

MAKERERE



UNIVERSITY

**ENHANCEMENT OF SOMATIC EMBRYOGENESIS IN
RECALCITRANT EAST AFRICAN HIGHLAND BANANAS**

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DECLARATION

I, Sadik Kassim, certify that this study is original and has not been submitted for any other degree award to any University before.

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DEDICATION

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ABSTRACT

Lack of appropriate *in vitro* protocols has limited advances in the use of tissue culture in rapid multiplication of planting material and development of reliable genetic transformations for AAA-EA banana. Embryogenic callus induction in AAA-EA banana has been difficult. Conventionally auxins have been used in MS medium with or without purine-based cytokinins for induction of embryogenesis. The low embryogenic response aside, the existing protocols have been cultivar dependent. The main objective of this study was to enhance plant regeneration through somatic embryogenesis in economically important AAA-EA banana cultivars for efficient propagation and genetic improvement. The specific study areas included: i) establishment of proliferation potential of AAA-EA banana cultivars in MS medium, ii) enhancement of embryogenic callus induction from scalps of AAA-EA banana cultivars through application of urea-type cytokinins, N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (TDZ) and N-(2-chloro-4-pyridyl)-N'-phenylurea (4-CPPU), and iii) establishment of appropriate basal salt mixture in presence of TDZ and 4-CPPU for embryogenic callus induction from immature male flower buds of AAA-EA banana cultivars.

Seven AAA-EA banana cultivars namely Mpologoma, Musakala, Mbwarzirume, Nfuuka, Nakinyika, Nakabululu and Kabula from different clone sets were used. Yangambi Km5 (AAA) and Sukali Ndizi (AAB) were included for comparison in some of the experiments. Proliferation potential and number of recoverable shoots from one shoot-tip explant of AAA-EA banana cultivars on the MS derived medium for banana multiplication were determined through normal shoot-tip culture. Various combinations of TDZ and 4-CPPU were tested for embryogenic callus induction from scalps and immature male flower buds. By substituting only the MS inorganic salts from the callus induction medium with the best TDZ and 4-CPPU combination, salt formulations: Chu (N₆), Eriksson, Gamborg B5, Nitsch, NLN, SH and White were tested for embryogenic callus induction from immature male flower buds of cultivars Mpologoma and Nakinyika.

The AAA-EA banana cultivars produced 3-4 new shoots in each subculture cycle and 57-169 recoverable shoots from one starting shoot-tip explant in 18 weeks. Non AAA-EA banana

cultivars, Sukali Ndizi and Yangambi Km5 produced 5 and 9 shoots from each subculture cycle and 322 and 352 recoverable shoots, respectively in the same period. Scalps were achieved on MS medium supplemented with TDZ and 4-CPPU in over 50% of the shoot-tip cultures within 4 subculture cycles especially in the medium containing equal proportions of 9 – 13 μM of TDZ and 4-CPPU. Depending on cultivar and, TDZ and 4-CPPU combination, embryogenic callus was formed directly on 2.5 – 20% of the scalp cultures on the auxin-free scalp induction medium. Cultivars Nakinyika, Nakabululu, Mbwazirume and Nfuuka developed regenerable embryogenic callus and recorded plant regeneration except Mpologoma. All the cultivars recorded between 4.6 - 22.2% embryogenic response from immature male flower buds on TDZ and 4-CPPU combinations. The callus induction medium supplemented with 10 μM TDZ+10 μM 4-CPPU induced more embryogenic response. When male flowers of cultivars Mpologoma and Nakinyika were cultured on the medium containing 10 μM TDZ+10 μM CPPU with the MS salts substituted by other salt formulations, they recorded 11.4% and 8.3% embryogenic response, respectively, on Gamborg B5, which was almost twice their response on pure MS medium.

Though the embryo to plant conversion rate was low, in both methods most of the studied cultivars showed embryogenic responses which in some instances were greater than those reported before. This suggests that a combination of TDZ and CPPU especially at 10 μM each can enhance somatic embryogenesis in a range of AAA-EA banana cultivars and more in interaction with Gamborg B5 salt formulation. Application and optimization of combined TDZ and 4-CPPU especially in Gamborg B5 salt formulation for routine induction of embryogenic callus and development of embryogenic cell suspensions, respectively, is thus worthwhile. This will improve plant regeneration and consequently propagation and genetic improvement of AAA-EA banana cultivars.

CHAPTER ONE

INTRODUCTION

1.1 East African highland banana among *Musa spp.*

Bananas (*Musa spp.*) belong to the genus *Musa*, order Zingiberales, family Musaceae. Based on the basic chromosome number and morphological characteristics especially orientation and arrangement of flowers in the inflorescence, the genus *Musa* is grouped into 5 sections: *Callimusa*, *Australimusa*, *Eumusa*, *Rhodochlamys* and *Incertae sedis* (Constantine and Rossel, 2001). The sections *Callimusa* and *Australimusa* contain species with a basic chromosome number of 10 ($2n = 20$) while *Eumusa* and *Rhodochlamys* have species with a basic chromosome number of 11 ($2n = 22$). The first four sections were proposed earlier (Cheesman, 1948) while the last section, *Incertae sedis* was proposed later (Argent, 1976) and has species with different unconfirmed basic chromosome numbers (Daniells *et al.*, 2001).

Edible bananas belong to section *Eumusa* and derived from at least two wild species, *Musa acuminata* Colla and *Musa balbisiana* Colla, the donors of A and B genome in bananas, respectively (Simmonds and Shepherd, 1955). They comprise parthenocarpic seed sterile diploid (AA) and autopolyploid (AAA, AAAA) clones of *M. acuminata* as well as allopolyploid clones from crosses between *M. acuminata* and *M. balbisiana* (AAB, ABB, AABB, AAAB, AB BB) (Roux *et al.*, 2008). The primary center of diversity for *M. acuminata* is Southeast Asia around Malaysia (Simmonds and Shepherd, 1955) whilst that for *M. balbisiana* Southern Asia specifically India (Daniells *et al.*, 2001). The allopolyploid clones were produced by hybridization in areas where the two species overlapped (Stover and Simmonds, 1987).

The East African highland banana (AAA-EA banana) is an autopolyploid triploid (AAA) (Pillay *et al.*, 2006). It evolved through mutation and chromosome restitution within the highlands of East Africa (Karamura, 1998), now referred to as its secondary centre of genetic diversity (Stover and Simmonds, 1987). Because of this, its genome is often denoted as AAA-EA to differentiate it from other bananas with AAA genome composition such as Cavendish,

Gros Michel and Yangambi Km5 (Karamura, 1998). The AAA-EA group of bananas comprises over 120 cultivars grouped into five clone sets namely Musakala, Nakabulubu, Nakitembe, Nfuuka and Mbidde (Karamura and Pickersgill, 1999). The first four clone sets are usually cooked and served in different food recipes while the last one is processed into juice or beer (Karamura and Pickersgill, 1999).

1.1.1 Importance of AAA-EA banana in East Africa

Banana is the fourth most important food crop in the world after rice, wheat and maize in terms of gross value of production estimated at 102 million metric tons harvested from 4.7 million hectares (FAOSTAT, 2012). Different banana genotypes dominate the various banana growing regions of the world. The AAA-EA banana is grown in the Great Lakes region of Eastern Africa covering parts of Uganda, Rwanda, Burundi, Tanzania, Kenya and Democratic Republic of Congo (DRC) (Smale, 2006). It dominates other banana genotypes or varieties grown in this region (Table 1.1).

Table 1.1 Popular banana types grown in the East African region by genome and use

Banana type/subgroup	Common Name	Genome	Use (Purpose)
AAA-EA banana	Matooke/Embidde	AAA-EA	Cooked/Juice/Beer
Cavendish	Banana	AAA	Dessert
Gros Michel	Bogoya	AAA	Dessert
Apple banana	Sukali Ndizi	AAB	Dessert
Plantain	Gonja	AAB	Roasted
Bluggoe	Kivuuvu	ABB	Cooked
Yangambi Km5	Km5	AAA	Juice/Beer
Pisang awak	Kayinja	ABB	Juice/Beer
Ney Poovan	Kisubi	AB	Juice/Beer

The AAA-EA banana is most popular in Uganda, Africa's first and world's second leading producer of bananas after India (AATF, 2009). The cooking AAA-EA banana is locally referred to as 'matooke' and it is so prominent in the diet of the people that banana is ranked

the most important staple and traded food crop in the country (Aliguma and Karamura, 2006). Each Ugandan consumes more than 400 Kg of banana, the highest per capita banana consumption in the world (Clarke, 2003; Sergeant *et al.*, 2004). Though mainly traded within the country, undocumented huge volume of banana is informally exported especially to neighboring Democratic Republic of South Sudan, Democratic Republic of Congo, Rwanda and Kenya. Annually, Uganda produces 10.6 million metric tons of banana (AATF, 2009) on over 1.6 million hectares or 37% of her arable land (FAO, 2005). About 75% of Ugandan families are engaged in banana production which is a source of income for over 30% of the Ugandan farmers (Oketch *et al.*, 2004).

Being a major source of energy and having ability to produce fruit year-round, the AAA-EA banana is reckoned as a sustainable food and income security crop in the East African region (CGIAR, 2008). The government of Uganda particularly lists banana top among the potential crops for achievement of “prosperity for all”. In this regard, in 2005, government designed a special project codenamed Presidential Initiative on Banana Industrialization Development (PIBID) to directly contribute to its value chain development and stimulate sustainable production (Muranga, 2007). Before this direct political intervention towards the promotion of the crop, a distinct program, the National Banana program was created under Uganda’s National Agricultural Research Organization (NARO) to work alongside member institutions of the Consultative Group on International Agricultural Research (CGIAR) especially the International Institute of Tropical Agriculture (IITA) and Bioversity International to improve the productivity of the crop using diverse novel approaches (CGIAR, 2008).

1.1.2 Constraints to production of AAA-EA banana

Production of bananas generally is limited by scarcity of quality planting materials. Conventionally, bananas are propagated by means of corms and suckers but these materials are slow to multiply, bulky and often contaminated with pests and disease pathogens (Vuylsteke, 2001). Bananas with AAA genome composition, including the AAA-EA banana, particularly have low natural proliferation ability leading to inadequate production of suckers to meet farmers’ demand (Baiyeri and Aba, 2005).

Besides the low natural proliferation ability, the AAA-EA banana is susceptible to pests and diseases; the most devastating pests being pests of roots and corms which can contaminate planting material including Banana weevil (*Cosmopolites sordidus*) and plant parasitic nematodes (*Radopholus similis*, *Pratylenchus coffeae*, *P. goodeyi* and *Helicotylechus multicinctus* (Tushemereirwe *et al.*, 2004); and foliar diseases including Banana Xanthomonas wilt (BXW) caused by *Xanthomonas campestris* *pv.* *musacearum* and Black Sigatoka caused by the fungus *Mycosphaerella fijiensis* Morelet (Tushemereirwe *et al.*, 2004). The AAA-EA banana is also drastically affected by soil nutrient deficiency and drought (AATF, 2003). These biotic and abiotic banana production constraints coupled with rampant poor agronomic practices have caused significant decline in banana production in the East African region during the last 20 years (Karamura *et al.*, 2004; Nantale *et al.*, 2008) especially in the traditional banana growing areas of central Uganda. In Uganda banana yields declined from 15 to 20 tonnes per hectare in 1970s to 6 tones per hectare by 2000 and it is estimated that in 2005, the country lost US\$ 35 million worth of bananas to Banana bacterial wilt (BBW) alone (AATF, 2009).

1.1.3 Constraints to genetic improvement of AAA-EA banana

The above impediments to banana production could be addressed sustainably through deployment of efficient regeneration protocols for mass production of disease-free banana planting material and development of improved varieties. Use of clean planting materials and, pest and disease resistant varieties is a vital component of an integrated pest management (IPM) strategy for control of banana pests and diseases (AATF, 2009). Availability of banana varieties that are drought tolerant and/or can thrive under poor soil conditions would increase both productivity and production of the crop. However, the AAA-EA banana is not readily responsive to regeneration protocols and yet its improvement through conventional breeding methods is difficult due to its ploidy, limited genetic diversity and long generation time. Besides, the AAA-EA bananas are characterized by high female and male sterility (Mukasa and Rubaihayo, 1993; Nyine and Pillay, 2007), and low seed viability (Ssebuliba *et al.*, 2006).

1.1.4 Somatic embryogenesis for improvement of AAA-EA banana

Somatic embryogenesis is of particular interest in crop improvement because an embryogenic callus can be incubated in a liquid medium on a rotary shaker to give a homogeneous embryogenic cell suspension (ECS) which can be used for genetic transformation and mass propagation. It has been vigorously pursued as a convenient means for genetic improvement in many crops which are naturally difficult to improve either due to their genome composition or life cycle (Khaleida and Al-Forkan, 2006) such as banana, sweet potatoes, pineapple, sugarcane, orchids and tree crops (Geo, 1985). However, the response of bananas particularly AAA-EA banana cultivars to somatic embryogenesis has been low.

Plant cell differentiation and morphogenesis *in vitro* is highly dependent on culture medium and conditions. The culture medium must contain all the chemical ingredients in proper proportions that plant tissue needs for growth including mineral elements, organic compounds such as vitamins, plant growth regulators and a carbon source (Kantharajah, 2001). A continuous supply of optimal amounts of these nutrients and growth regulators are required for induction and maintenance of callus culture. The presence of optimal proportions of growth regulators especially auxins is necessary for embryo initiation, both for the induction and maintenance of embryogenic cell suspension cultures (Gomez *et al.*, 2000). Besides the nutrient and growth regulator composition, callus induction and ECS maintenance is influenced by pH and osmotic condition of the medium as well as the physical factors such as light and temperature of the culture (Razdan, 1993).

1.2 Problem statement

Genetic improvement and mass propagation of EA- AAA banana using conventional methods is difficult due to its biology and low suckering ability. Application of somatic embryogenic techniques to facilitate its genetic improvement and mass propagation is limited by difficulties in callus induction and ECS development. Embryogenic calli/ECS have been obtained in different banana genome groups using auxins (2,4-D, IAA and NAA) either with or without purine-based cytokinins. Most of the EA-AAA banana cultivars have however been recalcitrant to these protocols.

Successful application of tissue culture techniques in crop breeding requires callus growth and plant regeneration potential of each crop species or variety to be determined (Khaleda and Al-Forkan, 2006). Embryogenic calli or embryogenic cell suspensions (ECS) have been generated using mainly immature male flower (Cote *et al.*, 1996; Grapin *et al.*, 1996; Ghosh *et al.*, 2009; Mohandas *et al.*, 2011) and scalp methods (Schoofs *et al.*, 1998; Ramírez-Villalobos and De García, 2008) in different banana genome groups including the AAA-EA banana (Namanya *et al.*, 2002; Sadik *et al.*, 2007). However, the frequency of embryogenic callus encountered on scalps of AAA-EA banana had been zero in most of the cultivars (Strosse *et al.*, 2003) while that from immature male flowers under standard *in vitro* callus induction procedures and in the presence of 0.8-1.0 mg l⁻¹ 2,4-D ranges from 0.01 to 0.02% though recently a frequency of 6.25% was recorded in cultivar Nakinyika following cold stress pre-treatment of the immature flowers (Arinaitwe and colleagues unpublished results). It has also been rare for two or more cultivars to show embryogenic response to the same protocol (Tripathi *et al.*, 2008a).

1.3 Justification of the study

Since propagation and improvement of AAA-EA banana using conventional methods are difficult due to its biology, it is imperative to pursue *in vitro* techniques for its multiplication and genetic improvement. Tissue culture techniques have been applied to enhance banana breeding efficiency (Vuylsteke *et al.*, 1997). Somatic embryogenic techniques particularly have been used to facilitate genetic transformation as an avenue to bypass challenges of polyploidy, low fertility, limited genetic variability and long generation time faced during banana breeding (Meenakshi *et al.*, 2007).

Several authors suggested protocols for transformation of banana through shoot tips (May *et al.*, 1995; Tripathi *et al.*, 2003, 2008a) to avoid the challenges of generating callus and establishing cell suspension cultures. Shoot tips are amenable to transformation regardless of ploidy or the genotype of the banana (Tripathi *et al.*, 2003, 2008a). But somatic embryogenic techniques offer unparalleled means for genetic improvement of banana. Plants regenerated from ECS frequently originate from a single cell and thus in case of transformation, it reduces occurrence of chimeric plants, a problem commonly encountered when using shoot tips as the

target material (Strosse *et al.*, 2003). Somatic embryogenic techniques also offer opportunities for protoplast culture/fusion, production of synthetic seed and spontaneous or induced mutagenesis to be applied. Besides facilitating genetic engineering through micro-injection, electroporation or Agrobacterium techniques, protoplast culture enables transfer of organelles such as mitochondria and/or chloroplasts between sexually incompatible parents and thus creation of novel hybrids (Pelletier, 1993). Further more, though there has been scanty information on *in vitro* proliferation potential of bananas especially AAA-EA banana cultivars, somatic embryogenesis techniques have higher potential for mass propagation of planting material than micropropagation through ordinary shoot-tip culture.

In vitro plant cell differentiation and morphogenesis is highly dependent on culture medium (Khaleda and Al-Forkan, 2006). Conventionally, auxins especially 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA) and naphthalene acetic acid (NAA) have been used either in combination with or without purine-based cytokinins for induction of embryogenesis in banana (Sannasgala *et al.*, 1991, Schoofs *et al.*, 1998; Sadik, 2007). Urea-type cytokinins, N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (thiadiazuron) (TDZ) and N-(2-chloro-4-pyridyl)-N'-phenylurea (forchlorfenuron) (4-CPPU) were reported to be highly active in regulating morphogenesis in tissue culture of many plant species (Victor *et al.*, 2004; Fiore *et al.*, 2004; Haruki *et al.*, 2007). They were also reported to mimic both auxin and cytokinin effects on growth and differentiation of cultured explants in different plant species (Murthy *et al.*, 1998). Despite their promising attributes for manipulation of morphogenesis or growth or cell differentiation, they have not been widely tried in the AAA-EA banana. On the other hand during the establishment of *in vitro* micro-propagation procedure of AAA-EA banana, only a few salt formulations were evaluated (Talengera *et al.*, 1994). Although Murashige and Skoog (1962) (MS) medium was adopted for the routine micropropagation of AAA-EA banana cultivars through shoot tip culture or direct organogenesis, there had been no justification that it was also the best for induction of embryogenesis.

