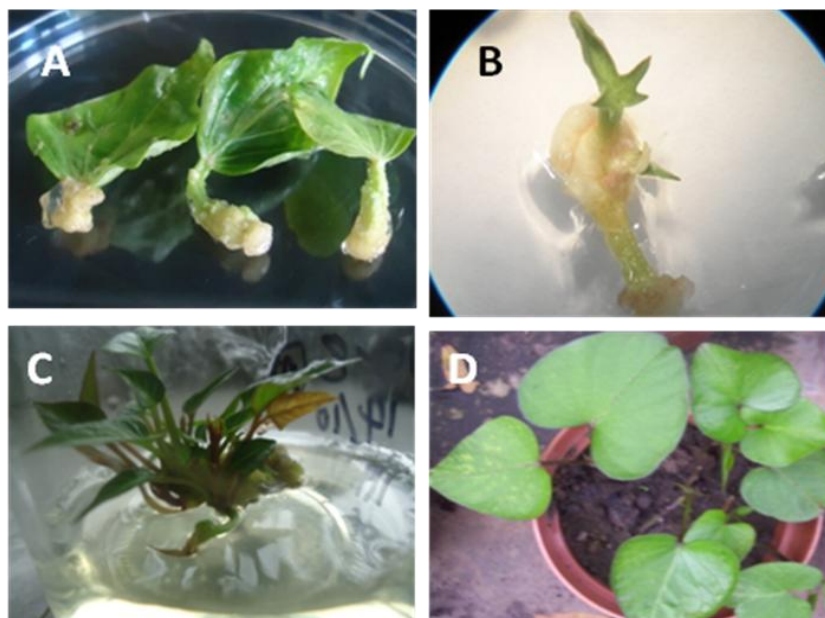
Figure 5.1: Adventitious shoot bud regeneration and plant development in *Ipomoea batatas*.

(**A**) Adventitious bud protrusion along the length of petiole of leaf explants after 2 weeks on bud induction MS media with 4.0 µM TDZ, followed by another week on MS medium supplemented with 0.25 µM NAA. (**B**) Elongation of adventitious buds leading to development of shoots on stem internode explants. (**C**) Development of multiple adventitious shoots on stem internode explants. (**D**) Growth of plants in soil 4 weeks after acclimatisation in the greenhouse.

All of the TDZ treatments induced multiple adventitious buds from the explants within the same 2 weeks (Table 5.1). In general, the number of stem explants that induced adventitious buds was higher than that for leaf explants. The highest number of buds was formed on 4.0 µM TDZ for both cultivars. The mean number of stem segments that formed buds on 4.0 µM TDZ was 67% for cv. Kyebandula and 59% for cv. Bwanjule. The lowest number of explants that induced adventitious buds was recorded for 0.5 µM TDZ. The number of buds per explants increased with TDZ concentration (Table 5.1). The number of explants producing adventitious buds and the number of buds per explant varied

significantly ($P \leq 0.001$) with both explant type and concentration of TDZ. Type of cultivar only showed a significant ($P \leq 0.01$) effect on the number of buds formed per explants.

Shoots were regenerated in all TDZ concentrations, from both types of explants and both cultivars; although overall the buds developing into shoots were low. The highest mean number of explants forming shoots was 17.5% on 2.0 µM TDZ for stem explants of cv. Kyebandula and 21.6% on 0.5 µM TDZ for cv. Bwanjule. This high shoot regeneration was achieved for stem explants. None of the investigated factors showed significant effect on number of explants regenerating shoots. Type of explant was the only factor that had a significant ($P < 0.001$) effect on number of shoots per explant.

Table 5.1: Effect of TDZ concentration (µM), cultivar and types of explant on bud induction and shoot regeneration frequency (%)

PGR (µM)	Cultivar	% Explants regenerating buds		Buds per explant (No.)		% Explants regenerating shoots		Shoots per explant (No.)	
		Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf
TDZ (0.5)	Kyebendula	44.4 ± 4.8a	22.2 ± 7.8a	4.7 ± 0.9a	2.3 ± 1.2a	12.1 ± 12.12a	16.7 ± 16.67a	0.7 ± 0.7a	0.3 ± 0.3a
TDZ (2.0)	Kyebendula	51.1 ± 2.9b	20.0 ± 5.1a	7.3 ± 0.33b	5.3 ± 1.2b	17.47 ± 2.34a	14.07 ± 7.07a	1.2 ± 0.6a	0.3 ± 0.3a
TDZ (4.0)	Kyebendula	66.7 ± 8.4c	41.1 ± 2.2b	9.3 ± 0.9c	6.0 ± 1.5b	8.22 ± 2.79a	3.03 ± 3.03a	1.7 ± 0.7a	0.3 ± 0.3a
TDZ (0.5)	Bwanjule	38.9 ± 6.2a	18.9 ± 2.9b	4.33 ± 0.7a	1.7 ± 0.9a	21.61 ± 14.2a	0.00 ± 0.00a	1.0 ± 0.6a	0.3 ± 0.3a
TDZ (2.0)	Bwanjule	47.8 ± 1.1b	20.0 ± 0.0b	4.3 ± 0.7a	1.7 ± 0.9a	9.21 ± 2.06a	5.56 ± 5.56a	1.0 ± 0.6a	0.3 ± 0.3a
TDZ (4.0)	Bwanjule	58.9 ± 4.4c	37.8 ± 5.6a	6.3 ± 1.2b	5.0 ± 0.6b	3.98 ± 2.03a	2.56 ± 2.56a	0.7 ± 0.3a	0.3 ± 0.3a

Values followed by the same letter in each column are not significantly (ns) different at the P ≤ 0.05 level (LSD test).

5.3.2 Effect of TDZ and NAA on adventitious bud induction and regeneration

Culturing explants on medium containing both TDZ and NAA reduced the frequency of explants regenerating adventitious buds and number of buds per explants. However, the same medium increased the mean number of explants forming shoots and the number of shoots per explant, compared with cultures where TDZ was supplied alone. In addition, like for treatments with TDZ alone, 4.0 µM TDZ was the best concentration at inducing explants to differentiate multiple buds. The bud induction frequency for stem explants was 66.7 and 58.9%, for cv Kyebandula and cv Bwanjule, respectively, when TDZ (4.0 µM) was supplemented alone. However, the bud induction frequency dropped to 58.8 and 48.9%, for cv Kyebandula and cv Bwanjule, respectively, when TDZ (4.0 µM) was supplemented together with NAA.

On the other hand, the presence of NAA improved the frequency of conversion of adventitious buds into shoots, especially in the presence of low concentration (0.5 µM) of TDZ. Shoot regeneration frequency improved from 12.1 to 22.6% for cv Kyebandula, when NAA was added to the TDZ-based medium. The increase in shoot regeneration frequency for cv Bwanjule under the same conditions was from 21.6 to 42.9%. Stem explants consistently performed better than leaf explants for all variables investigated (Table 5.2). Type of explants was significant ($P< 0.05$) in affecting the mean number of explants regenerating shoots and the number of shoots per explant. However, these two variables were not affected by type of cultivar and concentration of TDZ in NAA-containing medium. All the three factors, TDZ concentration, type of plant organ and cultivar, had a significant ($P< 0.05$) effect on the frequency of explants forming buds and the mean number of adventitious buds per explant (Table 5.2).

Table 5.2: Effect of TDZ concentration (µM) on bud induction and shoot regeneration frequency in the presence of NAA (0.25 µM)

PGR (µM)	Cultivar	% Explants regenerating buds		Buds per explant (No.)		% Explants regenerating shoots		Shoots per explant (No.)	
		Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf
TDZ (0.5)	Kyebendula	40.0 ± 9.62a	23.3 ± 3.85a	4.3 ± 0.3a	2.0 ± 0.6a	22.6 ± 8.5a	34.0 ± 3.3a	2.0 ± 0.0a	1.6 ± 0.3a
TDZ (2.0)	Kyebendula	41.1 ± 4.84a	23.3 ± 3.85a	8.0 ± 1.0b	5.7 ± 0.7b	28.3 ± 20.8a	38.0 ± 14.3a	2.7 ± 0.9a	2.3 ± 0.3a
TDZ (4.0)	Kyebendula	58.9 ± 7.29b	42.2 ± 2.22b	8.0 ± 1.5b	4.3 ± 1.5ab	21.2 ± 2.7a	11.1 ± 5.6a	2.0 ± 0.0a	1.2 ± 0.6a
TDZ (0.5)	Bwanjule	31.1 ± 4.01a	21.1 ± 2.22a	3.3 ± 0.7a	2.0 ± 0.6a	42.9 ± 14.3a	6.7 ± 6.7a	1.8 ± 0.2a	0.3 ± 0.3a
TDZ (2.0)	Bwanjule	40.0 ± 1.92b	15.6 ± 4.01b	2.3 ± 0.7a	2.0 ± 0.6a	17.5 ± 9.5a	38.1 ± 31.2a	1.7 ± 0.3a	2.0 ± 1.5a
TDZ (4.0)	Bwanjule	48.9 ± 11.60c	27.8 ± 5.88c	4.7 ± 1.5a	4.3 ± 1.9a	8.5 ± 4.3a	26.1 ± 8.4a	1.5 ± 0.9a	1.5 ± 0.3a

Values followed by the same letter in each column are not significantly (ns) different at the $P \leq 0.05$ level (LSD test).