Given the limited success with the existing regeneration protocols, it was logical to study the efficacy of TDZ and 4-CPPU with different salt formulations for embryogenic callus induction in AAA-EA banana cultivars. It was prudent to try both scalps and immature male

flowers because among the explants that have been used in somatic embryogenesis studies in banana, these two explants were reported to be the most responsive material for regenerable callus/ECS induction and yet they have parallel advantages (Meenakshi *et al.*, 2007). Other explants that have been tested include immature zygotic embryos (Marroqin *et al.*, 1993), immature male flowers (Cote *et al.*, 1996; Grapin *et al.*, 1996; Namanya *et al.*, 2002; Pérez and Rosell, 2008, Ghosh *et al.*, 2009; Mohandas *et al.*, 2011), female flowers (Grapin *et al.*, 2000) and somatic tissues such as scalps (Schoofs, 1997; Schoofs *et al.*, 1998; Strosse *et al.*, 2006; Sadik *et al.*, 2007; Ramírez-Villalobos and De García, 2008), *in vitro* leaf bases and corm slices (Novak *et al.*, 1989). Whereas the immature male flower method is less complex and takes a shorter time to induce callus, the scalp method remains a parallel alternative method for banana regeneration, since some bananas including the cooking banana cultivars such as 'Endirira' (AAA-EA) do not produce male flowers on which the male bud method depends (Schoofs *et al.*, 1998). Use of scalps to generate ECS also allows one to obtain explants from *in vitro* cultures or plants at any growth stage and thus in another way saves time, since most banana genotypes including the AAA-EA banana take long to flower. The purpose of this study, therefore, was to establish an efficient and consistent embryogenic callus induction protocol for inherently recalcitrant AAA-EA banana to enhance genetic improvement and propagule production.

1.4 Objectives

The main objective of this study was to enhance plant regeneration through somatic embryogenesis in economically important AAA-EA banana cultivars for efficient propagation and genetic improvement.

The specific objectives were to:

1. Establish the proliferation potential of AAA-EA banana cultivars in MS medium.
2. Enhance embryogenic callus induction from scalps of AAA-EA banana cultivars through application of suitable concentrations of TDZ and 4-CPPU growth regulators.
3. Establish appropriate basal salt mixture in presence of TDZ and CPPU for embryogenic callus induction from immature male flower buds of AAA-EA banana cultivars.

1.5 Hypotheses

1. Number of shoot buds and recovered shoots produced by AAA-EA banana in MS medium is cultivar dependent.
2. Percentage of embryogenic callus induced from scalps of AAA-EA banana is improved by TDZ and 4-CPPU growth regulators.
3. Percentage of embryogenic callus induced by TDZ and CPPU on male flower buds of AAA-EA banana cultivars is influenced by basal salt mixture.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Production of AAA-EA banana is affected by scarcity of planting material, pests, diseases, soil fertility and water stress among others. On the other hand, there are several impediments to its propagation and genetic improvement through conventional methods. This chapter is a review of related work towards development of *in vitro* techniques for propagation and improvement of AAA-EA banana. It discusses proliferation of banana *in vitro* and, efficacy of urea-type cytokinins TDZ and 4-CPPU and various salt formulations for callus induction and plant regeneration.

2.2 Banana micropropagation

Alternative means for multiplication of banana plants have been suggested including macropropagation and *in vitro* micropropagation (tissue culture). Macro-propagation involves field techniques where apical shoots of vigorously growing suckers are completely or partially decapitated to stimulate further sucker proliferation in the field, and detached corm techniques where corms preferably of maiden suckers are manipulated and placed in saw dust to produce plantlets in a screenhouse (Baiyeri and Aba, 2005). Although macropropagation appears to be simpler to conduct by farmers, it can propagate infected plants since low cost technologies have not been developed to enable farmers detect disease pathogens from the generated plantlets (Lorenzen, 2008).

On the other hand micro-propagation involves a collection of techniques used to grow plant cells, tissues or organs *in vitro*, in an aseptic and controlled environment. It is commonly done through shoot tip culture or somatic embryogenesis techniques (Strosse *et al.*, 2003) and has a number of advantages over other banana propagation methods to both banana growers and breeders. It enables more rapid multiplication of disease-free planting material in a smaller amount of space and has potential to yield several plantlets from a single isolated plant tissue (Vuylsteke, 2001). Micropropagated banana plants also establish more quickly, grow more

vigorously, have shorter and more uniform production period, and produce higher yields than propagules produced through other means (Vuylsteke, 2001). In fact, banana plants raised through tissue culture produce more suckers in the field than those propagated conventionally which in another way helps to overcome the problem of shortage of banana planting material (Aish *et al.*, 2003).

Micropropagation is a tool of particular interest to breeders of bananas and other vegetative crops such as pineapple and sugarcane (Geo, 1985). During plant breeding micropropagation can be used for conservation of breeding stock, multiplication of novel hybrids bred through conventional breeding methods or genetic transformation, regeneration of genetically modified cells or cells after protoplast fusion and generation of somaclonal variants from which selections can be made (Svetleva *et al.*, 2003). Micropropagation especially through somatic embryogenesis techniques is crucial for genetic improvement of banana.

2.2.1 Rate of proliferation of banana *in vitro*

Banana shoot proliferation *in vitro* is influenced by genotype/cultivar as well as growth regulator type and concentration (Arinaitwe *et al.*, 2000). In general genotypes with only A in their genome produce fewer shoots than those with one or two Bs in their genome (Strosse *et al.*, 2003). Cultivars with the same genome composition may differ in proliferation capacity (Arinaitwe *et al.*, 2000) and shoot tips from different rhizomes of the same cultivar may proliferate differently under *in vitro* conditions (Aish *et al.*, 2004). AAA-EA banana cultivars were reported to have low proliferation ability with small differences among them (Talengera *et al.*, 1994).

Usually cytokinins, particularly Benzylamino purine (BAP) has been used in MS medium for induction of shoot proliferation in AAA-EA banana (Talengera *et al.*, 1994). However, information on number of shoots especially recoverable shoots that could be produced from one starting shoot-tip explant after a given period and/or subculture cycle had been scanty. Aish *et al.* (2004) reported an average of 124 plants from each shoot tip of cv. Basrai (AAA) after five subculture cycles or months on MS basal medium supplemented with 5.0mg l⁻¹ BAP.

Similar information on proliferation of AAA-EA banana cultivars could enhance development of *in vitro* techniques for their multiplication.

2.2.2 Somaclonal variation as a limiting factor to recoverable banana shoots in vitro

Somaclonal variation is a phenotypic variation, either genetic or epigenetic displayed among plants regenerated from tissue culture or soma clones (Larkin and Scowcroft, 1981) resulting from both pre-existing variation within the explant and variation induced during the tissue culture phase (Leela *et al.*, 2003). It is manifested in different ways. Variation in karyotype, isoenzyme pattern, precocity for bearing, ploidy level, growth, yield, quality, pigmentation, disease resistance and tolerance to unfavorable soil conditions such as nutrient deficiency and climatic conditions such as drought were reported in somaclones of different plant species (Patil and Navale, 2000). Phenotypic variations such as rosy cluster of faint green leaves and twisted leaves have been reported in bananas (El-DougDoug *et al.*, 2007).

Though somaclonal variants may have desirable attributes and in the long run lead to selection of new varieties (Semal, 1986), they are condemned in commercial plantations because they distort uniformity and may be inferior to the preferred variety. Therefore prevention of their occurrence when multiplying bananas is critical. Occurrence and frequency of somaclonal variation is influenced by genotype and culture conditions including explant source, age of culture and concentration of growth regulators (Leela *et al.*, 2003). Since cultivar is usually fixed and the concentration of the adopted growth regulator can be standardized within a protocol, there is little to worry about these two factors. In fact practically there has been no evidence of the growth regulators routinely used in tissue culture at optimum concentrations directly affecting or increasing incidence of somaclonal variation in banana (Strosse *et al.*, 2003). But explant source and subculture cycle were reported to have substantial influence on somaclonal variation in banana (Strosse *et al.*, 2003). Therefore when selecting an explant and number of subculture cycles there should be considerable care and knowledge to avoid wrong choices and prolonged subculturing of the primary explants.

2.2.2.1 Effect of explant source and technique on somaclonal variation

Explant source (Razdan, 1993) and regeneration technique used (Merkle *et al.*, 1990) are critical variable for somaclonal variation. Highly differentiated tissues such as roots, leaves and stems are reported to produce more variations than meristemic tissues such as shoot tips and axillary buds (Duncan, 1997). However, somaclonal variation was reported to be higher in shoot tip derived cultures of cv. Grande Naine (5.3%) as compared to those derived through somatic embryogenesis using other plant parts (Schukin *et al.*, 1997). Similarly, Israeli *et al.* (1996) reported frequency of off-types among Cavendish banana plants produced via shoot tip culture to be higher than those obtained via somatic embryogenesis. Generally, Somatic embryogenesis is unique from regeneration via organogenesis (Merkle *et al.*, 1990). Somatic embryos tend to be less subject to somaclonal variation than organogenesis presumably because they do not tolerate it as it disrupts their ontogeny. Leela *et al.* (2003) suggested that hypomethylation of DNA in somatic cells during somatic embryogenesis induces a state of differentiation resembling that in the early zygotic embryos which leads to low somaclonal variation. Although there are also reports that somaclonal variation in plants generated from somatic embryogenic techniques is higher compared to the level observed in those produced through classical shoot tip culture (Strosse *et al.*, 2003), this review gives a clue to minimizing somaclonal variation due to explant influence during multiplication of planting material.

2.2.2.2 The effect of number of subculture cycles on somaclonal variation in banana

Culture age enhances variability among regenerated plants (Leela *et al.*, 2003) because *in vitro* culture conditions and rapid multiplication of a tissue affect genetic stability and increase mutations which lead to somaclonal variation (Duncan, 1997). This scenario is also observed in bananas (Reuveni and Israeli, 1990; Shepherd *et al.*, 1996; Zhenxun and Hongxian, 1997; Rodrigues *et al.*, 1998; Santos *et al.*, 2004; El-DougDoug *et al.*, 2007;).

2.3 Somatic embryogenesis in banana

The technique of cloning isolated single cells *in vitro* under appropriate conditions into whole plants is called somatic embryogenesis (Razdan, 1993). This potential of a cell to develop into a whole plant lies mainly in cellular differentiation and is due to the fact that genes

responsible for plant cell differentiation are present within all individual cells (Razdan, 1993). This implies that even inactive cells in differentiated tissues or organs are able to divide and grow under adequate culture conditions because the differentiated cell undergoes dedifferentiation during which the mature cell reverts to a meristematic state and forms undifferentiated callus tissue and then re-differentiation whereby the dedifferentiated cell or undifferentiated callus tissue forms a whole plant or plant organs (Razdan, 1993). Through manipulation of the culture condition, somatic cells from callus or freshly isolated explants can thus be induced to form somatic embryos.

The whole plant regeneration from callus tissue may occur either through organogenesis (shoot-bud differentiation) or somatic embryogenesis where the cell or group of cells initiate a development-pathway that leads to reproducible regeneration of non-zygotic embryos capable of germinating to form complete plants (Razdan, 1993). Then again somatic embryogenesis occurs in two ways: direct somatic embryogenesis and indirect somatic embryogenesis. During direct somatic embryogenesis, somatic embryos form directly on the surface of organized tissue such as a leaf or stem segment and the cells of the explant which develop into embryos behave as if they are predetermined for embryogenic development but only waiting for a suitable condition to allow its expression (V́ctor, 2001). Whilst in indirect somatic embryogenesis, embryos form indirectly via an intermediary step of callus or suspension culture requiring more complex medium and other factors to induce dedifferentiation and redifferentiation of the differentiated explant cells (Emons, 1994).

Although it is not clear which changes a somatic cell must undergo in order to become embryogenically capable, non-embryogenic cells can be induced to an embryogenic state by a variety of procedures including exposure to plant growth regulators, pH shock (including change in pH of the culture medium), heat/ temperature shock or treatment with various chemical substances, change in carbon source (from sucrose to glycerol), NH_4^+ concentration, light quality and subculture duration (V́ctor, 2001).

2.3.1 Role of hormones/growth regulators in somatic embryogenesis

Plant growth hormones are chemicals occurring naturally (endogenously) in plant tissues with regulatory rather than a nutritional role in growth and development (George *et al.*, 2008). They are generally active at very low concentrations and exist in several classes including auxins, cytokinins, gibberellins, ethylene and abscisic acid (Victor, 2001). They have synthetic versions with a biological activity equals to or exceeds that of their natural form (George *et al.*, 2008). The synthetic versions of plant growth hormones or substances are referred to as plant growth regulators and are added exogenous to *in vitro* cultures (George *et al.*, 2008). Though there are several classes of growth substances, plant differentiation *in vitro* is majorly regulated by auxins and cytokinins (Raemakers *et al.*, 1995).

Auxins are low-molecular weight organic substances, containing either an indole or an aromatic ring (George *et al.*, 2008). Besides indole-3-acetic acid (IAA), the most common natural auxin in plants there other natural auxins including 4-chloro-IAA, indole-3-butyric acid (IBA), phenylacetic acid (PAA) and precursors and metabolites of IAA such as indole-3-pyruvic acid, tryptamine and tryptophol which also have auxin effect George *et al.* (2008). The synthetic analogues of IAA include 2,4-dichlorophenoxyacetic acid (2,4-D), Naphthalene acetic acid (NAA) and Pichloram (2,4,5-T) (Victor, 2001).

Both naturally occurring auxins and their synthetic counterparts, in spite of their different structures, have similar biological properties and physiological effects (George *et al.*, 2008). According to the review of George *et al.* (2008) Forintance most of the IAA produced within plants is conjugated to other compounds to form esters, amides or glycosyl esters which are similar to synthetic auxins which can also be converted to conjugates with glucose (George *et al.*, 2008). Conjugation is a useful mechanism for storing IAA in cells and metabolizing excess auxin and thus stabilizing the level of free auxin in the plant (Normanly *et al.*, 2004). In conjugated molecule the auxin is protected from oxidative breakdown and can be released again through the action of enzymes (George *et al.*, 2008). The metabolism of auxin as well as the metabolism of any other hormone, consists of both biosynthetic and hormone-molecule modifying reactions. Biosynthesis of auxin is more intensive in meristematic regions and young growing organs such as rapidly growing leaves, apical buds, root tips, and developing

inflorescences and auxin is transported through the plant in special cells in vascular bundles (George *et al.*, 2008). The relative degree of activity of individual auxins in different growth processes thus depends on the plant, organ, tissue, cell, age and physiological state of the plant tissue (Davies, 2004) which on the other hand influence the rate of uptake and transport of the applied auxin to the target tissue, the inactivation (oxidation and/or conjugation) of the auxin within the explant, the level of the auxin in the explants and the sensitivity of the plant tissue to auxin.

George *et al.* (2008) reviewed the role of auxins extensively. In general auxins promote, mainly in combination with cytokinins, the growth of calli, cell suspensions and organs, and also regulate the direction of morphogenesis. At the cellular level, auxins control basic processes such as cell division and cell elongation. Since they are capable of initiating cell division they are involved in the formation of meristems giving rise to either unorganised tissue, or defined organs. In organized tissues, auxins are involved in the establishment and maintenance of polarity and in whole plants their most marked effect is the maintenance of apical dominance, mediation of tropisms, growth of stems, root development and conversion of stems into flowers and consequently fruits (Daphne and Michael, 2005). Among the plant growth regulators that mediate transition from somatic to embryogenic cells, auxins have mainly been used to induce embryogenesis. In Angiosperm monocots, primary embryogenesis was exclusively induced by auxin-supplemented media though a variety of growth regulators induced somatic embryogenesis in dicot species (Raemakers *et al.*, 1995). The auxins commonly used for induction of embryogenesis are 2,4-D, NAA, IAA, IBA, Picloram and Dicamba (Raemakers *et al.*, 1995).

Whereas auxins readily induce embryogenesis, further development of the induced somatic embryos is achieved by reducing or removing the auxin from the culture media because continued presence of auxin in the media leads to differential change in expression of the genes necessary to complete embryogenesis and instead the proembryogenic mass formed contains other mRNAs and proteins which generally inhibit the continuation of embryogenesis (George *et al.*, 2008). The removal of the auxin results into inactivation of a number of genes which allows embryogenesis to proceed. On the other hand polar transport of

auxin is essential for the establishment of bilateral symmetry during embryogenesis of somatic and zygotic embryos (Fischer and Neuhaus, 1996). Reduction or removal of the auxin particularly 2,4-D which usually increases the endogenous auxin level in the explant (Michalczyk *et al.*, 1992) from the culture medium allows establishment of a polar auxin gradient which stimulates somatic embryogenesis. Therefore natural phenomena such as conjugation of auxins with other compounds reduce the auxin levels in embryogenic callus to a level which induces active embryogenesis. However different auxins regulate embryogenesis in different ways. For instance ABA regulates genes involved in desiccation and maturation phases of embryogenesis by regulating carbohydrate metabolism via inhibition of α -amylase activity, stimulation of DNA synthesis and inhibition of peroxidase activity which causes degradation of IAA leading to formation of pre-globular structures (Rajasekaran *et al.*, 1987b).

Similar to auxins there are two distinct groups of compounds with cytokinin effect: N6-substituted adenine derivatives, the classical cytokinins and substituted urea compounds such as diphenylurea (Victor, 2001). The natural N6-substituted adenine cytokinin, Zeatin is produced in the root meristem and transported throughout the plant as Zeatin-riboside in the phloem. Both zeatin and its synthetic analogues, Benzyladenine (BA), N6-benzylaminopurine (BAP) and Kinetin stimulate DNA synthesis, cell division and a delayed response in undifferentiated tissue leading to formation of shoot primordia and or adventitious shoots (George *et al.*, 2008). They also delay senescence or the aging of tissues and are responsible for mediation of auxin transport throughout the plant. The cytokinin activity of substituted urea compounds such as N-phenyl-N'-1,2,3-thidiazol-5-ylurea (thidiazuron) (TDZ) has been reported to be similar to that of the most active cytokinins of adenine-type (George *et al.*, 2008).

Cytokinins have not been used primarily to induce embryogenesis though they have synergistic effect with auxins where a combination of the two can lead to callus formation depending on the proportion (Victor, 2001). Since cytokinins stimulate cell division, they could be important during the initial cell division phase of somatic embryogenesis (Danin *et al.*, 1993). However, though they have similar activity with adenine-based cytokinins,

ureatype cytokinins such as TDZ and N-(2-chloro-4-pyridyl)-N'-phenylurea (forchlorfenuron) (4-CPPU) were reported to promote growth of cytokinin-dependent callus cultures (George *et al.*, 2008). The unique influence of TDZ and 4-CPPU on morphogenesis in tissue culture was reported by different authors (Victor *et al.*, 2004; Fiore *et al.*, 2004; Haruki *et al.*, 2007) most of which suggest that they could function both as auxin and cytokinin. Therefore as the morphogenetic response of banana explants in culture is influenced by type besides the concentration of growth regulators (Nirmalya and De Langhe, 1985) it was necessary to study the effect of urea-type cytokinins on callus induction in banana.

2.3.1.1 Efficacy of TDZ and 4-CPPU for embryogenic callus induction from scalps

The type and concentration of growth regulators particularly of auxins are important factors to consider in callus induction and generation of ECS. The most commonly used auxin for induction of embryogenesis from scalps is 2,4-D (Schoofs *et al.*, 1997; Schoofs *et al.*, 1998; Sadik *et al.*, 2007). Schoofs *et al.* (1998) obtained ECSs from nodular callus generated by inoculating scalps on a semi-solid half strength MS mineral salt mixture supplemented with 5 μ M 2,4-D and 1 μ M Zeatin. The embryogenic cell aggregates appeared within 6 weeks with the cultivar Bluggoe (ABB) giving the best results. But some cultivars failed to produce any embryogenic cell material. Sadik *et al.* (2007) initiated cell suspensions by inoculating scalps directly in liquid medium comprising half strength MS mineral salts supplemented with 5 μ M 2,4-D and 1 μ M Zeatin. Cell suspensions were seen 2-3 weeks depending on the cultivar but there was poor embryogenic response in all the AAA-EA banana cultivars. This was possibly because 2,4-D and Zeatin at those concentrations were not strong enough to induce somatic embryogenesis in these inherently recalcitrant banana group. Earlier Sannasgala *et al.* (1992) obtained embryogenic cell suspensions by inoculating scalps in liquid medium of MS containing growth regulators 2,4-D, 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) and 6-Benzyl-adenine (BA). Whitish globular structures with embryogenic characteristics were released after the 3rd and 4th week but plant regeneration was poor.

Studies on effect of urea-type cytokinins on *in vitro* responses in various crops reveal better responses than other cytokinins. TDZ and 4-CPPU were reported to be more effective for the induction and morphogenesis of adventitious shoots than adenine-type cytokinin, BA in

Mesembryanthemum crystallinum (Haruki *et al.*, 2007). However, TDZ showed more strength in induction of axillary shoot proliferation in rose plants than CPPU (Singh and Syamal, 2001). In bananas, TDZ at relatively lower concentrations evoked higher degree of shoot proliferation than adenine based cytokinins (Arinaitwe *et al.*, 2000) while at relatively higher concentrations above 8 μM , TDZ induces scalps in bananas (Sadik *et al.* (2007).

Fiore *et al.* (2004) carried callus induction, somatic embryogenesis and plant regeneration in lemon (*Citrus limon* L.) and sweet orange (*C. sinensis* L.) on 16 different media, based on the nutrients and vitamins of Murashige and Tucker medium (MT) supplemented with different combinations of 2,4-D and 4-CPPU. Somatic embryos arose from callus at the surface of the explants 3 - 5 months after culture initiation and the percentage of embryo formation in both sweet orange and lemon were higher in the media containing 4-CPPU than those containing 2,4-D. These effects of TDZ and 4-CPPU on callus induction, somatic embryogenesis and plant regeneration have however, not been widely tested in bananas especially AAA-EA banana.

2.3.1.2 Efficacy of TDZ and 4-CPPU for embryogenic callus induction from immature male flower buds

There was scanty information on effect of TDZ or 4-CPPU on callus induction and ECS development from immature male buds of bananas. However, in other crops, urea-based growth regulators especially TDZ induced a diverse array of culture responses ranging from induction of callus to formation of somatic embryos (Murthy *et al.*, 1998). TDZ exhibits the unique property of mimicking both auxin and cytokinin effects on growth and differentiation of cultured explants, although structurally it is different from either auxins or purine-based cytokinins (Murthy *et al.*, 1998).

When intact peanut (*Arachis hypogaea* L.) seeds were incubated on medium containing 10 μM TDZ and 50 μM BAP the observations revealed that the regenerants induced by TDZ were somatic embryos while those induced by BAP were shoots (Victor *et al.*, 2004). Ying-Chun *et al.*, 2000) induced callus of *Phlaenopsis* *Nebulas* from seed derived protocorms on $\frac{1}{2}$ MS basal medium plus 0-1.0 mg l^{-1} (0-4.52 μM) TDZ and or 0-10 mg l^{-1} (0-45.24 μM) 2,4D.

They reported more callus on the medium supplemented with 0-1.0 mg l⁻¹ (0-4.52 µM) TDZ than with 0-10 mg l⁻¹ (0-45.24 µM) 2,4D.

Synergetic effect between TDZ and auxins or other cytokinins has been evident. A combination of 2,4-D and TDZ changed granular callus into a favorable friable form in *Oncidium* (Fang *et al.*, 2006). Tuong Huan *et al.* (2004) induced embryogenic calli from protocorm of *Cymbidium*, a hybrid orchid, on media supplemented with NAA or 2,4-D alone or in combination with TDZ. The medium containing the combination of 0.1 mg l⁻¹ NAA and 0.01 mg l⁻¹ TDZ formed more callus. However such interesting effects of TDZ or 4-CPPU on callus induction had not been tried in AAA-EA banana.

TDZ and 4-CPPU have been shown to be efficacious in morphogenesis of many dicot plant species however; information regarding their effect on *in vitro* regeneration of monocot species especially bananas is limited. This implies that in investigating their effects on *in vitro* regeneration of AAA-EA banana, it might be necessary to consider a wide range of their concentrations. Shan *et al.* (2000) observed plant regeneration from calluses derived from immature embryo culture of barley and wheat on a wide range of TDZ concentrations (0.045–45 µM).

2.3.2 Inorganic salts and their role during *in vitro* culture

Nutrient media for plant tissue culture are designed to allow plant tissues to be maintained in a totally artificial environment. Therefore most popular media formulations such as Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), White's medium (White, 1963), Gamborg's B5 medium (Gamborg *et al.*, 1968) and Schenk and Hildebrandt (SH) medium (Schenk and Hildebrandt, 1972) were formulated by analyzing nutrient components in particular plant species. Murashige and Skoog (MS) medium was developed for culture of tobacco and was formulated based on an analysis of the nutrient compounds present in the tobacco tissue (Murashige and Skoog, 1962). It has comparatively high salt levels, particularly of potassium (K) and Nitrogen (N). White's medium (White, 1963), was developed originally for the culture of tomato roots and has low salt levels. Gamborg's B5 medium (Gamborg *et al.*, 1968) was developed for soybean callus culture and contains a

much greater proportion of nitrate compared to ammonium ions. Schenk and Hildebrandt (SH) medium was developed for the culture of callus of both monocots and dicots (Schenk and Hildebrandt, 1972). Nitsch medium (Nitsch and Nitsch, 1969) was developed for anther culture and contains lower salt concentrations than MS medium, but not as low as in White's medium.

Salts as well as the forms in which they are supplied are very important (Gamborg *et al.*, 1968). Based on the quantities required by plants, mineral elements are grouped into two: macroelements and microelements (Razdan, 1993). Macroelements consist of Nitrogen (N), Potassium (K), Phosphorous (P), Calcium (Ca), Magnesium (Mg), and Sulphur (S). The microelements commonly used in plant tissue culture are Iron (Fe), Manganese (Mn), Zinc (Zn), Boron (Bo), Copper (Cu) and Molybdenum (Mo), Cobalt (Co) and Iodine (I).

Razdan (1993) and several other authors described the forms in which both macro- and microelements are added to the media as well as their functions. In brief nitrogen is added to plant nutrient media in oxidized nitrate ion (NO_3^-) form and/or reduced ammonium ion (NH_4^+) form as inorganic salts. Both amount of nitrogen as well as the relative amounts of NO_3^- and NH_4^+ are very important though the level of NH_4^+ is lower than that of NO_3^- in most media. Cultures of most species proliferate better on medium containing both nitrates and ammonium inorganic nitrogen sources. But some species can grow on a medium with ammonium alone provided one or more of the TCA cycle acids such as citrate, succinate and malate are included in the medium. The use of both nitrogen forms helps to maintain the pH of the medium and enhances plant response (Vinterhalter *et al.*, 2007). Nitrogen may also be added to medium in an organic form, as amino acids such as proline or glutamine or hydrolysates such as casein hydrolysate. Since organic nitrogen is already reduced i.e. in the form in which most nitrogen exists in the plant, it is taken up more readily than inorganic nitrogen (Vinterhalter *et al.*, 2007). Most inorganic nitrogen is converted to amino acids and then to proteins and thus nitrogen is essential for plant growth. The amount of potassium required by plants varies widely among different species but its concentration in media is generally correlated with that of nitrate and it plays an important role in balancing negative ions in plants as a major ion with a positive charge. Phosphorous is added to culture medium

as phosphate (PO_4^-) in sodium or potassium hydrogen phosphates and is an integral part of nucleic acids and other structural compounds. Calcium is supplied mostly as calcium chloride or calcium nitrate and is a co-factor of many enzymes and is particularly important in cell wall synthesis. Magnesium is usually added as magnesium sulfate in concentrations similar to that of calcium. In addition to balancing negative ions as a cation, it is an integral component of the chlorophyll molecule and is critical for the functioning of enzymes. Sulfur is supplied as the SO_4^- ion, generally with magnesium as the cation. It is a part of several amino acids and has an important function in protein structure.

Although microelements are added in small quantities some play vital role in tissue culture. For instance iron is added in a chelated form (Fe-EDTA) and is necessary for chlorophyll synthesis and functions in many oxidation/reduction reactions. However, Fe in combination with light synergistically promotes photodegradation of IAA (Dunlap and Robacker, 1988). Manganese is usually added as manganese sulfate and is required for enzyme reactions, particularly in respiratory and photosynthetic processes. Zinc is most commonly added to media as sulfate salt in concentrations similar to that of manganese and is required in many enzyme activities. Boron is supplied as boric acid in culture medium and is an essential element in lignin biosynthesis and metabolism of phenolic acids. Copper is added to culture medium as cupric sulfate in very low concentrations because at high amounts it can be toxic. It is critical in many enzyme reactions including the cytochrome oxidase system. Molybdenum is added as sodium molybdate in low concentrations in culture medium and functions in the transformation of nitrate to ammonium. Cobalt, Iodine and other elements including Nickel (Ni), Aluminum (Al) and Silicon (Si) have been added to some media formulations but have not been found to be essential for most plant species.

A basal salt formulation or media is complex consisting of many elements or components with some having divergent functions. Since there are considerable differences in requirements for different plant species and genotypes or cultivars, it is important to test several ideal media for particular plant species and genotypes for possible adoption of the most responsive one or wholesome or optimizing a few components in it (Kantharajah, 2001) for

the particular crop. The focus of this study therefore was to test different salt formulation for callus induction in AAA-EA banana.

2.3.2.1 Influence of salt formulation on callus induction

The influence of culture medium on callus/ECS induction in bananas was studied by several authors using both scalps (Schoofs *et al.*, 1998; Nahamya, 2000; Sadik *et al.*, 2007) and immature male flowers (Cote *et al.*, 1996; Ganapathi *et al.*, 1999; Gomez *et al.*, 2000). Though the goal of most of these studies was to test effect of auxins and cytokinins, they to some extent used some salt formulations and vitamins.

Schoofs *et al.* (1998) obtained scalps for induction of embryogenesis from several banana cultivars with low proliferation rate by maintaining proliferating meristems on MS mineral salt mixture supplemented with 1 μM IAA and 100 μM BAP and sub-culturing them at 4-8 weeks, depending on the cultivar. But too many sub-culture cycles (up to 9 depending on the cultivar) were required. In similar studies Sadik *et al.* (2007) using an MS derived medium described by Talengera *et al.* (1994) supplemented with various concentrations of BAP and TDZ, generated scalps from AAA-EA banana cultivars but there response was relatively low.

Several other studies were carried out on callus induction and ECS development from immature male buds of various banana genotypes. But only a few salt formulations were used. Cote *et al.* (1996) achieved embryo development in cv. Grande Naine (AAA) by plating the suspension on a medium of SH, vitamins of MS supplemented with 1.1 μM NAA, 0.2 μM zeatin, 0.5 μM kinetin, and 0.7 μM 2ip. In another study, Gomez *et al.* (2000) achieved embryo formation in hybrid cultivar FHIA – 18 (AAAB) using a medium of SH, vitamins of MS supplemented with 0.2 mg l^{-1} NAA, 0.05 mg l^{-1} kinetin and 0.05 mg l^{-1} zeatin. Secondary multiplication and maturation of the somatic embryos were achieved on basic MS medium supplemented with 0.3 mg l^{-1} BAP, 1 mg l^{-1} IAA; and 0.5 mg l^{-1} BAP and 2 mg l^{-1} IAA respectively. Ganapathi *et al.* (1999) also established embryogenic cultures using young male flowers on full strength MS medium and White's medium supplemented with 0.22 μM BA and 1.14 μM IAA. They found MS medium to be better than the White's medium in terms of

embryogenic callus induction. In general these studies clearly indicate that not many salt formulations were tested in bananas especially the AAA-EA bananas.

CHAPTER THREE

GENERAL METHODOLOGY

3.1 Genotypes and source of explants

The study was conducted in the Plant Tissue Culture Laboratory at Makerere University Agricultural Research Institute, Kabanyolo (MUARIK). Seven AAA-EA banana cultivars namely Mpologoma, Musakala, Mbwazirume, Nfuuka, Nakinyika, Nakabululu and Kabula from different clone sets as described by Karamura and Pickersgill (1999) were used. Yangambi Km5, an AAA banana, with origin from West Africa (Pillay *et al.*, 2006), and Sukali Ndizi, an exotic banana with genome AAB (Pillay *et al.*, 2003) were included for comparison in some of the experiments. The explants were obtained from the banana germplasm field at MUARIK and the banana fields of the National Banana Program at National Agricultural Research Laboratories (NARL), Kawanda.

3.2 Experiments

First *in vitro* shoot proliferation potential and number of recoverable shoots on the adopted MS derived banana multiplication medium was determined through normal shoot-tip culture. Then both scalp and immature male flower bud methods were pursued for somatic embryogenesis as illustrated diagrammatically below. In the scalp method, various combinations of TDZ and 4-CPPU were tested for scalp and callus induction. Immature male flower buds were excised and cultured on callus induction medium supplemented with different combinations of TDZ and CPPU alone or in presence of Indoleacetic acid (IAA) and Naphthalene acetic acid (NAA). Different salt formulations including Chu (N₆), Eriksson, Gamborg B5, Nitsch, NLN, SH and White were also tested for embryogenic callus induction through immature male flowers by substituting only MS inorganic salts (MS vitamins and other medium components retained) from the callus induction medium with the best TDZ and 4-CPPU combination.

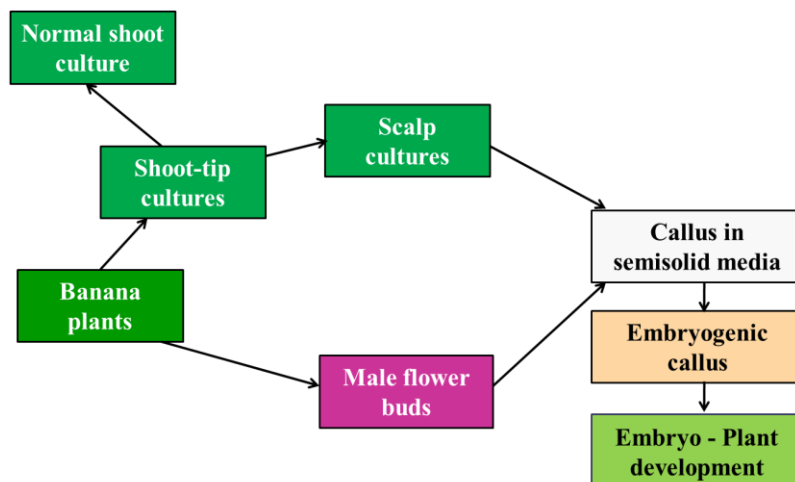


Figure 3.1 Diagrammatic illustration of the general methodology: From banana plants where shoot-tip explants were obtained to normal shoot culture or through scalp, callus and embryo induction to plant development or from banana plants where immature male flower explants were obtained through callus, embryo induction to plant development.

Shoot-tip explants used for determination of shoot proliferation potential and generation of scalps (Figure 3.1) were collected from young suckers of 40-100 cm height. Male flowers were collected 1 to 3 weeks after flowering from the same fields.