5.3.3 Effect of age of explants on adventitious bud induction and regeneration

Adventitious buds and shoots were induced from all five internodal positions investigated (Figures 5.2 and 5.3). Internode position 3 gave the best results; while the oldest internode (position 5) was the least responsive followed by the youngest (position 1) internode. Internode position was significant ($P < 0.05$) in affecting the mean number of explants differentiating buds, the number of buds per explant, the number of explants regenerating shoots and the number of shoots per explant.
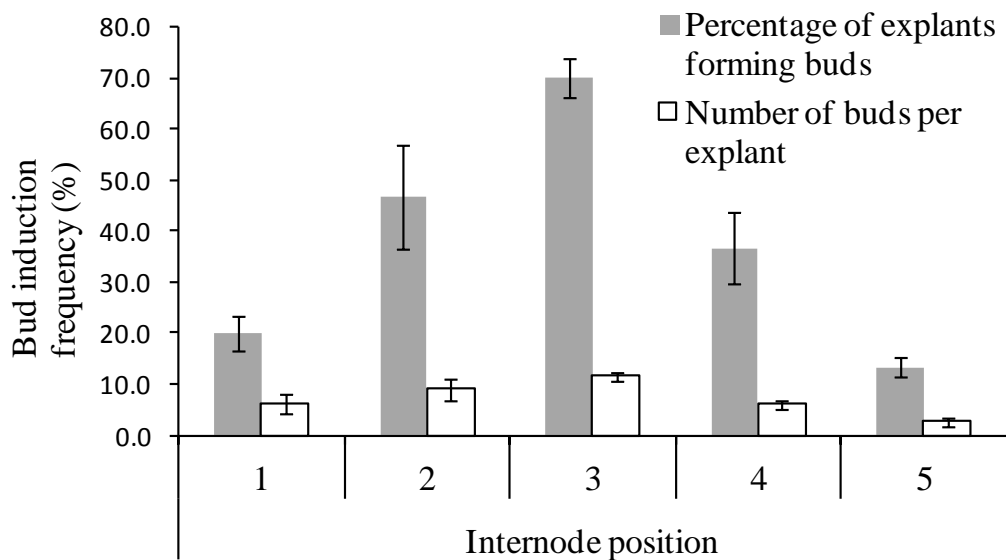
Figure 5.2: Effect of internode position on adventitious bud initiation.

Five internode positions were distinguished along the shoot with internode 1 being at the shoot apex and internode 5 being the lowest from the shoot apex. Data represent means from three replicates (30 explants in each). Vertical lines indicate standard errors of the means.

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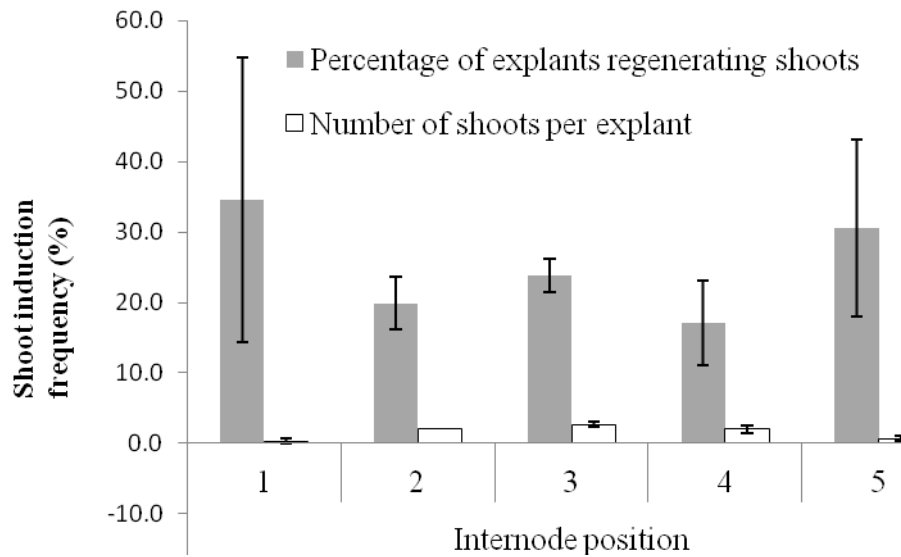
Figure 5.3: Effect of internode position on adventitious shoot regeneration.

Five internode positions were distinguished along the shoot with internode 1 being at the shoot apex and internode 5 being the lowest from the shoot apex. Data represent means from three replicates (30 explants in each). Vertical lines indicate standard errors of the means.

5.3.4 Effect of duration of explants on TDZ on adventitious bud formation

The best results were obtained when stem internode segments were cultured on bud induction medium (4.0 µM TDZ) for 7 days followed by cultures on medium containing 0.25 µM NAA. The 4.0 µM concentration of TDZ was best for adventitious bud induction according to initial experiments (above). The removal of TDZ from media without supplying NAA resulted in reduction of both bud and shoot regeneration. The highest frequency (61.1%) of explants differentiating adventitious buds was achieved when duration on TDZ was increased from 3 to 7 days before transfer to NAA medium (Table 5.3). The number of explants-forming shoots was also significantly (P< 0.001) higher for this treatment. However, the number of shoots per explant was not significantly affected by duration on TDZ or subsequent placement on either NAA or PGR-free medium. These results show that the frequency of explants inducing adventitious buds and the number of adventitious buds per explants was higher than the best of the experiments on effect of supplementing 0.25 µM NAA together with TDZ in medium (Tables 5.2 and 5.3).

Table 5.3: Effect of duration on medium supplemented with TDZ (4.0 µM) on bud induction and shoot regeneration frequency from stem explants

First media (Duration)	Second media	Frequency (% explants forming buds)	Buds per explant (No.)	Frequency (% explants forming shoots)	Shoots per explant (No.)
TDZ (3 days)	NAA	40.0 ± 12.0ba	6.0 ± 1.0b	18.2 ± 6.6c	1.0 ± 0.6a
TDZ (3 days)	PGR-Free	3.3 ± 3.3cb	1.3 ± 1.3c	0.0 ± 0.0c	0.7 ± 0.7a
TDZ (7 days)	NAA	61.1 ± 4.0a	13.0 ± 1.2a	55.8 ± 7.1b	3.0 ± 0.6a
TDZ (7 days)	PGR-Free	20.0 ± 3.3bc	3.7 ± 0.3ab	13.1 ± 7.2c	1.0 ± 0.6a

Values followed by the same letter under each variable are not significantly (ns) different at the $P \leq 0.05$ level (LSD test).

5.4　Discussion

Adventitious bud and shoot regeneration of sweetpotato (*Ipomoea batatas)* was achieved from two recalcitrant cultivars, Bwanjule and Kyebandula within 12 weeks (Table 5.1). However, the number of plants regenerated using the reported protocol were highly dependent on the number of explants investigated since the number of shoots regenerated per explant was considerably low. Nevertheless, the results achieved in this study are very important for the breeding of *I. batatas*, particularly African cultivars, which have been reported to be difficult to regenerate through both somatic embryogenesis and organogenesis; and are still considered recalcitrant to *in vitro* regeneration (Luo *et al.*, 2006). The plants regenerated in the present study do not originate from pre-formed buds but *de novo* buds induced by TDZ. It is important that direct regeneration without an intervening callus stage should be of single cell origin, avoiding possibility of chimeras after genetic transformation (Gong *et al.*, 2005).

Recently, there has been increased application of TDZ in somatic embryogenesis and adventitious regeneration of recalcitrant plants, particularly woody species (Corredoira *et al.*, 2008; Cuenca *et al.*, 2000; Sriskandarajah and Lundquist, 2009). However, the use of TDZ-based protocols for the regeneration of *I. batatas* has only been reported by few workers (Gosukonda *et al.*, 1995a; Gosukonda *et al.*, 1995b; Kumar *et al.*, 2007). When explants were cultured on TDZ-supplemented medium, multiple adventitious buds were induced along the length of explants. Some of the buds successfully elongated into shoots that developed into plants (Figure 5.1). These observations were common for all the TDZ concentrations (Table 5.1). Kumar *et al.* (2007) reported regeneration through adventitious

shoots on media containng cytokinins TDZ, benyladenine or 2-isopentenyladenine singly or TDZ combined with Indole acetic acid (IAA).

Although preliminary studies with 45.0 µM TDZ led to regeneration of sweetpotato from cultivar Kyebandula, most of the explants died after turning brown within 4 weeks (data not presented). In spite of the observation that all trials with TDZ led to regenerants, higher doses of TDZ inhibited further growth and development of the regenerants (Table 5.1). When Gosukonda *et al.* (1995a) evaluated various TDZ concentrations (0.0 to 0.4 mg L^{-1}) for the regeneration of sweetpotato, they found that 1.0 µM (0.2 mg L^{-1}) was optimal for regeneration of adventitious shoots from two of six cultivars grown in Americas. Later, Kumar *et al.* (2007) reported that 5.0 µM (1.0 mg L-1) TDZ was optimal for adventitious shoot regeneration from leaf explants of 'Tainong (T) 57', 'T 64' and 'T 66' sweetpotato cultivars from Japan. In this study, reduced concentration of TDZ generally led to improved shoot regeneration (Table 5.1). The same trend was observed for number of shoots per explants for cultivar Kyebandula.

Although the successful plant regeneration in this study is due to the use of TDZ, there was low frequency of conversion of adventitious buds into shoots in the presence of this plant growth regulator. Higher (2.5 and 5 µM) TDZ concentrations inhibited shoot elongation and stimulated callus production in *Phaseolus vulgaris* L. and *Vicia faba* L. (Mohamed *et al.*, 1992). Several authors reported that TDZ-induced buds do not readily elongate into shoots, a phenomenon that could be caused by high concentration of TDZ in the medium or prolonged persistence of TDZ in the explants tissues (Corredoira *et al.*, 2008; Cuenca *et al.*, 2000; Kumar *et al.*, 2007; Lyyra *et al.*, 2006). TDZ may be inhibiting shoot elongation due to its cytokinin activity leading to increased level of endogeneous cytokinin which inhibits effect of cytokinin oxidase and endogenous auxin (IAA) (Debnath, 2005).

When the auxin NAA (0.25 µM) was included in the current study, an increase in both mean number of explants forming shoots and the number of shoots per explants was recorded (Table 5.2). Similarly, auxins like IAA and NAA, have been shown to improve adventitious regeneration and shoot organogenesis in other TDZ-based protocols (Corredoira *et al.*, 2008; Sriskandarajah and Lundquist, 2009). Although the inclusion of the auxin NAA (0.25 µM) clearly led to an increase in the number of explants forming

shoots and shoots per explants in this study, the number of explants inducing buds and the number of buds per explants was affected (Table 5.2). This challenge was overcome by initially placing explants on medium with TDZ alone, followed by transfer to medium with NAA only (Table 5.3).

Although both cultivars investigated were able to regenerate plants, cultivar Kyebandula responded better than Bwanjule on all experiments. The different cultivar responses could be due to endogeneous differences in auxin and cytokinin content in the cultivars. George et al. (2005) argued that the concentration of endogeneous plant growth regulators which varies among species and cultivars has a significant effect on plant morphogenesis *in vitro*. It was also observed in the current study that stem explants were significantly better than leaf explants in both adventitious bud induction and shoot regeneration (Table 1). Gosukonda *et al.* (1995a) found leaf lamina of *I. batatas* to be the least responsive explants when compared to petioles. These workers also found TDZ better than both Zeatin riboside and Kinetin in inducing adventitious regeneration.

Although adventitious buds and shoots were induced in all intermodal segments, internode position 3 gave the best results; while the oldest (position 5) and the youngest (position 1) gave the lowest mean numbers of buds and shoots. In general, lower morphogenic capacity (adventitious shoots) is expected as the age of the donor plant increases. When experimenting with *I. batatas* leaf explants, other workers found that only second and third leaves were the best (Dessai *et al.*, 1995). Stem internode pieces from two *Fagus* species were also shown to have highest regenerative response from internodes proximal to the apical meristem, while those distal to the apex were the least productive (Cuenca *et al.*, 2000). Differences in regenerative abilities of explants of varying ages may be a result of differences in internal auxin, cytokinin and/or abscisic acid levels which in turn have a direct effect on plant morphogenesis *in vitro*.

A reliable adventitious regeneration protocol has been established for *I. batatas* cultivars, which have not been regenerated before. This protocol has potential to be extended to the regeneration of other economically important *I. batatas* cultivars. The ultimate goal is to apply this protocol in genetic transformation for improvement of *I. batatas* traits, especially for resistance to weevils.

CHAPTER SIX

TRANSIENT EXPRESSION OF *B*-GLUCURONIDASE IN RECALCITRANT UGANDAN SWEETPOTATO AND PUTATIVE TRANSFORMATION WITH TWO *CRY* GENES

6.1 Introduction

Transformation through *Agrobacterium* species is the most commonly used system since the transferred DNA has minimal rearrangement and there are only few transgene copies inserted into the plant genome (Choi *et al.,* 2007; Xing *et al.,* 2007). However, the process of transfer of foreign genes from *Agrobacterium* and integration into plant genomes is complex (Valentine, 2003). There are so many variables that affect *Agrobacterium*–mediated transformation, and no evidence of obvious combinations of these variables exists for broad application across plant species and cultivars (González *et al.,* 2008; Xing *et al.,* 2007). In addition, sweetpotato is considered recalcitrant to genetic transformation as different cultivars respond differently to transformation conditions (Yang *et al.,* 2011; Zang *et al.,* 2009). African cultivars have been reported to be particularly difficult to transform (Luo *et al.,* 2006; Tovar *et al.,* 2009).

Reporter genes have been used to develop transformation systems for cultivars that were not known to be amenable to genetic transformation (Xing *et al.,* 2007; Yu *et al.,* 2007). The common reporter genes that have been used to detect gene expression and protein localization *in vitro* include chloramphenicol acetyl transferase (CAT), green fluorescent protein (GFP) (Lawton *et al.,* 2000), luciferase (LUC), and β-glucuronidase (GUS) (Xing *et al.,* 2008). Although the *gfp* has been used in some cases (Lawton *et al.,* 2000), the GUS gene remains the most common reporter for sweetpotato transformation (Song *et al.,* 2004; Yang *et al.,* 2011; Yu *et al.,* 2007). The histochemical procedure used to demonstrate GUS activity in transformed plant tissue is very powerful even for resolving differences in gene expression between individual cell and cell types within tissues (Jefferson *et al.,* 1987).

The factors that are critical for successful genetic transformation include appropriate selective antibiotics, concentration of acetosyringone, duration of co-cultivation with

Agrobacterium, *Agrobacterium* concentration, light/dark treatment during co-culture, wounding of explants, infiltration of *Agrobacterium*, agitation during *Agrobacterium* infection, preculture and use of low temperature (González *et al.,* 2008; Xing *et al.,* 2007; Yu *et al.,* 2007). The aim of this study was to develop an *Agrobacterium*-mediated transformation system based on transient expression of *β*-glucuronidase (GUS) as a reporter gene.

6.2 Materials and Methods

6.2.1 Plant material

The Ugandan landrace Kyebandula was used in the optimisation of plant transformation conditions. *In vitro* cultures were prepared as described in Section 3.2. The cultures were used to supply whole leaf, stem internode pieces, petioles and primary root explants for co-culture with *Agrobacterium* at the National Agricultural Research Laboratories (NARL), Kawanda, Uganda.

6.2.2 Bacterial strain and plasmid for transient expression

Agrobacterium tumefaciens strain EHA105 (rifampicine resistant) harbouring the plasmid pCAMBIA1305.1 was used for transformation of cv. Kyebandula explants at NARL. The pCAMBIA1305.1 plasmid contained the β-glucuronidase (GUS) gene driven by the constitutive CaMV (cauliflower mosaic virus) 35S promoter (Figure 6.1). The transfer DNA (T-DNA) containing the GUS gene also had the hygromycin resistance gene under the double CaMV 35S promoter. *Agrobacterium* from liquid stock was streaked on solid LB medium in plates and left to grow for 3 days at 28°C in dark. The composition of LB medium was 10 g/L bacto-tryptone, 5 g/L yeast extract, 10 g/L sodium chloride and 15 g/L bacterialogical agar. The following antibiotics were added after the media was cooled; 100 mg/L Kanamycin, 25 mg/L rifampicin and 250 mg/L carbenicillin. A single colony of the *A. tumefaciens* from the three-day-old cultures was picked and grown overnight in 25 ml liquid LB medium on a shaker at 200 rpm and at a temperature of 28°C. The *Agrobacterium* cells were then centrifuged at 13,000 rpm for 10 min and the pellets were resuspended in liquid co-culture medium. Co-culture medium was composed of MS components, 30 g/L sucrose, 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 100 μM

acetosyringone. The cultures were shaken at 70 rpm for 1 hour before being used for infection of explants.
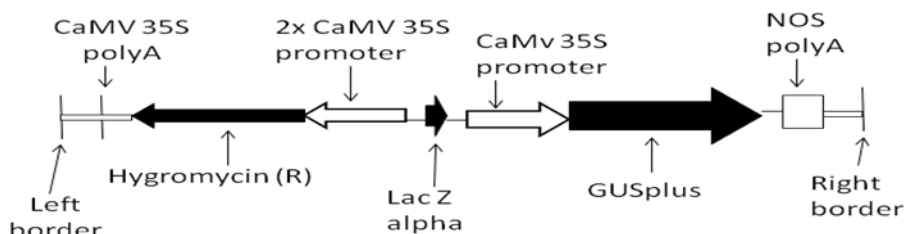
Figure 6.1: Schematic representation of T-DNA region of the pCAMBIA1305.1 plasmid

6.2.3   Histochemical localisation of GUS activity

Explants were immersed in liquid co-culture medium with various *Agrobacterium* optical density (OD) concentrations (0.4, 0.6, 0.8, 1.0) at 600 nm for 1 hr while shaking at 45 rpm. The explants were then removed from the liquid infection medium and blotted on filter-paper before placing on solid co-culture medium for 3 days in dark. The second experiment investigated the effect of co-culture duration on transformation frequency. This was conducted by immersing explants in co-culture medium with *Agrobacterium* OD concentration of 1.0 at 600 nm before placing the explants on solid co-culture medium for various days (1, 2, 3, 4, 5) depending on the experiment. This was followed by a third experiment in which explants infected with *Agrobacterium* at an OD concentration of 1.0 at 600 nm were placed on solid co-culture medium overlaid with filter-paper while another group of explants was placed on medium without filter-paper on it. The co-culture duration for this experiment was 4 days at 23°C in dark. The transformation frequency was determined by screening for transient GUS expression using histochemical localization of GUS activity after co-culture.