3.3 Data Analysis

The data on differentiated shoots, buds and total shoots as well as that on rate of scalp formation were subjected to ANOVA using GenStat 13th Edition (GenStat, 2010) and the means separated using the least significant difference (LSD) test at 5% level of significance. Data on callus formation was summarized in percentages using Excel computer program. Callus directly formed on the scalp induction media was summarized as percentage of explants or cultures with callus on the scalps being formed. Induced callus was summarized as a percentage of scalps that generated callus to the total number of scalps initiated on callus induction medium. Callus response from immature male flowers was presented as percentage of male flower explants that produced yellow callus to the total number of male flowers initiated on callus induction medium. The methodology including data analysis for each experiment is described in more detail in the respective chapters four, five and six.

CHAPTER FOUR

IN VITRO PROLIFERATION AND SHOOT RECOVERY AMONG THE EAST AFRICAN HIGHLAND BANANA

4.1 Introduction

In vitro micropropagation is an alternative mean of producing banana planting material. Micropropagation of banana, especially through shoot tip culture has of recent become a routine technology in many banana growing areas of the world including East Africa (AATF, 2009). A couple of commercial tissue culture laboratories have been established and the farmer adoption rate of tissue culture plants in the region has rapidly increased (AATF, 2003). Consequently, enormous research efforts have targeted enhancing the existing banana micropropagation protocols and adapting them to different AAA-EA banana cultivars (Makara et al, 2010). However, there had been scanty information on proliferation potential of AAA-EA banana cultivars *in vitro*. Under natural field conditions, AAA-EA bananas differ greatly in suckering ability (Karamura and Pickersgill, 1999). Hitherto, there had been no empirical data to compare *in vitro* proliferation of banana cultivars that naturally have low suckering ability *vis-a-vis* those with high suckering ability. Information on *in vitro* proliferation of AAA-EA banana cultivars would enhance development of micropropagation protocols for their multiplication and aid producers of tissue culture plants in planning, especially in deciding which cultivars would be economic to multiply. The objective of this study therefore, was to determine proliferation potential and the number of recoverable or weanable plantlets that can be generated from a single isolated shoot tip of AAA-EA banana cultivars with different suckering ability in the field.

4.2 Materials and Methods

Seven AAA-EA banana cultivars from different clone sets (Mpologoma and Musakala from Musakala clone set, Mbwazirume from Nakitembe clone set, Nfuuka and Nakinyika from Nfuuka clone set, Nakabululu and Kabula from Nakabululu and Mbidde clone sets, respectively) were studied. Non AAA-EA bananas, Yangambi Km5 (AAA) and Sukali Ndizi (AAB) were included for comparison. Cultivars in Musakala clone set have low suckering

ability while those in Nakitembe and Nakabululu clone sets have higher suckering ability (Karamura and Pickersgill, 1999). Other clone sets have moderate suckering ability.

4.2.1 Determining *in vitro* proliferation ability

Fifty shoot-tip explants of each cultivar were obtained from young suckers of 40-100 cm height uprooted from a banana mother garden at Makerere University Agricultural Research Institute, Kabanyolo (MUARIK). The suckers were trimmed to about 2-3 cm for both diameter and height. The explants were sterilized by immersing them for 20 minutes in 15 % v/v sodium hypochloride (a commercial bleach Jik containing 3.85% NAOCL was used) to which 0.2 % v/v of Tween 20 was added, and then in 75 % ethanol for 10 minutes before rinsing three times with distilled water. After sterilisation, the explants were further reduced to uniform size of 2 cm diameter and height, and carefully bisected longitudinally. The two halves of each shoot tip were initiated in a culture bottle containing 30 ml of the Murashige and Skoog (MS) medium modified for banana multiplication as described by Talengera *et al.* (1994).

The explants in culture bottles were placed in CRD in a growth room maintained at $26 \pm 2^{\circ}\text{C}$, in a light cycle of 16 hr with a photosynthetic photon flux (PPF) of about $60 \mu\text{E}/\text{m}^2\text{s}_1$ (Strosse *et al.*, 2003). Initially, the cultures were transferred to fresh multiplication medium each week for two weeks. At transfer 1 (T1) the dead or necrotic parts were cut off and each explant split again into two parts while at transfer 2 (T2) any isolatable shoot meristem was cut and cultured separately. Following T2, sub-culturing was done every 4 weeks for 4 cycles (S1–S4) before transferring the cultures to rooting medium. After 4 weeks on rooting medium, the cultures were weaned into steam sterilised soil substrate.

At each subculturing cycle, proliferating shoots and buds were counted and recorded before a random shoot from each bottle was transferred to the next subculture cycle. Other growth features such as shoot morphology and clustering were also observed. The number of differentiated shoots and buds were noted, and the total computed as the total shoot proliferation (TSP) potential per cycle. The data on differentiated shoots, buds and total

shoots were subjected to ANOVA using GenStat 13th Edition (GenStat, 2010) and the means separated using least significant differences (LSD) at 5% level of significance.

4.2.2 Determining recoverable shoots and derivation of predictive model for recoverable shoots

As the shoots multiplied and increased in number with culture cycle, a manageable proportion of twenty to thirty shoots were randomly picked and serially sub-cultured to determine the number of plantlets that could be generated from a shoot tip in a given time period. The number of recoverable shoots (RS) from an explant at end of a cycle was calculated as:

$RS = N \cdot P / S$. Where,

P = number of plantlets produced at the end of each cycle

S = number of shoots sub-cultured at each cycle to produce P plantlets

N = cumulative number of shoots cultured at that cycle

After linearising the data on recovered shoots and realizing a non-linear recoverable shoot growth pattern, the data were transformed into logarithmic scale to create new variables:

$$y = \ln Y,$$

where, Y is the number of recovered shoots and y is the transformed data. A least squares method was then used to fit the transformed data or new variables to a model using GenStat 13th Edition (GenStat, 2010):

$$y_{ij} = [(b_k + b_j)(x_i - 1) + b_q(x_i - 1)^2 + c].$$

Where,

y_{ij} = is the regression equation of cultivar j

b_k = average linear regression coefficient, is the average linear rate of growth in number of recoverable shoots for the cultivars

b_j = difference of the specific linear regression coefficient for cultivar j from b_k

- $b_k + b_j$ = individual linear regression coefficient for cultivar j, is the linear rate of growth in number of recoverable shoots produced from one shoot-tip explant of cultivar j per week or per unit change in time measured in weeks
- x_i = time in weeks to produce a given number of recoverable shoots from one shoot tip explant
- b_q = average quadratic regression coefficient, is the average quadratic rate of growth in number of recoverable shoots for the cultivars
- c = intercept point of the regression line, is the number of explants at T1 when the time (x) is 1 week (or the number of explants at initiation multiplied by 4)

The number of recoverable shoots produced from one shoot tip after a given time could, thus be predicted by converting the prediction for transformed data or transformed number of recoverable shoots (y) into untransformed data or actual number of recoverable shoots (Y) as follows:

$$Y_{ij} = e^{y_{ij}}$$

$$Y_{ij} = e^{[(b_k + b_j)(x_i - 1) + b_q(x_i - 1)^2 + c]} \text{ or } Y_{ij} = \text{Exp}[(b_k + b_j)(x_i - 1) + b_q(x_i - 1)^2 + c].$$

Where,

- Y_{ij} = number of recoverable shoots produced from one shoot tip explant of cultivar j after a given time in weeks
- e = 2.718282, is the base of the “natural logarithms”

The assumptions for this prediction model are that: recoverable shoots of bananas multiply in a linear function followed by a quadratic function; there is no contamination, the error in the transformed equation is normally distributed with mean 0 and other assumptions for regression hold.

The means of the multiplication rates for total shoots and recovered shoots of the cultivars were computed to determine the proportion of total shoots recoverable or effectiveness to generate plantlets from shoot buds. The linear regression coefficients (the rates of growth in

number of recoverable shoots) of the cultivars were separated using least significant differences (LSD) at 5% level of significance.

4.3 Results

4.3.1 *In vitro* shoot and bud proliferation

The number of differentiated shoots and buds produced by the different cultivars *in vitro* is given in Table 4.1. Significantly ($P < 0.05$) higher numbers of shoots were produced by Yangambi Km5 and Sukali Ndizi than the AAA-EA banana cultivars). All through the subculture cycles, Yangambi Km5 produced significantly ($P < 0.05$) the highest number of shoots among the studied cultivars, followed by Sukali Ndizi which also produced significantly ($P < 0.05$) higher number of shoots than the AAA-EA banana cultivars. The cultivars with high number of buds in general showed higher total number of shoots or proliferation ability.

The proliferation trend of the cultivars showed a linear increase and decrease in differentiated shoots and buds, respectively over the successive subculture cycles. The increase in differentiated shoots accounted for 92% of the sum of squares due to cycles, while the decrease in buds accounted for 89% of the sum of squares due to cycles (ANOVA table not shown). In general the AAA-EA banana cultivars produced between 3 and 4 total shoots while Sukali Ndizi and Yangambi Km5 produced 5 and 9 total shoots, respectively, at each subculture cycle. Among the AAA-EA banana cultivars Kabula had higher total shoots though its shoots were stunted and resulted into fewer recovered shoots.

Table 4.1. Number of shoots and buds of AAA-EA banana cultivars, and Yangambi Km5 and Sukali Ndizi cultivars at different subculture cycles on MS medium modified for banana multiplication

Cultivar	Subculture 1		Subculture 2		Subculture 3		Subculture 4	
	Shoots	Buds	Shoots	Buds	Shoots	Buds	Shoots	Buds
Mpologoma	2.4	1.6	2.5	2.0	2.5	0.8	3.6	1.4
Musakala	1.5	1.6	2.2	2.1	2.7	0.7	4.0	0.8
Mbwazirume	1.9	1.5	2.2	1.9	2.5	0.7	2.7	0.6
Nfuuka	2.3	1.5	2.6	1.0	1.9	1.2	2.9	1.0
Nakinyika	1.8	2.4	2.4	2.0	2.3	0.9	2.9	0.7
Nakabululu	1.3	2.8	2.0	1.1	1.9	0.5	3.5	1.0
Kabula	1.8	1.3	2.4	1.9	3.0	3.3	4.4	1.5
Yangambi Km5	4.2	5.2	4.3	5.1	6.5	2.8	6.4	1.8
Sukali Ndizi	3.9	1.5	3.8	1.7	3.4	0.8	4.3	1.5
LSD _(0.05)	0.80	1.10	0.71	1.11	0.78	0.78	0.93	0.66

4.3.2 Recoverable shoots

Differences in number of recoverable shoots among the cultivars were recorded as early as transfer 2 at two weeks after the initiation of the cultures (Table 4.2). By the 4th subculture cycle, the number of shoots recovered from one starting shoot tip ranged from 57 (for Kabula) to 169 (for Nakinyika) among the AAA-EA banana cultivars. Cv. Mbwazirume performed second to Nakinyika with 154 shoots while the other AAA-EA banana cultivars produced a moderate number of shoots. Sukali Ndizi and Yangambi Km5 produced significantly higher number of recoverable shoots (322 and 352, respectively) compared to the AAA-EA banana cultivars.

Table 4.2. Number of recoverable shoots of AAA-EA banana cultivars, and Yangambi Km5 and Sukali Ndizi cultivars generated from one starting shoot tip through serial multiplication (I – S4) on MS medium modified for banana multiplication

Cultivar	I	T1	T2	S1	S2	S3	S4
	2(E)	2(N _I)	N _{T1} *P/S	N _{T2} *P1/S	N _{S1} *P2/S	N _{S2} *P3/S	N _{S3} *P4/S
Mpologoma	2	4	5	12	28	69	110
Musakala	2	4	4	10	22	59	97
Mbwazirume	2	4	6	16	28	76	154
Nfuuka	2	4	8	18	34	73	113
Nakinyika	2	4	9	16	36	96	169
Nakabululu	2	4	7	13	25	60	96
Kabula	2	4	7	12	20	46	57
Yangambi Km5	2	4	6	21	70	211	352
Sukali Ndizi	2	4	8	18	81	201	322

E = Initial shoot tip explant at initiation (I)

N_I = Number of initiated shoot parts

S1, S2, S3 and S4 = Subculture 1, Subculture 2, Subculture 3 and Subculture 4.

N_{T1}, N_{T2}, N_{S1}, N_{S2}, N_{S3}, N_{S4} are the cumulative number of shoots generated at each cycle.

S = Number of shoots subcultured at each cycle.

P, P1, P2, P3 and P4 = Number of plantlets produced from sub-culturing S shoots at the end of each cycle.

The proportion of total shoots recovered (isolatable differentiated shoots that could regenerate regardless of size) or the ability of the cultivars to produce recoverable shoots was different (Table 4.3). Shoot recovery ranged from 36% (for Kabula) to 66% (for Mbwazirume) among the AAA-EA banana cultivars. Besides Kabula, all the AAA-EA banana cultivars had relatively high proportion (over 50%) of the total shoots recovered. Yangambi Km5 which had the highest proliferation rate had lower proportion (32%) of total shoots recovered. In general, there was a strong negative correlation between total shoot buds and % recovered shoots ($r = 0.94$).

Table 4.3. Multiplication rate for total shoot buds and recovered shoots of different cultivars after four subculture cycles on MS medium modified for banana multiplication showing their effectiveness in generating plantlets

Cultivar	Total shoot buds	Recovered shoots	Recovered shoots (%)
Mpologoma	4.14	2.20	53
Musakala	3.83	2.26	59
Mbwazirume	3.45	2.29	66
Nfuuka	3.54	1.96	55
Nakinyika	3.80	2.11	56
Nakabululu	3.48	1.95	56
Kabula	4.83	1.73	36
Yangambi KM5	9.04	2.88	32
Sukali Ndizi	5.18	2.71	52
LSD _(0.05)	0.617		

The morphology and shoot growth characteristics of the cultivars studied were conspicuously different (Figure 4.1). For instance Yangambi Km5 and Nakinyika cultures had clustered shoots accompanied with many leafy buds while Sukali Ndizi shoots were giant in stature with moderate number of buds. The cultures of cv. Mbwazirume and other AAA-EA banana cultivars had dominant and pointed shoots.

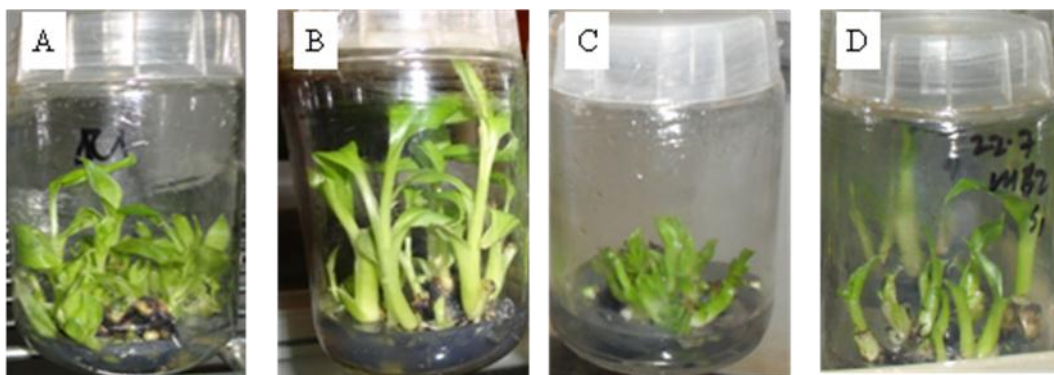


Figure 4.1 Characteristic shoot proliferation of banana cultivars on MS medium modified for banana multiplication: A) Yangambi Km5, B) Sukali Ndizi, C) Nakinyika, and D) Mbwazirume at subculture 1 on MS medium modified for banana multiplication.

4.3.3 Predictive model for recoverable shoots

The rates of multiplication of recoverable shoots produced from one shoot tip explant of the cultivars were significantly different as exhibited by the linear regression coefficients (Table 4.4). The AAA-EA banana cultivars had lower multiplication rate of recoverable shoots as compared to Yangambi Km5 and Sukali Ndizi. Among the AAA-EA banana cultivars Nakinyika, Mbwazirume and Nfuuka had higher multiplication rates followed by Mpologoma, Nakabululu, and Musakala. Kabula had the lowest multiplication rate of recovered shoots. All the regression lines (not shown) for recoverable shoots had high coefficients of determination ($R^2 > 90$) and thus were used to derive predictive models for the respective cultivars (Table 4.4). Since the AAA-EA banana cultivars had distinctly lower regression coefficients, their LSD was calculated without including the control cultivars which had higher and similar regression coefficients.

Table 4.4. Predictive model for recovered shoots of selected AAA-EA banana cultivars, Yangambi Km5 and Sukali Ndizi generated on MS medium modified for banana multiplication

Cultivar	Genotype	Predictive model	Regression coefficient
Nakinyika	AAA-EA	$Y = e^{[0.289(x-1) - 0.00386(x-1)^2 + 1.39]}$	0.289
Mbwazirume	AAA-EA	$Y = e^{[0.276(x-1) - 0.00386(x-1)^2 + 1.39]}$	0.276
Nfuuka	AAA-EA	$Y = e^{[0.270(x-1) - 0.00386(x-1)^2 + 1.39]}$	0.270
Mpologoma	AAA-EA	$Y = e^{[0.261(x-1) - 0.00386(x-1)^2 + 1.39]}$	0.261
Nakabululu	AAA-EA	$Y = e^{[0.253(x-1) - 0.00386(x-1)^2 + 1.39]}$	0.253
Musakala	AAA-EA	$Y = e^{[0.248(x-1) - 0.00386(x-1)^2 + 1.39]}$	0.248
Kabula	AAA-EA	$Y = e^{[0.227(x-1) - 0.00386(x-1)^2 + 1.39]}$	0.227
LSD _(0.05)			0.0354
Yangambi Km5	AAA	$Y = e^{[0.352(x-1) - 0.00463(x-1)^2 + 1.39]}$	0.352
Sukali Ndizi	AAB	$Y = e^{[0.350(x-1) - 0.00463(x-1)^2 + 1.39]}$	0.350

y = transformed number of recoverable shoots, Y = number of recoverable shoots, e = 2.718282 and x = time in weeks.