Following co-cultivation, the explants were harvested for GUS staining using histochemical GUS assay as described by Jefferson *et al.* (1987). The explants were washed in 70% ethanol for 2 minutes before incubation in fixation solution for 45 minutes at room temperature. The fixation solution was made by mixing 0.3% (v/v) formaldehyde,

10mM MES (pH 5.6) and 0.3 M mannitol. This was followed by washing the fixed explants with 50 mM phosphate buffer (pH 7.0) before incubation in substrate solution at 37°C for 24 hours. The substrate solution was made by mixing 50 mM sodium phosphate (pH 7.0), 5 mM potassium ferricyanide, 5 mM ferrocyanide, 10 mM EDTA, 50 mM ascorbic acid and 1 mM 5-bromo-4-chloro-3-indolyl-D-glucuronide (X-Gluc). Chlorophyll was removed by immersing the explants in a solution of methanol and glacial acetic acid (3:1) for 4 hours. The explants were then dehydrated in a series of ethanol (50, 70, and 95 %). The explants were then examined for expression of blue spots and photographed.

Each of the three experiments was carried out with 20 explants and repeated three times. Non-transformed explants were used as controls. This was achieved by placing the explants in liquid co-culture medium without *Agrobacterium* followed by treating as the transformed explants in subsequent stages. The data on frequency of explants showing GUS activity was transformed using the arcsine square root before analysis to stabilise the variance. The data presented here shows non-transformed data. Statistical analysis for the effect of the investigated parameters on GUS activity was performed using general linear model of analysis of variance (ANOVA) at 5% level of significance. Significancy between mean values was tested using the least significance difference (LSD) method at the 5% level.

6.2.4 Sensitivity of non-transformed explants to kanamycin

The sensitivity of untransformed explants to kanamycin was investigated by adding various concentrations of kanamycin (0, 25, 50, 75, 100, 125 mg/L) to callus induction medium. This was conducted in order to determine the optimal concentration for kanamycin for killing non-transformed plant cells. The callus induction medium (CIM) was MS medium with 30 g/L sucrose, 3 g/L phytagel, 1.0 mg/L 2,4-D, 250 mg/L cefotaxime and the different concentrations of kanamycin. The cultures were placed in dark at 28°C and the CIM was refreshed every 2 weeks. The survival of explants and formation of callus was assessed after 8 weeks in culture. The number of explants and replicates was as described above. The data were analyzed as described above (Section 3.5.3).

6.2.5 Transformation with weevil-resistance genes

The plasmid vector bearing the weevil-resistance genes was referred to as pCIP84. The plasmid vector was transformed into the *Agrobacterium tumefaciens* hypervirulent strain EHA105 (kanamycin resistant) through electroporation at the International potato centre (CIP), Lima, Peru (Kreuze *et al.,* 2009; Tovar *et al.,* 2009). This plasmid has a pCAMBIA2305.1 backbone. It has two weevil resistance genes, *ß-Amy:cry7Aa1* and *gSPOA1:cry3Ca1* with the *nptII* selectable marker gene in its transferred DNA (T-DNA) (Figure 6.2).
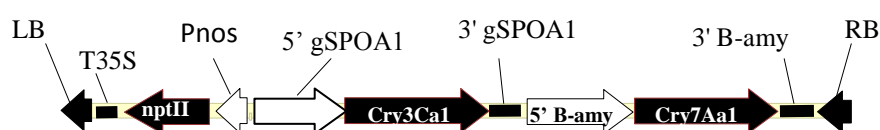
Figure 6.2: Schematic representation of T-DNA region of pCIP84 vector plasmid

6.2.6 PCR confirmation of weevil-resistance genes in plasmid pCIP84

The presence of *cry7Aa1* and *cry3Ca1* genes in the plasmid was confirmed upon receiving it at NARL. The *Agrobacterium* harbouring the plasmid pCIP84 was grown as described above, with Kanamycin (50 mg/L) added as the only antibiotic. Plasmid DNA was extracted from *Agrobacterium* cells using a plasmid extraction kit provided by Promega. A cleared lysate was achieved by pouring the *Agrobacterium* culture into a 2 ml tube and centrifugation at 13,000 rpm for 5 minutes. The available volume (20 ml) of the *Agrobacterium* culture could not fit into the 2 ml tube at once. Therefore only 1.5 ml of the *Agrobacterium* culture was added to the 2 ml tube at a time. Another 1.5 ml was added to the same tube after centrifugation of the bacterial cells and removal of the suspension. These were centrifuged as above and the process was repeated until all the *Agrobacterium* cells from the 20 ml culture were collected in the 2 ml tube. The pellet was then resuspended in 250 µL cell suspension solution. The rest of the DNA extraction process was conducted according to the instructions in the Promega kit.

The plasmid DNA was used in PCR to confirm integrity of *cry7Aa1* and *cry3Ca1* genes before embarking on transformation of sweetpotato at NARL. Equal amounts of 100 ng of

total DNA were amplified in 25 μL reactions using specific primers for the *cry7Aa1* gene: 5'-ACAACTCATCACCATACCAAAC-3' forward and 5'-AAGAGCAAGATGCAAGTTTG-3' reverse primers. These primers were expected to give products of 608 bp size. The specific primers for *cry3Ca1* gene were: 5'-CACCTATAGTAAAACCATTGGACAC-3' forward and 5'-TGCATGAAAGCCTTAAGAGG-3' reverse. These primers were expected to give fragments of 530 bp size. PCR amplifications for both *cry7Aa1* and *cry3Ca1* genes were performed with an initial denaturation at 93°C for 2 min, followed by 35 cycles at 93°C for 15 sec, 55°C for 30 sec, 72°C for 2 min, and final extension at 72°C for 7 min. PCR products were separated by electrophoresis on a 1.0 % (w/v) agarose gel for 45 min at a voltage of 80. The gel picture for the three separate tubes containing the same plasmid received from CIP, Lima, is shown in Figure 6.3.
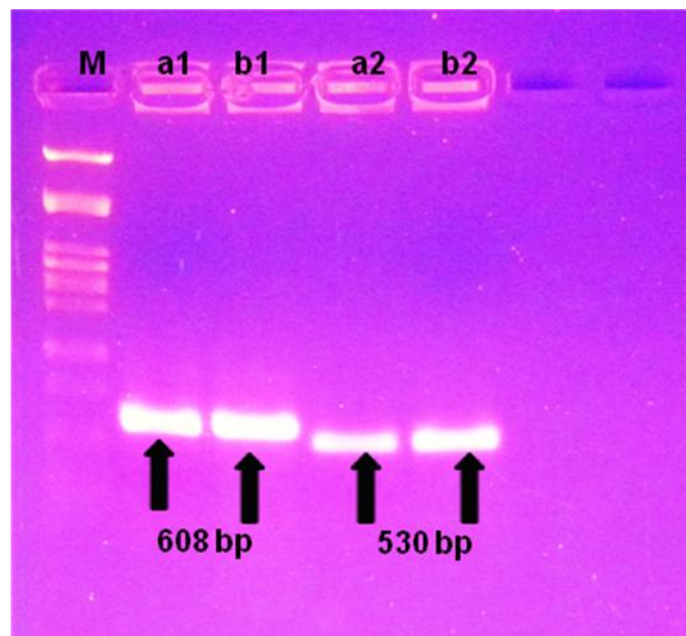

Figure 6.3: PCR amplification products of plasmid DNA.

Two culture tubes were supplied from CIP-Lima, containing *Agrobacterium* EHA105 with the pCIP84 vector construct. Lane M: DNA marker. Lanes a1 and a2 are *cry7Aa1* and *cry3Ca1* respectively, from one culture tube while Lane b1 and b2 are *cry7Aa1* and *cry3Ca1* respectively, from another culture tube.

6.2.7 Genetic transformation of sweetpotato with weevil-resistance genes

A single colony of *A. tumefaciens* strain EHA105: pCIP84 was cultured in 2ml of LB liquid containing 50 mg/L Kanamycin for 48 hr under conditions described above. Twenty µl of this culture were inoculated into 20 ml of fresh LB liquid (as above) and grown as above to an OD_{600} of 1.0. The culture was centrifuged at 2000 g for 10 min at 4°C. The bacterial pellet was resuspended in an equal volume of bud induction medium (BIM) and incubated for 1 hr at 28°C with rotary shaking at 100 rpm. BIM was composed of MS basal salts, sucrose (30 g l^{-1}), myo-inositol (0.1 g l^{-1}) and sweetpotato vitamin stock (1 ml l^{-1}). The media was adjusted to pH 5.8 before autoclaving as above. TDZ (4.0 µM) and acetosyringone (100 µM) were supplemented to the medium after autoclaving. Freshly harvested *in vitro* stem pieces (0.6 – 1.0 cm) from internode positions 2, 3 and 4 of cv. Kyebandula were incubated in the bacterial suspension with rotary shaking at 100 rpm for 20 min at 28°C, and then blotted dry on sterile filter paper. Three plant transformation experiments were conducted with each having 100 explants.