4.4 Discussion

4.4.1 *In vitro* shoot and bud proliferation

The low *in-vitro* proliferation ability of AAA-EA banana cultivars and lack of significant differences in proliferation among them was reported earlier (Talengera *et al.*, 1994) and thus was not surprising. Besides, this banana group has been reported to be less responsive to regeneration protocols (Sadik *et al.*, 2007) compared to other genotype groups. But the outstanding proliferation rates of Yangambi Km5 (with an AAA genome) and Sukali Ndizi (with an AAB genome) may be of interest to researchers. Conventionally cultivars having only A in their genome have been reported to produce fewer shoots and buds compared to those cultivars having one or two Bs in their genome (Strosse *et al.*, 2003). It is also reported that the origin of the A genome among the AAA-EA bananas is different from that of other commonly grown banana cultivars with A genome (Simmonds and Shepherd, 1955). This

could suggest that the A genome of *Musa acuminata* Colla in the AAA-EA banana is less responsive to *in vitro* proliferation using the available culture media formulations and protocols.

Although cv. Nakabululu did not perform so well after producing a significantly higher number of buds in subculture 1, it was generally observed that high bud proliferation especially during the early subculture cycles influenced subsequent proliferation of the cultures of all the cultivars and consequently their overall proliferation ability (Table 1; Table 2). Buds are precursors for shoot development and since they are not fully differentiated, it was likely that they continued to divide when exposed to a fresh medium during sub culturing. This implies that their decrease in number with subculture cycle indicated an inherent overall decline in proliferation rate with increase in subculture cycle or age of the culture. Formation of meristems and their subsequent development is controlled by endogeneous auxins within the apices whose decline with age triggers differentiation of buds into shoots and terminates further bud development (George *et al.*, 2008). However, as the mean number of shoots was still increasing by the fourth subculture cycle, the process of sub-culturing could be continued to produce numerous plants from a single explant. But abnormal plants especially somaclonal variants might arise and/or increase to unacceptable numbers due to onset of mutations. Considerable increase in frequency of variants has been reported in bananas when maintained in tissue culture for over five months or 5 subculture cycles (Rodrigues *et al.*, 1998; El-DougDoug *et al.*, 2007). The age of the culture and or number of subculture cycles affect genetic stability due to spontaneous mutations which accumulate with time (Duncan, 1997; Leela *et al.*, 2003).

4.4.2 Recoverable shoots

There was a wide variation in ability of the cultivars to produce recoverable shoots. Interestingly, the cultivars with high proliferation rate displayed less efficiency to produce recoverable shoots (Table 4.3). This was associated with the number and morphology or growth characteristics of their shoot cultures. For instance cv. Kabula produced numerous stunted shoots which were difficult to isolate and subculture while cv. Mbwarzirume had moderate number of dominant and pointed shoots (Figure 4.1) which were easy to separate

and subculture. Yangambi Km5 produced numerous tiny differentiated shoots that were difficult to recover and subculture compared to Sukali Ndizi with relatively fewer dominant shoots. Since the explants were of uniform size, the variation in number of shoots among the cultivars was probably due to differences in their response to culture conditions. Differences in cultivar proliferation rate have been attributed to differences in growth regulator uptake, translocation rate to meristematic regions, and metabolic processes among genotypes (Blakesley, 1991; Youmbi *et al.*, 2006).

The above observations imply that *in vitro* proliferation ability and particularly efficiency to produce recoverable shoots of the different AAA-EA banana cultivars could be improved by varying the culture conditions during the subsequent subculture cycles. Cultivars with high proportion of buds to differentiated shoots and/or numerous tiny shoots require enhanced shoot elongation and dominance. This could be achieved by reducing the amount of cytokinin and other growth inducing substances in the medium in the subsequent subculture cycles while adding gibberellins especially GA3 which enhances growth and elongation of dwarfed or stunted banana plantlets (Damasco *et al.*, 1996; Sanna *et al.*, 2008), a phenomenon which has also been demonstrated in common bean (Veltcheva and Svetleva, 2005) and cotton (Divya *et al.*, 2008).

On the other hand, those cultivars with low proportion of buds to differentiated shoots require enhanced bud proliferation which could be achieved by increasing the amount of cytokinin and adding other growth inducing substances such as abscisic acid in the subsequent subculture cycles (Kantharajah, 2001). Application of more active cytokinins with carry-over effect such as Thidiazuron (TDZ) sustained bud proliferation in bananas for some time (Makara *et al.*, 2010). Cytokinins produce delayed response in undifferentiated tissue (Mineo, 1990) which favors formation of shoot buds (Razdan, 1993).

4.4.3 Predictive model for recoverable shoots

In general the study suggested that *in vitro* banana proliferation and ability to recover weanable shoots from shoot buds of AAA-EA bananas do not depend on the cultivar's suckering ability *in vivo*. The number of recoverable shoots *in vitro* was influenced by the cultivar's shoot growth characteristics. Thus a model for estimating number of plants that

could be produced from a shoot-tip explant after a given time would be useful to those involved in micropropagation of AAA-EA banana cultivars. This study derived a regression model that could be used to predict the number of recoverable plantlets from one shoot-tip explant of AAA-EA banana cultivars after a given time period. For example through calculation using the model, the number of shoots that could be recovered from a shoot-tip explant of cv. Nakinyika after 18 weeks ($Y = e^{[0.289(x-1) - 0.00386(x-1)^2 + 1.39]} = 178$). The number recorded from the experiment was 169 (Table 4.2). For cv. Mbwasirume ($Y = e^{[0.276(x-1) - 0.00386(x-1)^2 + 1.39]} = 142$). The one obtained from the experiment was 154 (Table 4.2). The difference was attributed to changes in shoot growth characteristics of each cultivar. For any given AAA-EA cultivar, the linear regression coefficients above would be used depending on its clone set. Regression models have been used to predict plant multiplication rates (Mendes *et al.*, 1999) and thus the model could be used to give a fair estimate. But the underlining principle while developing the prediction model was that bananas may be safely sub-cultured up to the 4-6th subculture cycle corresponding to 4.5 - 6.5 months. Thus care must be taken when predicting beyond this period as there may be increased changes in proliferation or growth pattern depending on cultivar.

4.5 Conclusion

In vitro shoot proliferation in East African highland bananas on the widely adopted MS medium modified for banana multiplication varies among the cultivars. The cultivars that inherently displayed high shoot bud proliferation recorded low % recovered shoots (which limited their potential to produce much more shoots) suggesting that *in vitro* proliferation is cultivar dependent while the recovery of shoots from shoot buds of AAA-EA bananas is cultivar independent. Based on shoot culture growth characteristics, the number of recoverable shoots from one shoot-tip explant in a given time can be predicted using a regression model. Using the predictive model, the number of recoverable shoots from one shoot-tip explant for instance of cv. Mpologoma at 5.5 months (5 subculture cycles) and 6.5 months (6 subculture cycles) would be 176 and 245, respectively. This implies that in practice one would require 7 starting shoot-tip explants to subculture for 5.5 months to generate enough planting material to plant one hectare at spacing of 9 by 9 metres.

CHAPTER FIVE

EFFICACY OF TDZ AND 4-CPPU FOR EMBRYOGENIC CALLUS INDUCTION FROM SCALPS OF RECALCITRANT EAST AFRICAN HIGHLAND BANANA

5.1 Introduction

In vitro plant cell differentiation and morphogenesis is highly dependent on culture medium (Khaleda and Al-Forkan, 2006). Optimum proportion and/or concentration of growth regulators especially auxins is necessary for successful induction and maintenance of embryogenic callus or cell suspension cultures (Gomez *et al.*, 2000). N-phenyl-N'-1,2,3-thidiazol-5-ylurea (thidiazuron) (TDZ) and N-(2-chloro-4-pyridyl)-N'-phenylurea (forchlorfenuron) (4-CPPU), both substituted phenylurea compounds, are reported to be highly active in regulating morphogenesis in the tissue culture of many plant species (Victor *et al.*, 2004; Fiore *et al.*, 2004; Haruki *et al.*, 2007). They are known to exhibit a unique property of mimicking both cytokinin and auxin effects on growth and differentiation of cultured explants (Murthy *et al.*, 1998). At low concentrations TDZ and 4-CPPU stimulate shoot proliferation, simulating cytokinin activity while at high concentrations they induce callus, typical of an auxin activity (Vinayak *et al.*, 2009). Their effect seems to be distinctive for each respective crop variety, suggesting that they could be suitable for determining variety specific regeneration protocols (Vinayak *et al.*, 2009). The aim of this study, therefore, was to test the efficacy of a combination of TDZ and 4-CPPU growth regulators for enhancing embryogenic callus induction in recalcitrant AAA-EA bananas through scalps.

5.2 Materials and Methods

5.2.1 Plant material

Five AAA-EA banana cultivars namely Mbwaizirume, Mpologoma, Nakabululu, Nakinyika and Nfuuka from different clone sets were selected for this study based on farmers' variety preference. Shoot-tip explants were extracted from young suckers 40-100 cm tall from a field at Makerere University Agricultural Research Institute, Kabanyolo (MUARIK). They were sterilized and cultured on the Murashige and Skoog (MS) medium modified for banana

multiplication (Talengera *et al.*, 1994) up to transfer 2 to produce adequate clean shoots for generating scalps.

5.2.2 Generation of scalps

In vitro derived shoot-tips from transfer 2 were put on the MS medium modified with various concentrations of TDZ and 4-CPPU growth regulators to generate scalps as designated below:

TDZ and 4-CPPU combination	Designation
5µM TDZ + 5µM 4-CPPU	M1
7µM TDZ + 7µM 4-CPPU	M2
9µM TDZ + 9µM 4-CPPU	M3
11µM TDZ + 11µM 4-CPPU	M4
13µM TDZ + 13µM 4-CPPU	M5
26µM TDZ	M6
26µM 4-CPPU	M7

These rates were selected based on different studies on effect of TDZ in banana (Sadik *et al.*, 2007) and other crops (Ying-Chun *et al.*, 2000).

Fifty shoot-tips of each cultivar were used in each hormone treatment and placed in a completely randomized design (CRD) in a dark growth room maintained at $26 \pm 2^{\circ}\text{C}$. The cultures were serially subcultured every four weeks until scalps were formed. The rate of scalp formation in the various media was determined by counting the number of shoots and buds in each subculture cycle until scalps were formed. Callus directly formed on the scalps being generated was also recorded. Other growth features or characteristics such as corm-like growth when observed were noted.

5.2.3 Callus induction and embryo development

The scalps were excised under dissecting microscope and cultured on callus induction medium derived by combining 5µM 2,4-D (2,4-Dichlorophenoxyacetic acid) (Schoofs *et al.*, 1997) with TDZ and 4-CPPU singly and in combination at 5µM, 10µM and 15µM as

hormone treatment. Fifty scalps of each cultivar were used in each treatment. All the cultures were placed in a CRD in a dark growth room maintained at $26 \pm 2^{\circ}\text{C}$. They were observed every two weeks for four months to observe embryogenic responses. Any embryogenic callus achieved was transferred to the MS banana multiplication medium supplemented with $5\mu\text{M}$ benzyl amino purine (BAP). Embryo to plant development was observed for at least 28 days. The plantlets regenerated together with the number of normal roots (with hair roots) they possessed were recorded.

5.2.4 Data analysis

The data on rate of scalp formation was subjected to analysis of variance (ANOVA) using GenStat 13th Edition (GenStat, 2010). The means were separated using least significant differences (LSD) at 5% level of significance. Comparisons were done on number of shoots and multiple buds across cultivars, subculture cycles and growth regulator combination. Callus directly formed on the scalp induction media was summarized as percentage of explants or cultures with callus on the scalps being formed. Induced callus was summarized as a percentage of scalps that generated callus to the total number of scalps initiated on callus induction medium.

5.3 Results

5.3.1 Scalp formation

Scalp formation in all the cultivars varied across hormone treatment and subculture cycle. There were significant differences in mean shoot and bud proliferation on the different combinations of TDZ and 4-CPPU (Table 5.1). At subculture 1, medium M5 ($13\mu\text{M}$ TDZ+ $13\mu\text{M}$ 4-CPPU) induced significantly ($P < 0.05$) more multiple buds with fewer shoots in cultivars Nakinyika, Nakabululu and Nfuuka, which favors scalp formation. Medium M1 ($5\mu\text{M}$ TDZ+ $5\mu\text{M}$ 4-CPPU) induced significantly ($P < 0.05$) higher number of multiple buds in cv. Mpologoma but it induced high shoot proliferation which does not favor scalp formation. However, at subculture 2, treatment M1 ($5\mu\text{M}$ TDZ+ $5\mu\text{M}$ 4-CPPU) induced significantly ($P < 0.05$) higher number of multiple buds in cultivars Mbwarzirume, Nakinyika

and Nakabululu while cv. Nfuuka continued to produce significantly ($P < 0.05$) higher numbers of multiple buds in Treatment M5 (13 μ M TDZ+13 μ M 4-CPPU).

There was significant increase in mean multiple bud proliferation from subculture 1 to subculture 3 in all the cultivars but at varying levels depending on hormone treatment (Table 5.1). In cycle 3, cv. Nakinyika had significantly ($P < 0.05$) the highest mean multiple bud proliferation on medium M6 (26 μ M TDZ), followed by Nakabululu on M3 (9 μ M TDZ+9 μ M 4-CPPU) and Mbwarzirume on M5 (13 μ M TDZ+13 μ M 4-CPPU). Cultivars Mpologoma and Nfuuka produced the same number of multiple buds on medium M4 (11 μ M TDZ+11 μ M 4-CPPU) and M7 (26 μ M 4-CPPU), respectively.

Scalps were achieved in all TDZ and 4-CPPU combinations (Table 5.2). With exception of cultivars Mpologoma and Mbwarzirume, scalps were formed in over 50% of the cultures of the various cultivars by the fourth subculture cycle. Mpologoma produced fewer ($\leq 50\%$) scalps in most of the media while Mbwarzirume produced fewer scalps (37.5%) only in medium M7 (26 μ M 4-CPPU). Cultivars Nakinyika and Nakabululu produced high number of scalps ($\geq 70\%$) in all the media or treatments.

Table 5.1. Cultivar shoot and multiple bud proliferation on different combinations of TDZ and 4-CPPU (M1, M2, M3, M4, M5, M6 and M7) at first, second and third subculture cycles

Cycle	Cultivar	Organ	Mean number of shoots and multiple buds							LSD (0.05)
			M1	M2	M3	M4	M5	M6	M7	
1	Mbwazirume	Shoots	1.5	1.0	1.3	1.4	1.5	1.6	1.1	0.244
		M. buds	3.9	1.6	2.8	2.9	3.2	2.7	2.4	0.626
	Mpologoma	Shoots	1.4	1.3	0.8	1.5	1.4	1.1	1.4	0.244
		M. buds	1.3	2.0	1.4	1.7	1.4	0.9	0.8	0.626
	Nakabululu	Shoots	1.3	1.7	1.1	1.3	1.2	2.4	1.5	0.244
		M. buds	3.0	3.6	5.3	4.2	6.5	2.1	4.5	0.626
	Nakinyika	Shoots	2.0	1.4	1.1	0.9	0.8	2.4	1.4	0.244
		M. buds	3.2	2.0	4.0	4.3	7.8	2.4	4.2	0.626
2	Mbwazirume	Shoots	1.9	1.4	0.9	0.7	0.3	1.1	2.2	0.244
		M. buds	1.5	3.2	3.1	4.0	4.3	2.9	1.9	0.626
	Mpologoma	Shoots	0.3	0.8	0.6	0.7	0.4	0.6	0.6	0.244
		M. buds	5.7	4.2	4.2	3.6	3.7	2.1	2.1	0.626
	Nakabululu	Shoots	0.7	0.6	0.5	0.6	0.0	0.6	1.4	0.244
		M. buds	2.3	3.1	2.7	2.1	2.6	2.0	1.0	0.626
	Nakinyika	Shoots	0.7	1.0	1.1	0.8	1.2	1.6	1.8	0.244
		M. buds	4.6	2.2	3.6	2.2	4.0	3.1	3.9	0.626
3	Mbwazirume	Shoots	0.3	0.3	0.3	0.5	0.4	1.8	1.3	0.244
		M. buds	5.3	2.0	3.5	2.9	4.5	2.2	3.7	0.626
	Mpologoma	Shoots	0.7	1.0	0.5	0.4	0.4	1.0	1.3	0.244
		M. buds	3.3	3.9	3.2	4.5	5.2	3.6	2.4	0.626
	Nakabululu	Shoots	1.7	2.3	0.7	1.0	0.6	0.9	2.0	0.244
		M. buds	5.8	6.2	4.4	8.8	9.1	8.6	6.9	0.626
	Nakinyika	Shoots	2.4	2.2	0.7	1.5	1.7	1.4	0.6	0.244
		M. buds	7.2	5.0	6.8	8.3	4.9	4.5	6.1	0.626
4	Mbwazirume	Shoots	1.4	0.8	0.7	1.6	1.4	0.8	1.4	0.244
		M. buds	8.6	6.6	9.3	8.5	8.6	8.5	8.4	0.626
	Mpologoma	Shoots	1.1	1.5	0.2	0.6	0.7	0.7	2.5	0.244
		M. buds	8.3	6.9	8.5	8.4	8.2	9.4	9.8	0.626
	Nakabululu	Shoots	1.4	1.3	1.4	1.1	0.3	1.0	1.0	0.244
		M. buds	8.2	7.8	7.6	7.7	7.3	7.2	8.3	0.626
	Nakinyika	Shoots	1.7	2.3	0.7	1.0	0.6	0.9	2.0	0.244
		M. buds	5.8	6.2	4.4	8.8	9.1	8.6	6.9	0.626

Cycle LSD_(0.05) = 0.160 for shoot and 0.410 for multiple buds

Cultivar LSD_(0.05) = 0.206 for shoot and 0.529 for multiple buds

M. buds= multiple buds.

Table 5.2. Cultivar percentage ideal scalps on the different combinations of TDZ and 4-CPPU at the fourth subculture cycle

Cultivar	Percentage ideal scalps under different media treatment						
	M1	M2	M3	M4	M5	M6	M7
Mbwazirume	75.0	60.0	60.0	70.0	70.0	66.7	37.5
Mpologoma	47.1	40.0	50.0	70.0	60.0	50.0	31.3
Nakabulu	70.6	75.0	82.4	72.2	71.4	75.0	87.5
Nakinyika	88.2	60.0	70.0	70.0	70.0	100.0	100.0
Nfuuka	76.5	82.4	73.3	94.1	69.2	50.0	50.0

5.3.2 Callus and embryo development

As the scalps formed on the media containing various combinations of TDZ and 4-CPPU, compact yellowish, pinkish and cream callus tissue developed on them (Figure 5.1). The percentage of scalp cultures that had callus on them varied with hormone treatment and cultivar (Table 5.3). By cycle 4 (achieved through serial transfer an explant from each of the primary explants), all the cultivars produced callus on their scalp cultures even on the medium containing the lowest concentration of TDZ and 4-CPPU (5 μ M TDZ + 5 μ M 4-CPPU). Cultivar Nakinyika had the highest percentage of cultures with callus (20%) on medium M5 (13 μ M TDZ + 13 μ M 4-CPPU) and M6 (26 μ M TDZ), Mpologoma (20%) on M6 (26 μ M TDZ), Nfuuka (17.5%) on M1 (5 μ M TDZ + 5 μ M 4-CPPU), Nakabululu (17.5%) on M7 (26 μ M 4-CPPU) and Mbwazirume (12.5%) on M6 (26 μ M TDZ). All the cultivars performed well on MS supplemented with 26 μ M TDZ with up to 12.5 - 20% of their scalp cultures having callus on them by cycle 4 (Table 5.3).

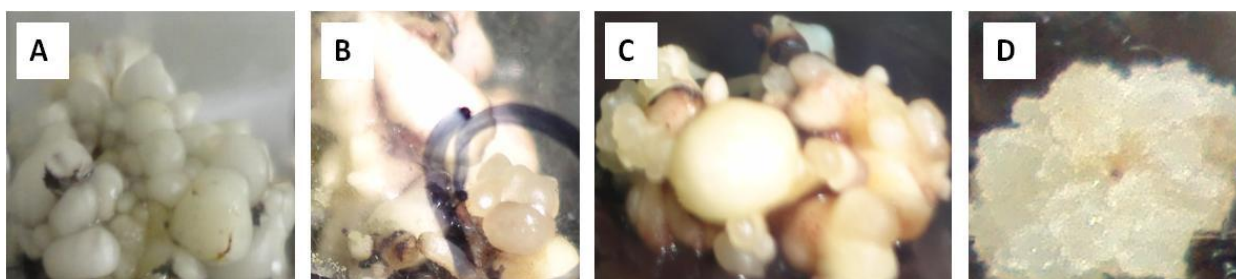


Figure 5.1 Embryogenic callus formation on scalps of cultivar Nfuuka on 9 μ M TDZ + 9 μ M 4-CPPU at the third subculture cycle: (A) Ideal scalp, (B) Callus initiation, (C) Embryo development (D) Secondary embryo development.

Table 5.3. Mean percentage callus formation on scalp cultures of AAA-EA banana cultivars on different combinations of TDZ and CPPU by the fourth subculture cycle

Cultivar	Mean percentage callus under different media treatment						
	M1	M2	M3	M4	M5	M6	M7
Mbwazirume	10.0	10.0	2.5	5.0	10.0	12.5	2.5
Mpologoma	2.5	5.0	2.5	10.0	17.5	20.0	2.5
Nakabululu	2.5	5.0	10.0	10.0	10.0	15.0	17.5
Nakinyika	7.5	5.0	7.5	7.5	20.0	20.0	17.5
Nfuuka	17.5	12.5	7.5	10.0	5.0	15.0	2.5
Treatment LSD _(0.05) = 6.905; Cultivar LSD _(0.05) = 5.836							

As the callus on scalps aged and became necrotic by cycle 4, some embryos developed on it and differentiated directly into shoot (Figure 5.2). This was observed in cv. Nakabululu on 13 μ M TDZ+13 μ M 4-CPPU. When the callus from the different cultivars was removed and cultured on a hormone free medium, it differentiated into somatic embryos and shoots (Figure 5.3) regardless of initial hormone treatment implying that it was all homogeneously embryogenic.

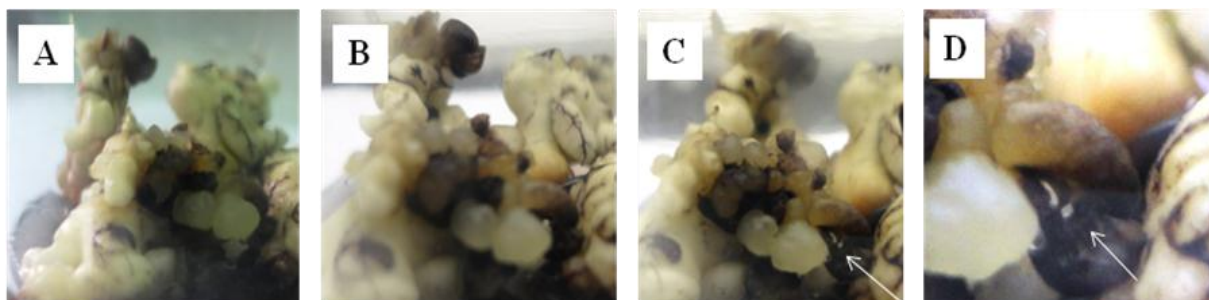


Figure 5.2 Direct embryogenesis in cv. Nakabululu on 13 μ M TDZ+13 μ M 4-PPU: (A) Callus on scalp, (B) Embryo initiation on callus, (C – D) embryo to shoot development.

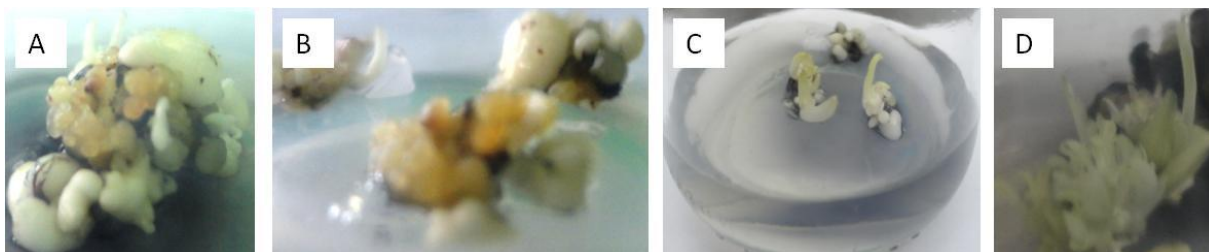


Figure 5.3 Differentiation of callus on scalps of cv. Nakinyika into somatic embryos and shoots on transfer to hormone free MS medium: (A) Callus on scalp (B) Embryo development, (C) Callus differentiation into shoot (E) Scalp tissue differentiation into shoot.

When the callus from the different cultivars was removed and transferred to MS medium containing 5 μ M BAP, it only regenerated into plantlets (Figure 5.4) in a few cultures of cultivars Nakinyika, Nakabululu, Nfuuka and Mbwazirume (Table 5.4). Of the plantlets regenerated 5 out of 12 and 4 out of 5 plantlets of cultivars Nakinyika and Mbwazirume, respectively, developed normal roots (Table 5.4) and were weaned (Figure 5.5). But when the scalps were excised and cultured on a medium with 5 μ M 2,4-D to generate callus they all died.

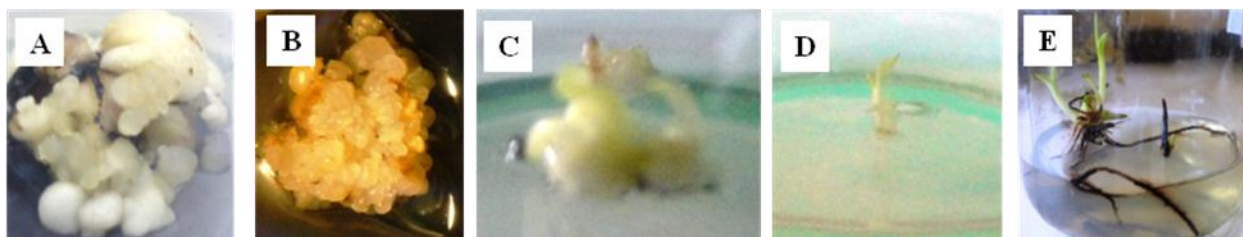


Figure 5.4 Different stages of plant regeneration through direct embryogenesis from scalps of cv. Mwazirume after transfer of embryogenic callus to MS medium with 5 μ M BAP: (A) Embryogenic callus development, (B) Somatic embryo development, (C – D) embryo to plant development, (E) Root development on the plantlets under the same medium.

Table 5.4 Number of plantlets and their roots of various AAA-EA banana cultivars regenerated out of twenty (20) embryogenic callus cultures induced by TDZ and 4-CPPU growth regulators

Cultivar	Number of plantlets regenerated	Number of plantlets with roots	Mean root number
Mwazirume	5	4	6.0
Mpologoma	0	0	0
Nakabulu	10	1	2.0
Nakinyika	12	5	5.4
Nfuuka	2	0	0



Figure 5.5 Plants of cvs Nakinyika and Mbwazirume regenerated through direct embryogenesis induced by TDZ and 4-CPPU after transfer of the embryogenic callus to MS medium with 5 μ M BAP under acclimatization in soil.

5.4 Discussion

5.4.1 Scalp formation

Cultivars Nakinyika and Nakabululu produced more multiple buds on treatment M5 (13 μ M TDZ+13 μ M 4-CPPU) at subculture 1 but at subculture 2, they produced more multiple buds on treatment M1 (5 μ M TDZ+5 μ M 4-CPPU). This was probably due to changes in effect of the growth regulators with subculture cycle especially TDZ which was reported to have carry-over effect (Makara *et al.*, 2010). Concentrations of TDZ and 4-CPPU above 7 μ M appear to suppress shoot apical dominance and favor multiple bud proliferation. However, in cv. Mbwazirume, mean bud proliferation on 5 μ M TDZ + 5 μ M 4-CPPU was significantly higher than on 7 μ M TDZ + 7 μ M 4-CPPU suggesting that combined TDZ and 4-CPPU at low concentration permits vigorous proliferation of both shoots and buds depending on cultivar. TDZ is active in inducing adventitious and axillary shoot proliferation (Ashraf *et al.*, 2004; Youmbi *et al.*, 2006) which are precursors for scalp formation. Similarly, 4-CPPU induced multiple axillary shoot proliferation in *Impatiens walleriana* L. (Subotić *et al.*, 2008).

Multiple bud proliferation was significantly ($P < 0.05$) higher on 13 μ M TDZ+13 μ M 4-CPPU than on 26 μ M TDZ and 4-CPPU when used individually. The interaction of TDZ and 4-CPPU to induce more multiple bud proliferation when used in combination at lower concentration (13 μ M) compared to when used singly at concentration of 26 μ M suggests both synergistic and additive effects and implies that high levels of each of them can be avoided. Achievement of plant regeneration at lower concentrations of combined TDZ and 4-CPPU could be advantageous in reducing somaclonal variation. In bananas, somaclonal variations of different types have occurred due to high concentrations of growth hormones such as 6-benzylaminopurine and dicamba (Leela *et al.*, 2003).

5.4.2 Callus development and embryogenesis

Embryogenic callus was directly formed on the scalp induction medium containing TDZ and 4-CPPU. Commonly, embryogenesis is induced after inoculating the ideal scalps onto medium containing 2,4-D or other auxins (Schoofs *et al.*, 1998; Sadik *et al.*, 2007; Ramírez-Villalobos and De García. 2008). The formation of embryogenic callus under TDZ and 4-CPPU treatments as described in this study conforms to reports that at high concentrations, these urea-type cytokinins exhibit an auxin activity and thus could induce embryogenesis (Vinayak *et al.*, 2009). Chhabra *et al.* (2008) suggested that at high concentrations, TDZ imposes stress on the explant and could redirect its development from shoot organogenesis to somatic embryogenesis. At concentrations higher than 2 μ M, TDZ induced direct somatic embryogenesis on cotyledonary node explants of recalcitrant lentil (Chhabra *et al.*, 2008). Earlier, TDZ was reported to have induced higher somatic embryogenesis in protocalli than even 2,4-D (Lehminger-Mertens and Jacobsen, 1989).

Although TDZ and 4-CPPU exhibit an auxin activity and induce somatic embryos in cultures grown on media devoid of auxins, the mechanism of these urea-type cytokinins to induce embryogenic callus in bananas are of interest as earlier reports suggested that somatic embryogenesis could be induced in dicot species on cytokinin-supplemented media (Raemakers *et al.*, 1995) but auxins solely mediated the transition from somatic to embryogenic cells in angiosperm monocots (Víctor, 2001). TDZ induced somatic embryogenesis in rice but after a short treatment with 2,4-D (Aparna and Rashid, 2004). This

implies further research on whether auxins solely mediate transition from somatic to embryogenic cells in angiosperm monocots is also necessary.

Cultivar Nfuuka formed callus on medium M1 with low levels of TDZ and 4-CPPU. Relating to the name 'Nfuuka' "meaning ever changing" in one of the dialects in Central Uganda, the response of cv. Nfuuka suggested that it is mutatic and may on all occasions respond to a wider range of treatments than other AAA-EA banana cultivars. However, the increase in callus formation on low levels of TDZ and 4-CPPU with subculture cycle was attributed to carry-over effect of urea-type cytokinins especially TDZ (Makara *et al.*, 2010).

The differentiation of callus on scalps generated on TDZ and 4-CPPU into somatic embryos and shoots is in agreement with other reports. Razdan (1993) reported that a whole plant regeneration from callus tissue may occur either through organogenesis (shoot-bud differentiation) or somatic embryogenesis where the cell or group of cells initiate a development-pathway that leads to reproducible regeneration of non-zygotic embryos capable of germinating to form complete plants. There was more embryo development when the callus was removed and cultured on a hormone free medium probably due to reduction in auxin activity resulting from the high concentrations of TDZ and 4-CPPU. Though the process of somatic embryogenesis is often initiated in media containing high levels of auxins, later reduction in auxin concentration usually stimulates further embryo development since lower auxin concentrations induce pro-embryogenic cells to divide and develop into embryos (Sharp *et al.*, 1980).

The scalps formed on media containing TDZ and 4-CPPU died when transferred on to medium containing 5 μ M 2,4-D possibly due to excessive cumulative auxin activity effect (George *et al.*, 2008). Since urea-type cytokinins especially TDZ have carry-over effect (Makara *et al.*, 2010), their auxin activity together with that of 2,4-D could have been too high for the stimulation of the cells in the scalp tissue to dedifferentiate and form callus but rather killed them. However, somatic embryos were produced from stem explants of Golden Pothos (*Epipremnum aureum*) on MS medium supplemented with 0.5 mg l⁻¹ (2.26 μ M) 2,4-D and 2 mg l⁻¹ (8.07 μ M) CPPU, and 0.5 mg l⁻¹ (2.26 μ M) 2,4-D and 2.0 mg l⁻¹ (9.08 μ M) TDZ (Zhang

et al., 2004). This suggests that lower concentration of 2,4-D is required for scalps generated on media containing TDZ.

Among the explants that have been used in somatic embryogenesis in bananas such as immature zygotic embryos, immature male flowers, female flowers, scalps, leaf bases and corm slices, scalps were reported to be among the most responsive material for initiating regenerable embryogenic cell suspension cultures (Meenakshi *et al.*, 2007). One impediment to using the scalp method to generate callus in AAA-EA bananas has been the long time required to achieve ideal scalps. Up to 9 subculture cycles on a medium containing 100 μM BAP (Schoofs *et al.*, 1998) or 6 subculture cycles on a medium containing 10 μM TDZ (Sadik *et al.*, 2007) were required. Recently Ramírez-Villalobos and De García (2008) reduced the production time of embryogenic scalps from tetraploid somaclonal variant CIEN-BTA-03 of the Cavendish subgroup from 8-14 months (reported for the other tetraploids of this subgroup (Strosse *et al.* 2006)) to 6 months by growing shoot apices in 25 mg l^{-1} BA plus 0.217 mg l^{-1} IAA but the mean percentage of explants with scalps was only 38%. Though tetraploid bananas generally take longer to develop than triploids, in this study, scalps of AAA-EA bananas were achieved in over 50% of the scalp cultures within 4 subculture cycles on TDZ and 4-CPPU. The influence of TDZ on proliferation in AAA-EA bananas had been reported (Arinaitwe *et al.*, 2000; Sadik *et al.*, 2007; Makara *et al.*, 2010). This is the first report on combined effect of TDZ and CPPU on scalps and regenerable embryogenic callus induction in AAA-EA bananas.

5.5 Conclusion

Embryogenic callus was formed on 2.5 – 20% of the scalp cultures depending on cultivar and interaction of TDZ and 4-CPPU combination. Cultivars Nakinyika, Nakabululu, Mbwazirume and Nfuuka developed regenerable embryogenic callus and recorded plant regeneration through direct embryogenesis. Early plant regeneration at lower concentrations of combined TDZ and 4-CPPU could reduce occurrence of somaclonal variation. These results thus suggest that the percentage of embryogenic callus induced from scalps of AAA-EA banana cultivars is increased by TDZ and 4-CPPU growth regulators.