The explants were placed on sterilized filter paper overlaid on solid BIM. Solid BIM was made by adding 3 g/L of phytagel to liquid BIM and autoclaving as above. Co-cultivation was carried out for 4 days at 23°C in the dark. After co-cultivation, the explants were washed in antibiotic wash medium (liquid BIM with 500 mg l^{-1} cefotaxime) for 10 min, rinsed three times in sterile water, and then blotted dry on sterile filter paper. The explants were then placed on solid BIM with 4.0 µM TDZ, 250 mg l^{-1} cefotaxime and 100 mg l^{-1} Kanamycin for 3 days. They were placed horizontally on the medium and partially pressed into the medium. The explants were then transferred to bud elongation medium (BEM) comprised of MS salts (4.3 g l^{-1}), sucrose (30 g l^{-1}), myo-inositol (0.1 g l^{-1}), phytagel (3 g l^{-1}), 0.25 µM NAA, 250 mg l^{-1} cefotaxime and 100 mg l^{-1} kanamycin. The petri dishes containing the cultures were placed in dark for 4 weeks at 28°C before transfer to 16 h photoperiod under same temperature. The cultures were transferred to fresh bud elongation medium every 4 weeks. Regeneration of the putative transformed plants was achieved through adventitious shoot formation on the same medium within 12 weeks.

6.2.8 DNA extraction and PCR analysis of putatively transgenic plants

DNA from ten putatively transformed plants was used in initial analysis of the putatively transformed plants. Four grams of plant tissue was ground in liquid nitrogen, added to 20

mL of CTAB extraction buffer (100 mM Tris-HCl, 25 mM EDTA, 1.4 M NaCl and 2% CTAB) with 200 μL of 2-mercaptoethanol in a 50-mL polyethylene tube and incubated at 65°C in a water bath for 40 min, with occasional opening of the tubes to reduce build-up of pressure. An equal volume of chloroform/isoamyl alcohol (24:1) was mixed with the extract by inverting the tubes for 5 min and spun at 16,000 g for 5 min. The upper phase was transferred to a new 50-mL tube and 0.6 volumes of isopropanol (-20°C) was added. DNA was precipitated by placing tubes at -20 °C for 10 min and spun at 16,000 g for 5 min. The supernatant was discarded. The pellet was washed twice with 75 % ethanol and dried. The pellet was then dissolved in 30 μL nuclease-free water and digested with 1 μL of 10 mg/mL RNase for 15 min at 37°C. The PCR conditions for *cry7Aa1* were similar to those used for analysis of integrity of the pCIP84 vectors (above). It is preferred that transgenic plants are not contaminated with the *Agrobacterium* used in genetic transformation. This is to ensure that the PCR results of the *cry* genes are not due to bacteria presence on plant tissue, but due to transformation of the plants. This step is also important to minimise future biosafety concerns. The presence of *Agrobacterium* in the putatively transformed plants were detected by PCR using primers for *virD2* gene which is found in the *Agrobacterium* but is not in the T-DNA of the plasmid pCIP84. The primers for *virD2* are: 5'-ATGCCCGATCGCGCTCAAGT-3' forward and 5'-CCTGACCCAAACATCTCGGCT-3' reverse. PCR conditions for *virD2* gene were: initial denaturation at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 45 s, and final extension at 72°C for 10 min. The PCR products were separated by electrophoresis on a 1.0 % (w/v) agarose gel for 45 minutes at a voltage of 80. The expected fragment size from the PCR was 338 bp.

6.3   Results

6.3.1   Effect of concentration of *Agrobacterium*

Transformation frequency was determined through the number of explants showing transient GUS expression using histochemical localization of GUS activity. GUS staining after 3 days co-culture showed that *Agrobacterium* concentration at an OD of 1.0 at 600 nm gave the highest transformation frequency for all the sweetpotato organs namely primary roots, petioles, whole leaves and stem internode pieces (Figure 6.4). This concentration of *Agrobacterium* was significantly (p < 0.001) better than the rest in

improving transformation frequency. All types of plant organs investigated showed capacity to be transformed under the conditions (Figure 6.5). Petiole explants performed significantly ($p < 0.001$) lower than the rest of the explants types. There was no significant difference among the remaining explants types (stem internodes, whole leaves and primary roots) in influencing transformation frequency.
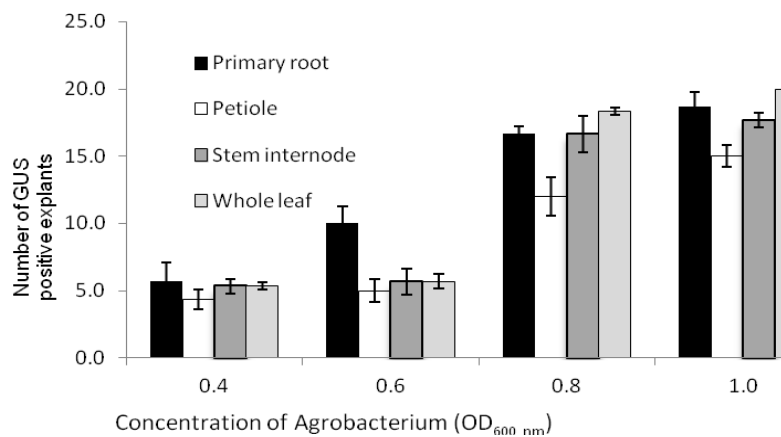
Figure 6.4: Effect of concentration (OD_{600} nm) of *Agrobacterium* on transient GUS expression.
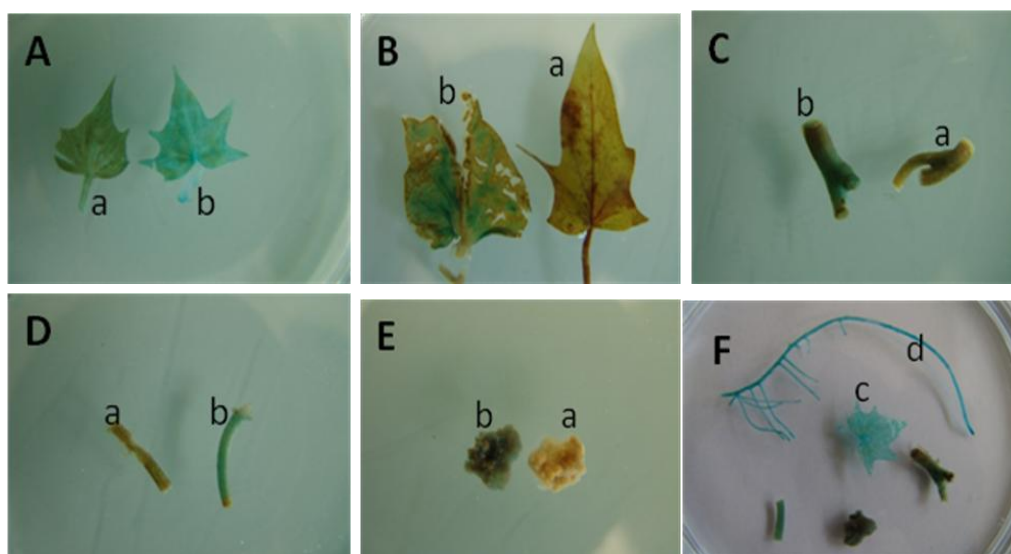
Figure 6.5: Different types of explants of cultivar Kyebandula showing transient GUS activity after 5 days of co-cultivation with *Agrobacterium tumefaciens* EHA105 (pCAMBIA1305.1).

The transformed explants (a) showed blue colour as positive indication of transient GUS expression. Non transformed controls (b) did not show blue colour. (A) Young leaves (4 weeks old); (B) Old leaves (7 weeks old); (C) Stem nodes; (D) Petioles; (E) Callus; (F) Comparatively high Gus activity in primary roots (d) and young leaves (c).

6.3.2 Effect of duration of co-culture

The data shows that the best transformation frequency was achieved with co-culture duration of 4 days for both whole leaf and stem internode explants (Figure 6.6). Similar to the experiment on concentration of *Agrobacterium*, the two types of explants namely whole leaves and stem internode pieces did not have a significant ($p \leq 0.05$) difference in influencing transformation frequency. However, the duration of co-culture had a significant ($p < 0.001$) effect on transformation frequency. The best transformation results were obtained when explants were co-cultured for 4 days, although there was not significant ($p < 0.001$) reduction in transformation frequency when explants were cultured for 3 days. Extending co-culture duration to more than 4 days resulted in a loss of transformation frequency, which was significant ($p < 0.001$) even when only one day was added.

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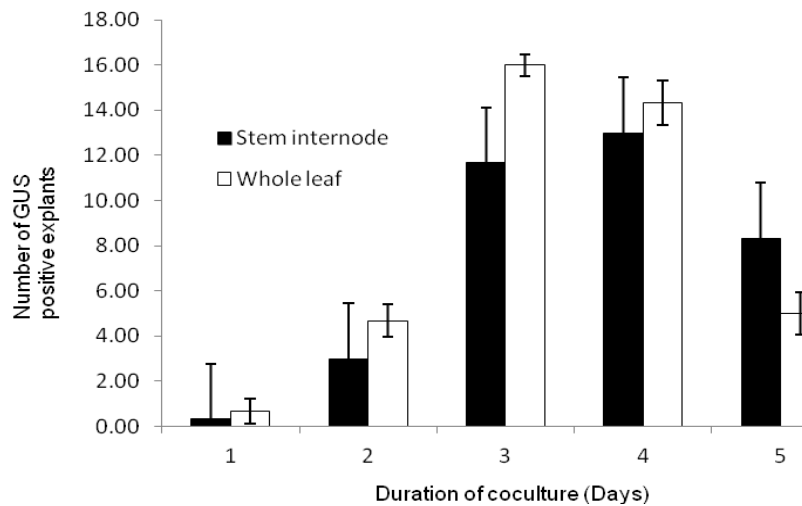
Figure 6.6: Effect of cocultivation time (days) on transient GUS expression.