CHAPTER SIX

EFFICACY OF TDZ AND 4-CPPU IN DIFFERENT SALT FORMULATIONS IN EMBRYOGENIC CALLUS INDUCTION FROM IMMATURE MALE FLOWERS OF AAA-EA BANANA

6.1 Introduction

Culture medium nutrient components and growth regulator combination greatly influence plant cell differentiation and morphogenesis *in vitro* (Kantharajah, 2001). Auxins, particularly 2,4-dichlorophenoxyacetic acid (2,4-D) have been widely used in combination with cytokinins such as Benzyl adenine (BA) (Sannasgala *et al.*, 1992), Zeatin (Schoofs *et al.*, 1998; Ramírez-Villalobos and De García, 2008) and Benzyl amino purine (BAP) (Sadik *et al.*, 2007) for initiation and maintenance of embryogenic cell suspension (ECS) in banana. Whereas urea-type cytokinins, N-phenyl-N'-1,2,3-thidiazol-5-ylurea (thidiazuron) (TDZ) and N-(2-chloro-4-pyridyl)-N'-phenylurea (forchlorfenuron) (4-CPPU) have desirable attributes for embryogenic callus induction from plant explants (Murthy *et al.*, 1998; Haruki *et al.*, 2007), they have not been tried for induction of somatic embryogenesis in banana especially through immature male flower buds. On the other hand a number of salt formulations were described by different authors including MS (Murashige and Skoog, 1962), Chu N6 (Chu, 1975), Eriksson (Eriksson, 1965), Gamborg B5 (Gamborg *et al.*, 1968), Nitsch (Nitsch and Nitsch, 1969), NLN (Lichter, 1982), SH (Schenk and Hilderbrandt, 1972) and White (White, 1963) for use in tissue culture. During the establishment of *in vitro* micro-propagation procedure for the AAA-EA banana, only a few salt formulations were evaluated (Talengera *et al.*, 1994). Although MS medium was adopted for routine micropropagation of the AAA-EA banana through shoot tip culture, there has been no justification that its salt mixture is also the best for callus induction or ECS development. The aim of this study, therefore, was to test efficacy of TDZ and 4-CPPU, and different salt formulations for embryogenic callus induction in AAA-EA banana cultivars through immature male flowers.

6.2 Materials and Methods

6.2.1 Plant material

Five AAA-EA banana cultivars namely Mbuzirume, Mpologoma, Nakabululu, Nakinyika and Nfuuka from different clone sets were used in this study. Sukali Ndizi (AAB) which is responsive to regeneration protocols was included as a check. Male flowers were collected 1 to 3 weeks after flowering from the field banana germplasm at Makerere University Agricultural Research Institute, Kabanyolo (MUARIK) and the banana fields of the National Banana Program at National Agricultural Research Laboratories (NARL), Kawanda. They were reduced to 0.8 – 2 cm within 6 hours after collection and the reduced immature male flowers were surface sterilized in 70% ethyl alcohol for 2 minutes before they were initiated within two hours.

6.2.2 Effect of TDZ and 4-CPPU on callus induction

Using a scalpel with fine blades, immature flower bud clusters were excised under dissection microscope from the 18th - 8th position and cultured on callus induction medium containing 5 μ M 2,4-D supplemented with different combinations of TDZ and CPPU alone or in presence of IAA and NAA as designated below:

TDZ and 4-CPPU combination	Designation
5 μ M TDZ+5 μ M 4-CPPU	H1
10 μ M TDZ+10 μ M 4-CPPU	H2
15 μ M TDZ+15 μ M 4-CPPU	H3
5 μ M TDZ+5 μ M 4-CPPU+1mg ⁻¹ IAA+1mg ⁻¹ NAA	H4
10 μ M TDZ+10 μ M 4-CPPU+1 mg ⁻¹ IAA+1mg ⁻¹ NAA	H5

In each treatment fifty male flowers of each cultivar were initiated on separate petridishes and placed in a completely randomized design (CRD) in a dark growth room maintained at 26 $\pm$ 2°C. The cultures were observed every two weeks for 8 months. Yellow callus (YC) and embryogenic callus (EC) developing from the flower buds was recorded. Any organ-like structure developing on the callus or alongside embryos was noted.

6.2.3 Callus induction on different basal salt mixture substituted on callus induction medium supplemented with TDZ and 4-CPPU

By substituting only MS inorganic salts (MS vitamins and other medium components retained) from the callus induction medium supplemented with 10 μ M TDZ+10 μ M 4-CPPU (the most efficacious combination in pure MS medium), salt formulations of Chu (N₆), Eriksson, Gamborg B5, Nitsch, NLN, SH and White were tested for embryogenic callus induction in AAA-EA banana cultivars. Briefly immature male flower buds of two cultivars Mpologoma and Nakinyika (selected based on their earlier contrasting responses and availability of male flowers) were prepared as described above and cultured on semisolid callus induction media containing the above salt formulations, full strength MS vitamins, 30gl⁻¹ sugar, 5 μ M 2,4-D and 10 μ M TDZ+10 μ M 4-CPPU at pH 5.7. Fifty male flowers of each cultivar were used in each salt mixture treatment and placed in a CRD in a dark growth room maintained at 26 $\pm$ 2°C. The cultures were observed every two weeks for at least 8 months. The number of flower buds on each petridish that responded (grew or differentiated into callus) was recorded. Yellow callus, embryogenic callus and organ-like structures developing on the callus or alongside embryos were recorded. Any callus with embryos achieved from both experiments was transferred onto the MS derived banana multiplication medium described by Talengera *et al.* (1994) but hormone free and embryo to plant development observed for two months.

6.2.4 Data analysis

The data on callus formation were summarized in percentages using Excel computer program. Yellow callus response was presented as percentage of male flower explants that produced yellow callus to the total number of male flowers initiated on callus induction medium. Embryogenic callus response was expressed as proportion of callus cultures with embryos. The data on number of immature male flower bud clusters that responded on the various basal salt mixtures was subjected to ANOVA using GenStat 13th Edition (GenStat, 2010). The means were separated using least significant differences (LSD) at 5% level of significance. Comparisons were done on number of flower bud clusters that responded across cultivars and salt formulations.

6.3 Results

6.3.1 Effect of TDZ and 4-CPPU on callus induction

The response of the immature male flower buds of the different cultivars on the callus induction medium containing various combinations of TDZ and 4-CPPU did not vary during the first two months except some cultures of cv. Mpologoma on medium H1 (5 μ M TDZ+5 μ M 4-CPPU) dried without forming yellow callus. From 3-8 months, there were differences in response among the cultivars on the different growth regulator combinations (Table 6.1). Between 3-4 months the cultures of Mpologoma and Mbwarzirume showed embryogenic response on H2 (10 μ M TDZ+10 μ M 4-CPPU) (formed a white translucent callus with somatic embryos on its surface) of up to 4.62% and 5.72% respectively. The cultures of other cultivars had not yet formed embryogenic callus. Between 5-6 months, Nakabululu and Nakinyika showed embryogenic response of 4.76% on H3 (15 μ M TDZ+15 μ M 4-CPPU) and 5.26% on H5 (10 μ M TDZ+10 μ M 4-CPPU+10 mg l⁻¹ IAA+10mg l⁻¹ NAA) respectively. From 7-8 months, Nakabululu showed 22.22% embryogenic response on H2 (10 μ M TDZ+10 μ M 4-CPPU) while Mpologoma and Nfuuka had 2.38% and 9.09% embryogenic response respectively on H3 (15 μ M TDZ+15 μ M 4-CPPU). Most of the callus was white and translucent and formed on primary yellow callus (Figure 6.1). About 5% of the embryogenic calli had friable tissue. Most of the flower buds of Sukali Ndizi did not differentiate into callus instead grew excessively into floral structures. A few flower buds of AAA-EA banana cultivars also grew without differentiating into callus particulars on low TDZ and 4-CPPU concentrations.

Table 6.1. Percentage yellow callus (%YC) and embryogenic callus (%EC) formed from immature male flowers of different banana cultivars on callus induction medium supplemented with various combinations of TDZ and 4-CPPU

Cultivar	Hormone treatment	Period in months taken to induce embryos					
		3-4 months		5-6 months		7-8 months	
		%YC	%EC	%YC	%EC	%YC	%EC
Mpologoma	H1	66.7	0.0	23.3	0.0	3.3	0.0
	H2	100.0	4.6	76.5	0.0	0.0	0.0
	H3	100.0	0.0	42.9	0.0	23.8	2.4
	H4	100.0	0.0	0.0	0.0	0.0	0.0
	H5	96.8	3.2	32.3	0.0	0.0	0.0
Mbwazirume	H1	67.8	1.7	65.5	1.7	0.0	0.0
	H2	97.1	5.7	97.1	5.7	61.3	0.0
	H3	100.0	0.0	100.0	0.0	4.4	0.0
	H4	100.0	0.0	0.0	0.0	0.0	0.0
	H5	100.0	0.0	0.0	0.0	0.0	0.0
Nfuuka	H1	100.0	0.0	100.0	0.0	100.0	0.0
	H2	100.0	0.0	92.9	0.0	42.9	0.0
	H3	72.7	0.0	72.7	0.0	54.6	9.1
	H4	0.0	0.0	0.0	0.0	0.0	0.0
	H5	100.0	0.0	0.0	0.0	0.0	0.0
Nakabululu	H1	100.0	0.0	70.0	0.0	70.0	0.0
	H2	80.0	0.0	77.8	0.0	44.4	22.2
	H3	100.0	0.0	95.2	4.8	90.5	9.5
	H4	100.0	0.0	100.0	0.0	18.2	0.0
	H5	0.0	0.0	0.0	0.0	0.0	0.0
Nakinyika	H1	100.0	0.0	100.0	0.0	0.0	0.0
	H2	100.0	0.0	69.2	0.0	0.0	0.0
	H3	100.0	0.0	100.0	0.0	67.7	0.0
	H4	34.2	0.0	0.0	0.0	0.0	0.0
	H5	79.0	0.0	63.2	5.3	63.2	5.3
Sukali Ndzi	H1	100.0	0.0	83.3	0.0	0.0	0.0
	H2	100.0	0.0	0.0	0.0	0.0	0.0
	H3	100.0	0.0	100.0	0.0	66.7	0.0
	H4	100.0	0.0	90.9	0.0	0.0	0.0
	H5	100.0	0.0	100.0	0.0	0.0	0.0

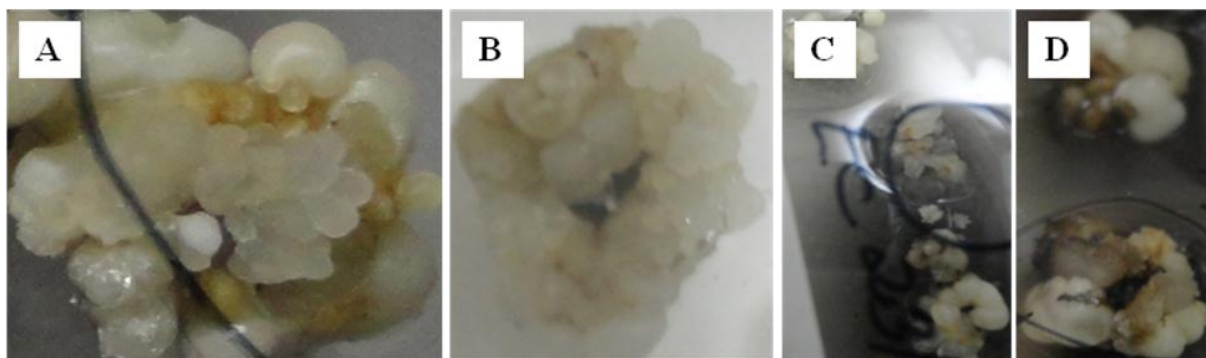


Figure 6.1 Different stages of callus development from immature male flower buds of cv. Nfuuka on callus induction medium supplemented with 15 μ M TDZ+15 μ M 4-CPPU: A) white translucent callus on primary yellow callus, B) white callus, C) Callus with primary embryos, D) Embryogenic callus with friable tissue.

6.3.2 Callus induction on different basal salt mixture substituted on callus induction medium supplemented with TDZ and 4-CPPU

In the first two months there were no differences in response of the cultivars on the various salt mixtures except 5.88% of the cultures of cultivar Mpologoma formed callus with somatic embryos on Gamborg B5 basal salt mixture which increased in 3-4th months to 11.43% (Table 6.2). Between 3-4 months, 8.33% and 3.33% of cultures of cultivar Nakinyika formed embryos on Gamborg B5 and White basal salt mixtures respectively. The cultures of both cultivars on Nitsch, NLN and SH salt mixtures dried within 4 months without forming any somatic embryos. Those on Chu N6 and Eriksson salt mixtures dried in 6 months while those on Gamborg B5 and White salt mixtures dried after 8 months. On pure MS medium cultures of cultivar Mpologoma had 4.62% embryogenic response between 3-4 months and dried in 6 months while cultures of cv. Nakinyika dried after the same period without showing any embryogenic response.

Table 6.2. Percentage yellow callus (%YC) and embryogenic callus (%EC) formed from immature male flowers of cv.s Mpologoma and Nakinyika on various basal salt mixtures supplemented with 10µM TDZ+10µM 4-CPPU

Cultivar	Basal salt mixture	Period in months taken to induce embryos					
		3-4 months		5-6 months		7-8 months	
		%YC	%EC	%YC	%EC	%YC	%EC
Mpologoma	Chu N6	100.0	0.0	83.9	0.0	0.0	0.0
Nakinyika	Chu N6	82.6	0.0	82.6	0.0	0.0	0.0
Mpologoma	Eriksson	90.0	0.0	90.0	0.0	0.0	0.0
Nakinyika	Eriksson	100.0	0.0	84.4	0.0	0.0	0.0
Mpologoma	Gamborg B5	100.0	11.4	97.1	11.4	97.1	11.4
Nakinyika	Gamborg B5	86.2	8.3	86.2	8.3	86.2	8.3
Mpologoma	Nitsch	100.0	0.0	0.0	0.0	0.0	0.0
Nakinyika	Nitsch	97.1	0.0	0.0	0.0	0.0	0.0
Mpologoma	NLN	94.3	0.0	0.0	0.0	0.0	0.0
Nakinyika	NLN	91.4	0.0	0.0	0.0	0.0	0.0
Mpologoma	SH	97.5	0.0	0.0	0.0	0.0	0.0
Nakinyika	SH	53.9	0.0	0.0	0.0	0.0	0.0
Mpologoma	White	87.2	0.0	87.2	0.0	87.2	0.0
Nakinyika	White	100.0	3.3	100.0	3.3	100.0	3.3
Mpologoma	MS	100.0	4.6	76.5	0.0	0.0	0.0
Nakinyika	MS	100.0	0.0	69.2	0.0	0.0	0.0

The cultures on the various salt formulations responded differently (Table 6.3). There was significantly ($P < 0.05$) higher survival and differentiation of the flower buds into callus on Chu N6 and White basal salt mixtures than on other salt mixtures except flower bud cultures of cultivar Nakinyika had also higher survival in MS medium. The survival and differentiation of the flower buds of both cultivars on Nitsch was significantly ($P < 0.05$) low. The response of the cultures of both cultivars on Eriksson (ER) and SH was moderate.

Cultures of cultivar Nakinyika had significantly ($P < 0.05$) lower survival than those of Mpologoma on Gamborg B5 (Gamb.), Nitsch and NLN basal salt mixtures.

Table 6.3. Mean number of male flower buds that differentiated into callus on various salt mixtures substituted on callus induction medium with 10 μ M TDZ+10 μ M 4-CPPU

Cultivar	Basal salt mixture								LSD (0.05)
	Chu N6	ER	Gamb.	Nitsch	NLN	SH	White	MS	
Mpologoma	7.26	5.53	6.28	3.35	6.54	5.84	7.94	5.91	1.219
Nakinyika	7.26	5.09	4.59	3.39	4.75	6.41	7.94	7.94	
LSD (0.05)	0.610								

The morphology and growth characteristics of the cultures also varied among the basal salt mixture. The flower buds of both cultivars grew normally on Gamborg B5 and White salt mixtures and differentiated into callus some of which had visible embryos (Figure 6.2). The embryos survived for over 6 months though some of the callus cultures had blackening at the base. Organ-like structures also grew on some of the calli.

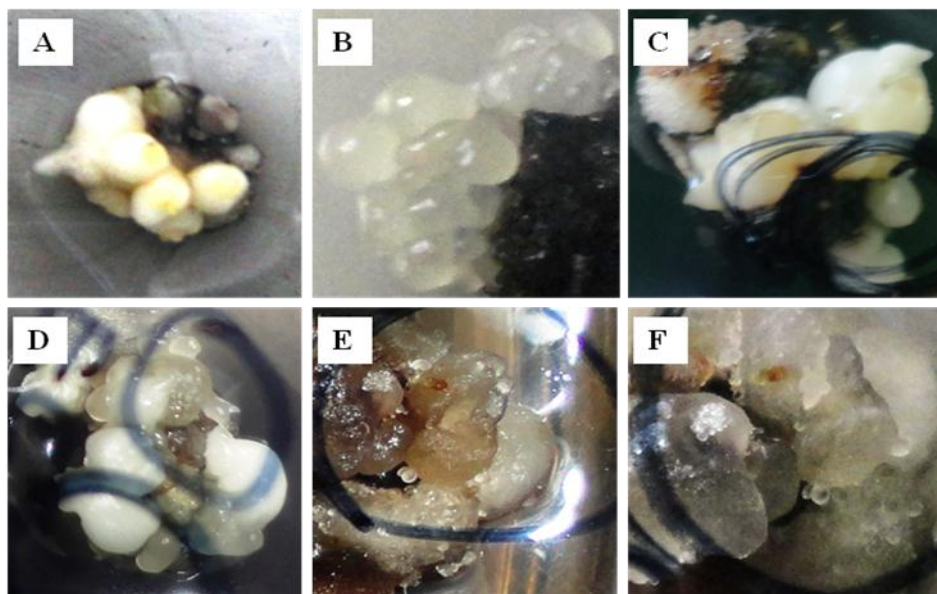


Figure 6.2 Different stages of callus development from immature male flower buds of cv. Mplogoma on Gamborg B5 basal salt mixture substituted on callus induction medium supplemented with 10 μ M TDZ+10 μ M 4-CPPU: A) flower bud differentiation into callus, B) white callus, C) development of embryos together with organ like structures, D) development of secondary embryos, E) Embryogenic callus with friable tissue, F) Callus with somatic embryos at different stages.