6.3.3 Effect of placement on filter-paper

An *Agrobacterium* concentration at an OD of 1.0 at 600 nm and a co-culture duration of 4 days on co-culture medium overlaid with filter-paper significantly (p = 0.001) improved transformation efficiency (Figure 6.7). There was little *Agrobacterium* growing around the explants and the filter-paper in contact with the explants. In the experiment without filter-paper, the explants were in direct contact with the medium and *Agrobacterium* could be seen growing around the explants and the medium for most explants. There was no significant difference between whole leaf and stem internode pieces in influencing transformation efficiency.
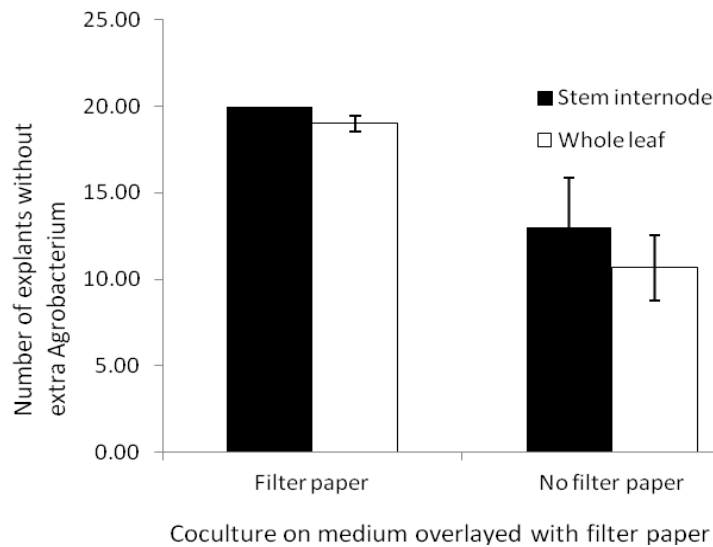
Figure 6.7: Effect of placing explants on filter paper during cocultivation on elimination of excess *Agrobacterium* from explants.

6.3.4 Sensitivity of explants to kanamycin

Both the type of plant organ and the kanamycin concentration were shown to significantly ($p < 0.001$) influence survival of explants on CIM. Kanamycin concentration of 100 mg/L or higher completely inhibited survival and callus formation from whole leaf explants which turned brown due to necrosis within 6 weeks. There were a few stem explants that showed survival on CIM supplemented with 125 mg/L kanamycin after 8 weeks although most of them were not able to continue forming callus (Figure 6.8). Therefore a kanamycin concentration of 70 mg/L was used for whole leaves and that of 100 mg/L was used for stem internode pieces in subsequent experiments.
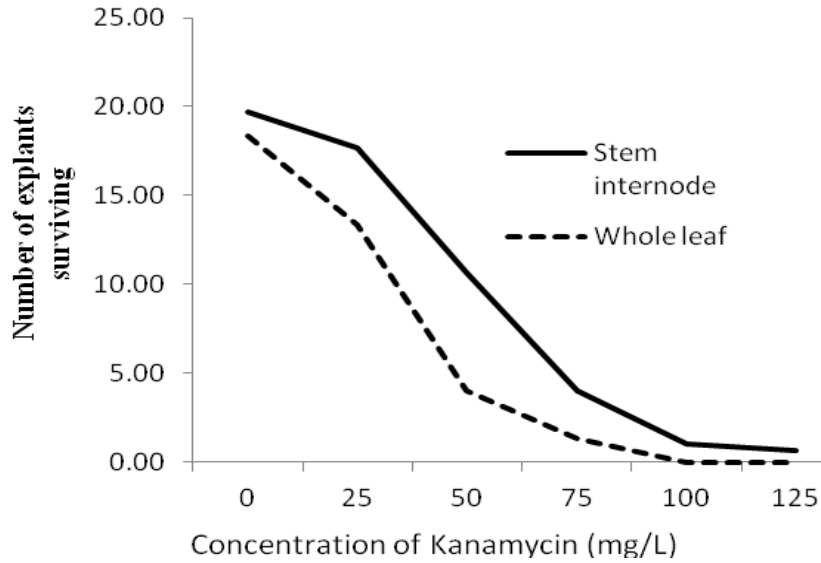
Figure 6.8: Effect of Kanamycin concentration (mg/L) on survival of explants.

6.3.5 Regeneration of putatively transformed plants

After three days of co-culture on bud induction medium, explants started expanding. The swelling was more pronounced at the cut ends of the explants where callus was also formed within two weeks after co-culture. None of the induced calli regenerated shoots. In the same period, multiple adventitious shoot buds were observed along the length of most explants. The frequency of explants with adventitious buds after 4 weeks was recorded as 54 % while the mean number of buds per explants was seven. Some buds started to elongate within the four weeks of placement on selection medium. However most adventitious buds failed to elongate into shoots and remained green or died with the explants after 12 weeks. After 8 weeks, the shoots that had developed from the elongating buds were cut out from the explants and placed on sweetpotato propagation medium where they rooted easily by the twelfth week. There were 11, 2 and 5 plants regenerated in experiments 1, 2, and 3, respectively. In total, eighteen independent plants were regenerated. The per cent of explants regenerating shoots was 6.0 %. One to two shoots were formed per explants for those explants that formed shoots. The regenerated plants were further multiplied by cutting into nodal segments on the sweetpotato propagation medium without antibiotics.

6.3.6 PCR analysis of putatively transformed plants

Among the 18 putatively transformed plants, 10 were used for initial PCR analysis and the expected fragment length for *cry7Aa1* (608 bp) was found in 8 them. It was absent in the nontransformed control. Two of the eight plants that were positive to PCR with *cry7Aa1* primers were also positive to primers for *virD2* gene producing a fragment of 338 bp length (Figure 6.9).
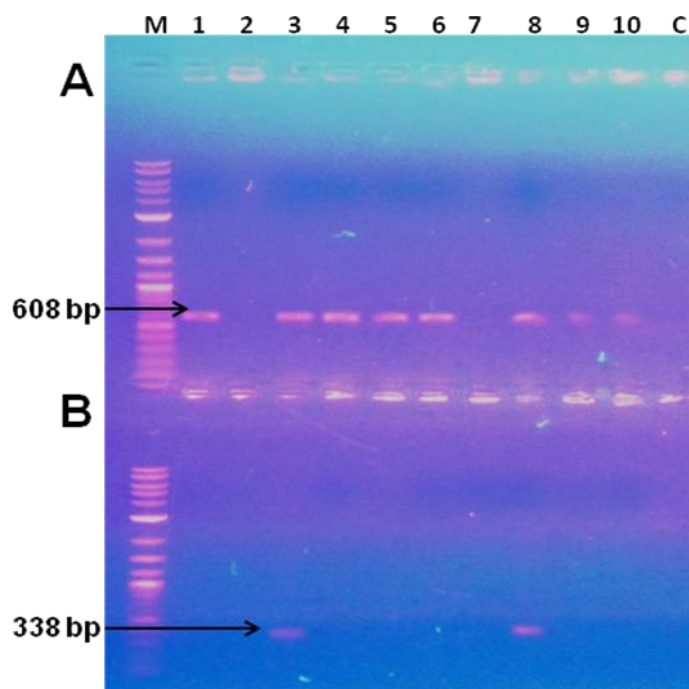
Figure 6.9: Molecular analysis of putatively transgenic plants.
(**A**) PCR analysis for cry7Aa1. Lane C: Untransformed plant as negative control. Lane M: DNA marker. Lanes 1, 3-6, 8-10: Cry 7Aa1-positive plants. Lane 2 and 7: Cry 7Aa1-negative plants; (**B**) PCR analysis for virD2 gene. Lane 3 and 8: virD2-positve plants. Lanes 1, 2, 4-7, 9, 10: virD2-negative plants.

6.4 Discussion

The objective of the present study was to establish a method for high transformation frequency of the Ugandan landrace cultivar 'Kyebandula'. The transformation method combined with optimised regeneration protocol led to the first success on genetic transformation of cv. Kyebandula for weevil-resistance. Some of the important factors that determine transformation frequency are concentration of bacteria, length of co-cultivation and elimination of excess bacteria (Cheng *et al.,* 1997). The transformation frequency was

determined by screening for transient GUS expression using histochemical localization of GUS activity after co-culture. There was no significant difference between the two plant organs, namely whole leaves and stem internode pieces, in their response to *Agrobacterium* concentration, co-culture duration and co-culture on filter paper. However, the expression of GUS in both of these commonly used explant types was lower than that for primary roots. From the data, it is concluded that *Agrobacterium* OD concentration of 1.0 at 600 nm is the best for the transformation of these explants from cv. Kyebandula.

Xing *et al.* (2007) did not find a significant difference when various *Agrobacterium* OD concentrations (0.6, 0.8, 1.0) at 600 nm were used for infection, although the best transformation in that work was achieved when OD concentration was 0.8. The same work demonstrated that higher OD reduced transformation efficiency due to over-infection of plant cells by the large number of *Agrobacterium* cells at high OD concentration (Xing *et al.,* 2007). However in the present work the *Agrobacterium* OD concentration of 1.0 at 600 nm was significantly better than any lower concentration investigated. For similar plant organs used as explants in this study, Song *et al.* (2004) used an *Agrobacterium* OD of 0.8 to 1.0 while Luo *et al.* (2006) and Kreuze *et al.* (2008) used an OD of 0.4 – 0.6. This could partly explain why Song *et al* (2004) obtained higher transformation efficiency than Luo *et al.* (2006). Yang *et al.* (2011) used an OD value of 1.0 while an OD of 0.5 was used in the work of Yu *et al.* (2008) who reported comparatively high transformation efficiency than any previous work (Yu *et al.,* 2007). The cultivars reported in the above work are Jewel (Luo *et al.,* 2006), Huachano (Kreuze *et al.,* 2008), Lizixiang (Yu *et al.,* 2007), Xu55-2 (Xing *et al.,* 2007) and Beniazuma (Song *et al.,* 2004), while Yang *et al.* (2011) used thirteen cultivars adapted to China. It is interesting to note that Yang *et al.* (2011) who used a high *Agrobacterium* OD value of 1.0 also recommended a longer co-culture duration (4 to 7 days) as compared to most reports which recommend 3 days (Song *et al.,* 2004; Yu *et al.,* 2007).

Normally 2-3 days are standard for co-cultivation in most transformation protocols (Xing *et al.,* 2007). In the current work, 4 days was the best co-cultivation time for cv. Kyebandula. Xing *et al.,* (2007) also found optimal co-culture time was 4 days for cell suspensions of cv. Xu55-2. Some reports (Prakash *et al.,* 1991) recommended co-culture of more than 3 days while others (Otani *et al.,* 1998) found 2 days sufficient.

The recurrence of *Agrobacterium* has been reported as a major problem in previous studies and Kreuze *et al* (2008) discarded 5 to 18 % of explants due to excessive bacterial overgrowth. Song *et al.* (2004) did not report any *Agrobacterium* overgrowth when they placed filter-paper on co-culture medium before placing explants. In the present work, the placement of explants on filter-paper followed by washing in antibiotic medium eliminated the excess *Agrobacterium* almost completely (Figure 6.7). To our knowledge this is the first report to compare these two approaches and demonstrate that the placement of filter-paper on co-culture medium has a direct and significant effect on suppression of excess *Agrobacterium*. It is possible that when the explants are placed directly on medium, the co-culture medium supports the growth of *Agrobacterium* to an extent that has the same effect as long co-culture duration (above). It is evident that high *Agrobacterium* recurrence is associated with low transformation efficiency.

An efficient selection method is required for the production of transgenic plants. Based on the data in the current study it is concluded that kanamycin of 100 mg/L is sufficient for selection of transformed callus from stem internode pieces of cv. kyebandula. The results with whole leaf explants were more conclusive than those for stem internode segments which showed slightly more resistance to kanamycin (Figure 6.8). The deployment of kanamycin for selection of transformed cells is not straightforward. Although kanamycin has been used successfully to select transformed sweetpotato cells by some groups (Moran *et al.,* 1998), other groups demonstrate that kanamycin has a negative effect on regeneration while others opted not to use kanamycin in the first 5 days after co-culture (Sheng-Jun *et al.*, 2004). Xing *et al.* (2008) found that high dose of kanamycin decreased the livelihood and proliferation of embryogenic callus of cultivar Xu55-2 from China. This group found that 10 mg/L Kanamycin was sufficient for selection of transformed cell aggregates of cv. Xu55-2 and suggested that cultivar has an effect on survival in presence of Kanamycin (Xing *et al.,* 2008). This suggestion is supported by the findings of another group which demonstrated that cultivar Yulmi has higher intrinsic resistance to kanamycin (200 mg/L) (Shin *et al.*, 2007). Generally, most researchers have demonstrated that sweetpotato cells are less sensitive to kanamycin and therefore a high dose (25-100 mg/L) of this antibiotic is required to kill non-transformed cells and leave out only transformed cells on medium (Luo *et al.*, 2006; Prakash and Veradarajan, 1992; Song *et al.*, 2004).

The optimised transformation conditions led to successful regeneration of putatively transformed plants. This is the first report on genetic transformation of a popular Ugandan landrace cultivar. Luo *et al* (2006) reported that important Chinese and African cultivars are difficult to transform. African cultivars that were investigated were not mentioned in their report and it was not indicated whether the experiments with African cultivars were on somatic embryogenesis or organogenesis. Later, Tovar *et al.* (2009) reported that they obtained only one regenerating transgenic shoot from African cv. Wagabolige and no regeneration from cv. Tanzania after investigating about 10,000 explants of each cultivar with a somatic embryogenesis protocol.

In this study a total of 18 plants were regenerated through adventitious shoots from a total of 300 stem internode pieces. Importantly, there were shoots regenerated from each experiment supporting the reproducibility of the protocol. However, the conversion of adventitious buds into shoots remains a problem as a large number of adventitious buds failed to elongate into shoots. This phenomenon could be due to the effect of TDZ as observed in previous studies with *Vaccinium vitis-idaea* and *Paulownia tomentosa* Steud. (Corredoira *et al.,* 2008; Debnath, 2005).

PCR results with *cry7Aa1* primers showed that 2 of the ten randomly selected plants were not transformed. Song *et al.* (2004) reported that 100 mg/L of kanamycin was effective in selection of transformed plants when they used stem internodes as explants. However, Luo *et al* (2006) encountered non-transformed escapes under similar conditions. This latter group argued that the strong regeneration capacity of stem segments seems to compete with the regeneration from the rare transformed cells (Luo *et al.*, 2006). Later, another group proposed that the regeneration of non-transformed plants on selection medium is because non-transformed cells can sometimes survive among transformed cells within a single callus clump (Yu *et al.,* 2007). Similarly in the present work the plants that were not transformed but were able to survive on medium with kanamycin could have done so due to the reasons promoted by Yu *et al* (2007) and Luo *et al* (2005). At the present point the 8 plants are considered as putatively transformed awaiting southern hybridization to confirm stable integration of the *cry* genes into the sweetpotato genome and also the copy number of the genes in the events.

The two plants that showed PCR-positive to *virD2* primers showed that the *Agrobacterium* used for genetic transformation survived on them. The implication of this is that it is likely that the PCR-positive results with *cry7Aa1* gene is due to the pCIP84 plasmid in the *Agrobacterium* and not the transgenic nature of the plants. These two plants are currently being propagated on medium with the antibiotic cefotaxime to eliminate the *Agrobacterium.* From previous studies it looks too difficult to entirely eliminate *Agrobacterium* from plant tissue after transformation (Ogawa and Mii, 2007). Ogawa and Mii (2007) found that although *Agrobacterium* was not evident on media after suppression with antibiotics, the PCR with *virC* on 16-weeks-old shoots was positive. This could indicate that the *Agrobacterium* they used for transformation survived on the tissue of the regenerated plants as they developed, although the *Agrobacterium* could not survive directly on the media on which the plants were growing.

This report is new in that it is based on readily accessible explants of an African cultivar that has not been reported in previous transformation work. It is known that *Agrobacterium* transformation has a negative effect on regeneration efficiency (Song *et al.,* 2004; Xing *et al.,* 2008). Therefore preliminary regeneration studies to this work needed to be coupled with successful transformation to be more valuable. In regeneration experiments with TDZ, prior to transformation, the best results showed 61.0 % explants formed adventitious buds, 39 buds per explant (mean), 56.0 % explants forming shoots and 9 shoots per explant (mean) for those explants that formed shoots (Chapter 5). When the regeneration conditions were incorporated in the transformation process the results for all the parameters dropped to 54.0 % explants forming adventitious buds, 7 buds formed per explant (mean), 6.0 % explants forming shoots and one shoot per explant (mean) for those explants that formed shoots.

The current data demonstrates the ability to genetically transform cv. Kyebandula based on transient expression of the GUS reporter gene. The transformation conditions optimised through transient expression were applied in the transfer of weevil-resistance genes into cv. Kyebandula. The genetic transformation protocol reported here has potential to be extended to other sweetpotato cultivars. Southern hybridization is the next most important step in the plants that have been regenerated in this work.

CHAPTER SEVEN

GENERAL DISCUSSION

Sweetpotato has for long been considered recalcitrant to somatic embryogenesis (Yang *et al.,* 2011). Most of the reported protocols are cultivar specific, have low regeneration frequencies which are also difficult to reproduce (Santa-Maria *et al.,* 2009; Yang *et al.,* 2011; Yu *et al.,* 2007). The most important challenge is the different responses of cultivars to *in vitro* conditions, requiring optimisation of different plant growth regulators and other factors (Al-Mazrooei *et al.,* 1997; Santa-Maria *et al.,* 2009). The two methods that have been applied in the regeneration of transgenic sweetpotato are somatic embryogenesis and shoot organogenesis. Somatic embryogenesis is more preferred for application in genetic transformation due to high efficiency of transformation while the major advantage with shoot organogenesis is that it requires short culture periods and is relatively less cultivar-specific. To-date, no single protocol has dominated in the production of transgenic sweetpotato.

In this study embryogeneic callus, *de novo* roots and adventitious shoots were achieved from cultivars that have not been regenerated before. The ease of regeneration with the control non-African cultivars Jonathan, Jewel and Huachano confirmed previous reports that African cultivars are difficult to regenerate (Luo *et al.,* 2006; Tovar *et al.,* 2009). However the data from the above studies emphasizes the fact that recalcitrance is a relative term since different cultivars have shown to regenerate under some conditions and fail in other conditions where others perform better. Based on literature search, the results presented here are the first for the reported 'recalcitrant' Ugandan cultivars. The successful regeneration of cv. Kyebandula and Bwanjule was achieved when work on other recalcitrant species, especially woody plants was taken into consideration (Corredoira *et al.,* 2008; Cuenca *et al.,* 2000; Sriskandarajah and Lundquist, 2009).

The auxin 2,4-D has demonstrated high potential to induce somatic embryogenesis (Table 3.2 and 5.1). Some Ugandan cultivars also regenerated roots in the presence of 2,4-D (Table 4.3 and Figure 4.5). The embryogenic callus induced could be useful for initiation of cell suspensions to enable genetic transformation of African cultivars while the method

for *de novo* regeneration of roots has potential application in the regeneration of plants or hairy root cultures for cultivars that are recalcitrant to shoot regeneration. The induction of somatic embryogenesis with 2,4-D appears to be preferable in view of existing literature (Yang *et al.,* 2011). Although the data with TDZ gave the best response in terms of adventitious shoot regeneration, the regeneration of sweetpotato through somatic embryogenesis is still an option to pursue. This is mainly because somatic embryogenesis leads to high efficiency of selection of transgenic plants on medium (Song *et al.,* 2004; Yang *et al.,* 2011; Yu *et al.,* 2007).

'Whole leaf' explants gave the best results in somatic embryogenesis studies. However, the comparison was only with petioles and leaf discs (Chapter 4). Therefore, stem internode pieces which gave best results in adventitious shoot regeneration cannot be ruled out from somatic embryogenesis experiments. Song *et al.* (2004) found this as the best explant in somatic embryogenesis experiments although Luo *et al.* (2006) opted to use 'whole leaf' explants in shoot organogenesis studies. The shoot regeneration within 12 weeks in the experiments that involved TDZ is the first report, based on current literature. This regeneration with a few media changes and no intervening callus is less costly and reduces the chance of somaclonal variation. Importantly, the plants regenerated in the present study do not originate from pre-formed buds but *de novo* buds induced by TDZ. It is important that direct regeneration without an intervening callus stage should be of single cell origin, avoiding possibility of chimeras after genetic transformation (Gong *et al.,* 2005). Although there has been increased application of TDZ in somatic embryogenesis and adventitious regeneration of recalcitrant plants (Corredoira *et al.,* 2008; Cuenca *et al.,* 2000; Sriskandarajah and Lundquist, 2009), this has not been widely extended to sweetpotato, except for a few cases (Gosukonda *et al.,* 1995a; Gosukonda *et al.,* 1995b; Kumar *et al.,* 2007).

In order to efficiently integrate the regeneration protocol into the genetic transformation of a Ugandan cultivar, experiments to optimize parameters for transformation were conducted. The transformation frequency was determined by screening for transient GUS expression using histochemical localization of GUS activity after co-culture. From the data, it is concluded that *Agrobacterium* OD concentration of 1.0 at 600 nm is the best for the transformation of these explants from cv. Kyebandula. In this work, 4 days was the best

co-cultivation time for cv. Kyebandula. Xing *et al.,* (2007) also found optimal co-culture time was 4 days for cell suspensions of cv. Xu55-2. However, some reports (Prakash *et al.,* 1991) recommended co-culture of more than 3 days while others (Otani *et al.,* 1998) found 2 days sufficient. Some groups have used filter-paper during co-culture (Song *et al.,* 2004) while other do not place explants on filter paper during co-culture (Kreuze *et al.,* 2008). There was no published data to explain the effect of filter paper in the transformation process. The current study successfully demonstrated that placing explants on filter-paper significantly (Figure 6.7) reduces extra *Agrobacterium* during co-culture and its re-currence after co-culture and therefore indirectly improves transformation efficiency.

The transformation of plants through *Agrobacterium* species is the most commonly used system since the transferred DNA has minimal rearrangement and there are only few transgene copies inserted into the plant genome (Choi *et al.,* 2007; Xing *et al.,* 2007). However there are so many variables that affect *Agrobacterium*–mediated transformation, and no evidence of obvious combinations of these variables exists for broad application across plant species and cultivars (González *et al.,* 2008; Xing *et al.,* 2007). Furthermore, despite wide use of *Agrobacterium* in genetic transformation, sweetpotato has shown a high genotype dependent response with a low transformation and regeneration efficiency in most of these studies (Luo *et al.,* 2006; Sheng-Jun *et al.,* 2004; Song *et al.,* 2004). A critical challenge is that regeneration system in non-transformed sweetpotatoes can not be directly applied to transformed system coupled with *Agrobacterium* (Garcia *et al.,* 2000; Gosukonda *et al.,* 1995a; Otani *et al.,* 1998). When regeneration is coupled to transformation, the numbers of regenerated plants drop dramatically, presumably due to some modification caused by *Agrobacterium* infection step (Song *et al.,* 2004; Xing *et al.,* 2008). This background imposed the experiments on transformation efficiency after optimizing regeneration protocol for Cv. Kyebandula and Bwanjule.

In the regeneration experiments with TDZ, prior to transformation, the best results showed 61.0 % explants formed adventitious buds, 13.0 buds per explant (mean), 56.0 % explants forming shoots and 3 shoots per explant (mean) for those explants that formed shoots (Table 5.3). When the regeneration conditions were incorporated in the transformation process the results for all the parameters dropped to 54.0 % explants forming adventitious buds, 7 buds formed per explant (mean), 6.0 % explants forming shoots and one shoot per

explant (mean) for those explants that formed shoots. This data confirmed that there was need to combine optimisation of both regeneration and transformation for the sweetpotato cultivars that have not been reported in previous transformation work.

The optimized TDZ-based regeneration protocol was combined with the established transformation parameters, with co-culture step involving *Agrobacterium* harboring *cry7Aa1* gene. PCR results with *cry7Aa1* primers showed that 8 plants were putatively transformed while another 2 were not transformed plants that survived on selection medium (100 mg/L kanamycin). Luo *et al.* (2005) and Song *et al.* (2008) reported that 100 mg/L of kanamycin was effective in selection of transformed plants. Nonetheless, the high presence of escapes encountered in the work of Luo *et al* (2006) caused them to use whole leaves instead of stem internode pieces. These authors argued that the strong regeneration capacity of stem segments seems to compete with the regeneration from the rare transformed cells.

CHAPTER EIGHT

GENERAL CONCLUSIONS AND RECOMMENDATIONS

African cultivars have been demonstrated to be difficult to regenerate and therefore limiting application of genetic transformation to improve their attributes (Luo *et al.,* 2006; Tovar *et al.,* 2009). The main objective of the studies reported here was to develop a protocol for regeneration and genetic transformation that could enable transformation of selected Ugandan sweetpotato cultivars with weevil-resistance genes.

By successfully reducing the time for regeneration of the non-African cultivar Huachano, the results above demonstrate that a major setback in regeneration through somatic embryogenesis has been overcome. Although the regeneration of African cultivars through somatic embryogenesis remains a challenge, the embryogenic callus reported above could be useful for the initiation of embryogenic cell suspensions which are not readily available for transformation of most sweetpotato cultivars (Yang *et al.,* 2011). The method for *de novo* regeneration of roots, optimized in this work, may be applicable in the regeneration of plants or hairy root cultures. A possibility exists that the use of other types of plant organs, such as meristems, as explants and various concentrations of 2,4-D or other auxins could improve somatic embryogenesis in the cultivars investigated.

In general, an efficient regeneration protocol was achieved through adventitious shoots induced on medium supplemented with TDZ. Overall, the results confirm that most *I. batatas* cultivars that were thought to be recalcitrant can be regenerated following optimisation of media composition. For the *Agrobacterium*-mediated transformation, the most important factors are in the early stages leading to transfer of the transgene from the *Agrobacterium* and integeration in the plant genome. The transformation protocol optimised using transient GUS expression has already shown practical application in genetic transformation of Ugandan sweetpotato cultivars with genes that have potential to improve resistance to weevils.

Based on the current data from the studies in this thesis, it is recommended that optimisation of regeneration protocols continues in search for a genotype-independent

protocol. Although the protocols of somatic embryogenesis are lengthy and more complex, it is recommended that these protocols be explored further, particularly those based on cell suspensions as reported recently by most groups in China (Yang *et al.,* 2011; Yu *et al.,* 2007; Zang *et al.,* 2009). The putatively transformed plants regenerated in this study are few for expression analysis since *Agrobacterium* insertion into plant geneome is random and a large number of independent events increases the chance of producing a plant with high transgene expression. Therefore, it is recommended that the number of plants regenerated be increased before embarking on detailed expression studies. Currently, the early regeneration of plants can be achieved with the optimised adventitious regeneration protocol based on TDZ.

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