The cultures on Chu N6 and Eriksson grew vigorous but most of them had water cells especially in cv. Nakinyika (Figure 6.3). A few flower buds differentiated into yellow callus and grew normally until they dried due to age. The cultures on Nitsch, NLN and SH salt mixtures had slow growth characterized with blackening at the base of the initiated flower buds. Most of the flower buds on these three salt formulations dried at an early stage without showing any sign of embryogenic response (Figure 6.3). When the callus of the various cultivars on the different salt formulations substituted on the callus induction medium supplemented with TDZ and 4-CPPU was removed and cultured on a hormone free MS medium, it regenerated into shoots through both organogenesis and somatic embryogenesis (Figure 6.4) regardless of salt formulation confirming embryogenic competence.

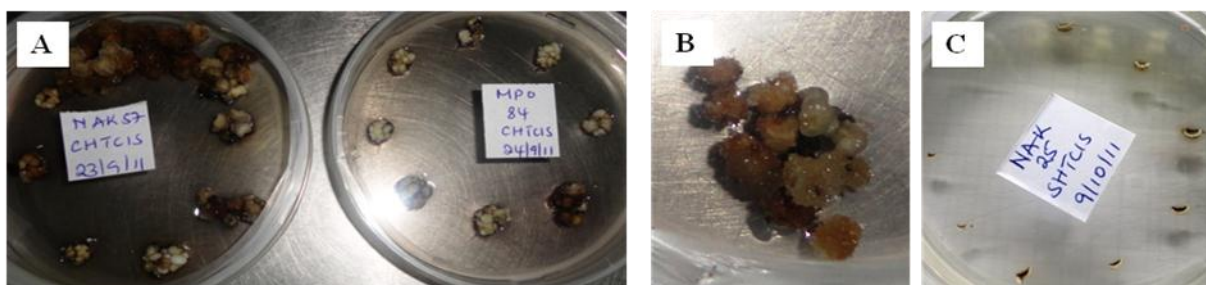


Figure 6.3 Growth characteristics of immature male flower buds of cv.s Mplogoma and Nakinyika on Chu N6 and SH basal salt mixtures substituted on callus induction medium supplemented with 10 μ M TDZ+10 μ M 4-PPU: A) flower bud differentiation into water cells, B) Prolifertaion of water cells, C) Early dried flower buds on SH salt mixture.

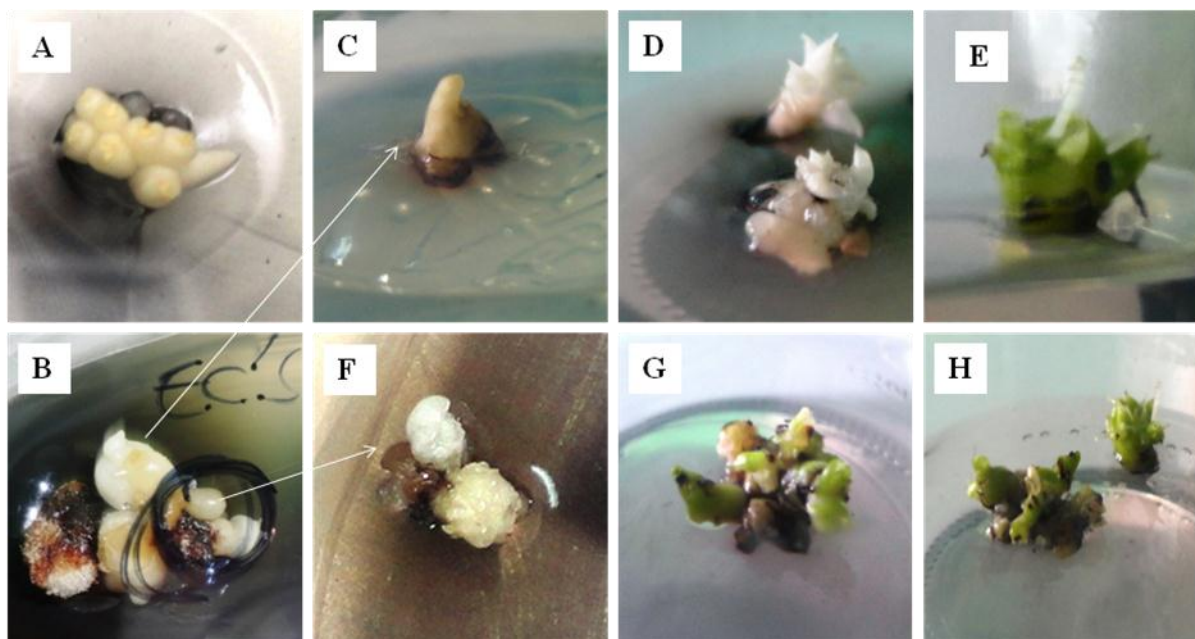


Figure 6.4 Plant development through organogenesis and somatic embryogenesis from immature male flower buds of cv. Mplogoma on Gamborg B5 basal salt mixture substituted on callus induction medium supplemented with 10 μ M TDZ+10 μ M 4-PPU: A) flower bud cluster, B) Differentiation into embryos and shoot-like structure, C-E) Direct organogenesis on hormone free medium, F-H) plant development through embryogenesis after transferring the embryogenic callus on hormone free medium.

6.4 Discussion

6.4.1 Callus induction on callus induction medium supplemented with TDZ and 4-CPPU

Embryogenic callus formation among the cultivars on the callus induction medium supplemented with various combinations of TDZ and 4-CPPU varied. However all the AAA-EA banana cultivars studied showed embryogenic response implying that a combination of TDZ and 4-CPPU with 2,4-D is efficacious for callus induction from male flower buds of AAA-EA banana cultivars. Favorability of combinations of urea-type cytokinins with auxins for induction of embryogenesis was reported in other banana genotypes. Divakaran *et al.* (2011) generated embryogenic callus from bract explants of diploid ($2n = 22$) banana cultivars on Murashige and Skoog medium supplemented with TDZ (0.045–9.00 μM). When they transferred the embryogenic callus on MS basal medium supplemented with biotin and or glutamine it produced somatic embryos and consequently regenerated normal plants with little somaclonal variation and survival rate in soil of 90%. In other crops, combined effect of TDZ and 2,4-D induced somatic embryogenesis in non-responsive caryopses of rice (Aparna and Rashid, 2004). Tuong Huan *et al.* (2004) achieved more embryogenic calli induction from protocorm of *Cymbidium*, a hybrid orchid, on medium containing a combination of NAA and TDZ than on NAA alone. A Combination of 2,4-D and TDZ also changed granular callus into a favorable friable form in *Oncidium* (Fang *et al.*, 2006). Fiore *et al.* (2004) reported higher percentage of embryo formation in both sweet orange (*C. sinensis* L.) and lemon (*Citrus limon* L.) on 4-CPPU when they used media supplemented with different combinations of 2,4-D and 4-CPPU to induce callus and somatic embryogenesis.

All the cultivars (except Nfuuka) formed more somatic embryos on the medium supplemented with 10 μM of each of TDZ and 4-CPPU than on the medium supplemented with 5 μM or 15 μM of each of them. The low response of the cultures on the callus induction medium supplemented with 15 μM of each of TDZ and 4-CPPU was attributed to excessive auxin effect. At high concentrations urea-type cytokinins behave typical like auxins and thus the auxin activity from them added to that of 2,4-D could have been too high to induce embryogenic response. This implies cv. Nfuuka which responded best on the medium

supplemented with 15µM of each of TDZ and CPPU require higher auxin activity to stimulate embryogenesis in it.

Instead of differentiating into callus on the medium supplemented with 5µM of each of TDZ and 4-CPPU some of the flower buds proliferated into new floral tissue. Hernandez and Rosell-Garcia (2008) reported inflorescence proliferation in dwarf Cavendish male flower on medium supplemented with 5µM TDZ. At low concentrations urea-type cytokinins exhibit cytokinin activity which favors tissue proliferation. It means at 5µM, TDZ and 4-CPPU added none or minimal auxin activity to that of 2,4-D which was not sufficient to induce embryogenic response. It further implies that cv. Sukali Ndizi (AAB) whose cultures had excessive proliferation on all the treatments require much higher auxin supply and thus needed higher TDZ and 4-CPPU amounts to stimulate embryogenesis. Banana genotypes with AAB appear to require higher auxin concentration to induce embryogenesis probably due to their heterogenomic hybrid nature especially the presence of the B genome which is usually associated with higher tolerance to stress. Embryogenic callus and somatic embryos were generated from male flower buds of banana cv. Rasthali (AAB) on MS medium supplemented with 18.10 µM 2,4-D, 5.37 µM NAA and 5.71 µM IAA (Sukhada *et al.*, 2010).

6.4.2 Callus induction on different basal salt mixture substituted on callus induction medium supplemented with TDZ and CPPU

Under the same MS vitamins and hormone treatment embryogenic response was observed on Gamborg B5, MS and Whites basal salt mixtures but none on Chu N6, NLN, Nitsch and SH salt formulations. Other authors reported on callus induction and ECS development from immature male buds of different banana genotypes on some of these salt formulations. Cote *et al.* (1996) achieved embryo development in *Musa* AAA cv. Grande Naine (AAA) by plating its cell suspension on a medium of SH with MS vitamins. Similarly Gomez *et al.* (2000) achieved embryo formation in hybrid cultivar FHIA-18 (AAAB) using a medium of SH with MS vitamins. When Ganapathi *et al.* (1999) initiated young banana male flowers on full strength MS medium and White's medium, they found MS medium to be better than the White's medium in terms of embryogenic callus induction. But in this study no cultivar

responded on SH while cultivar Nakinyika showed embryogenic response on white basal salt mixture but not on MS medium.

Notwithstanding cultivar differences, the performance of the various basal salt mixtures was probably influenced by their interaction with TDZ and CPPU in the media. For instance urea-type cytokinins especially TDZ in synergy with Ca^{2+} stimulates ethylene production (Wing-Kin and Shang, 1986) which most likely caused the cultures on the basal salt mixtures with high calcium content especially MS and NLN to dry early (Table 6.2). Gamborg B5 basal salt mixture has low calcium content compared to the other salt mixtures; perhaps that was why both its callus and embryos survived for longer time than cultures on the other salt mixtures (Table 6.2).

On the other hand both cultivars Mpologoma and Nakinyika formed more embryos on Gamborg B5 basal salt mixture than on other salt mixtures including on MS medium one of the most commonly used media for plant tissue culture. Compared to the other salt formulations, Gamborg B-5 basal salt mixture has also the lowest total nitrogen content of which 93% is in nitrate form and only 7% in ammonical form. Since banana cultures have a high demand for nitrogen (Purseglove, 1972; Stover and Simmond, 1987) and mostly prefer ammonical nitrogen (Marchal, 1990) the limited amount of nitrogen in it possibly created a stress which favored embryo formation. It was probably because of the same reason that the White basal salt mixture which has also lower total nitrogen imposed stress and induced embryogenic response in cv. Nakinyika.

The trend in survival and response of the flower buds on each petridish was not clear. However the survival of flower buds in the culture medium was linked with their embryogenic response. The cultures of both Mpologoma and Nakinyika on Gamborg B5 salt mixture survived for the same period. But cultivar Mpologoma whose flower buds had significantly ($P < 0.05$) higher survival rate showed higher embryogenic response. The higher survival of the flower buds of cv. Mpologoma on the callus induction medium possibly increased their chances for embryogenic response. It implies that individual flower bud

survival and duration of survival of the cultures on the various salt formulations determined embryogenic response.

When the callus of the various cultivars from the different salt formulations substituted on callus induction medium supplemented with TDZ and CPPU was transferred into hormone free MS derived medium, it developed into shoot through both organogenesis and somatic embryogenesis (Figure 6.4). An explant can regenerate into an entire plant either through organogenesis whereby shoots and roots simply form sequentially or somatic embryogenesis whereby a cell or group of cells from the explant initiate and develop viable embryos (Victor, 2001). The review by Victor (2001) reveals that both organogenesis and somatic embryogenesis can occur in the same explant but originating from particular tissue layers or cells within the explant. Besides differences in physiological state of the flower buds or tissues and cells within them, the occurrence of organogenesis and somatic embryogenesis together in this study was attributed to the presence of TDZ and CPPU in the media and their interaction with the various salt mixtures. Urea-based growth regulators especially TDZ was reported to induce a diverse array of culture responses including induction of callus, formation of somatic embryos and other structures (Murthy *et al.*, 1998). TDZ induced both direct shoot organogenesis and somatic embryogenesis on cotyledonary node explants of lentil (*Lens culinaris* Medik.) (Chhabra *et al.*, 2008). This is possibly because of its unique property of mimicking both auxin and cytokinin effects on growth and differentiation of cultured explants (Murthy *et al.*, 1998).

Previously auxins particularly 2,4-D was used in combination with purine-based cytokinins such as BA, BAP and Zeatin for induction of embryogenesis in AAA-EA banana. Besides low embryogenic responses it was rare for more than two cultivars to show embryogenic response on a single treatment though embryogenic response in banana was reported to be genotype dependent (Youssef *et al.*, 2010). In this study where the callus induction medium was supplemented with urea-type cytokinins TDZ and CPPU, all the studied AAA-EA banana cultivars produced regenerable embryogenic callus especially on the callus induction medium supplemented with 10 μ M TDZ+10 μ M CPPU. When cultivars Mpologoma and Nakinyika were cultured on the same medium containing 10 μ M TDZ+10 μ M CPPU but the MS salts

substituted with the other salt formulations, 11.4% and 8.3% of their cultures respectively had embryos on Gamborg B5 salt formulation almost twice their response on pure MS medium. Since the male flowers were collected randomly from the same fields and therefore, there were no instances of differences in embryogenic response due to differences in developmental stage of the flower buds (Youssef *et al.*, 2010), these responses were rated to those remarkably reported in other genotypes such as by Ghosh *et al.* (2009). Recently when Ghosh *et al.* (2009) established embryogenic cell suspension culture of Cavendish cultivar Robusta (AAA) using immature male flowers they reported embryogenic callus response of 10.3%.

Though some cultures proliferated instead of forming regenerable embryogenic callus, given the limited availability of immature flowers and/or long time required to wait for banana plants to flower, the induced floral tissue could be used as a source of flower buds for more callus induction. Perez-Hernandez and Rosell-Garcia (2008) found newly induced floral tissues from banana inflorescence cultured on 5 μ M TDZ to be embryogenically competent.

6.5 Conclusion

The callus induction medium supplemented with a combination of TDZ and CPPU induced various levels of embryogenic response from immature male flower buds of AAA-EA banana cultivars Mbwarzirume, Mpologoma, Nakabululu, Nakinyika and Nfuuka. The callus induction medium supplemented with 10 μ M TDZ+10 μ M CPPU induced more embryogenic response. Embryogenic response on Gamborg B5 salt mixture substituted on the callus induction medium supplemented with 10 μ M TDZ+10 μ M CPPU doubled especially in cv. Mpologoma. The results thus suggest that the percentage of embryogenic callus induced by TDZ and 4-CPPU on immature male flower buds of AAA-EA cultivars is influenced by basal salt mixture and in the presence of these two growth regulators, Gamborg B5 salt formulation is more appropriate for callus induction followed by White and MS salts depending on cultivar.

CHAPTER SEVEN

GENERAL DISCUSSIONS

This study determined proliferation potential and number of recoverable or weanable plantlets that could be generated from a single isolated shoot tip of AAA-EA banana cultivars with different suckering ability in the field on the adopted MS derived medium for banana multiplication. Understanding the trend in *in vitro* proliferation of the AAA-EA banana cultivars would enhance development of micropropagation protocols for their multiplication and aid producers of tissue culture plants in planning especially by estimating number of shoot-tip explants to initiate in culture to produce a given number of plantlets. It also pursued somatic embryogenic techniques which have higher potential for mass propagation of planting material and possible use of the tissues for genetic transformation. Specifically urea-type cytokinins TDZ and 4-CPPU, and different salt formulations were tested for embryogenic callus induction in AAA-EA banana cultivars through scalps and immature male flowers.

The AAA-EA banana cultivars produced 3-4 new shoots in each subculture cycle and 57-169 recoverable shoots from one starting shoot-tip explant in 18 weeks. The study thus revealed a variation in *in vitro* shoot proliferation among the studied AAA-EA banana cultivars but the trend was different from that of their suckering ability in *in vivo*. Since differences in cultivar proliferation rate have been attributed to differences in growth regulator uptake, translocation rate to meristematic regions, and metabolic processes among genotypes (Blakesley, 1991; Youmbi *et al.*, 2006), the inconsistency in proliferation *in vitro* and suckering *in vivo* implied that though they belong to the same genome group, AAA-EA banana cultivars differ in growth regulator uptake, translocation and metabolic processes. The ability of the cultivars to produce recoverable shoots was influenced by the number and morphology or growth characteristics of their shoot cultures suggesting that both proliferation and efficiency to produce recoverable shoots of AAA-EA banana cultivars could be improved by varying culture conditions or adjusting the amount of cytokinin and other growth inducing substances in the medium during the subsequent subculture cycles. As the multiplication rate of recoverable shoot depended on the cultivars' shoot growth characteristics, the number of

recoverable shoots from one shoot-tip explant of each cultivar in a given time could be predicted using a regression model and thus a producer of tissue culture plants could be able to project the number of plants to produce from a given number of shoot tip explants.

The results from somatic embryogenic techniques which have higher potential for micropropagation and are more preferred in case of genetic transformation were promising both from the scalp and immature male flower methods. One impediment to using the scalp method to generate callus in AAA-EA banana had been the long time required to achieve ideal scalps. On TDZ and 4-CPPU combination, scalps of AAA-EA bananas were achieved in over 50% of the shoot-tip cultures within 4 subculture cycles. Besides long time required generating scalps there has been low embryogenic response on the existing protocols. On the TDZ and 4-CPPU treatment, embryogenic callus was formed on 2.5 – 20% of the scalp cultures depending on cultivar. Cultivars Nakinyika, Nakabululu, Mbwazirume and Nfuuka developed regenerable embryogenic callus and recorded plant regeneration through direct embryogenesis. The plantlets of cultivars Nakinyika and Mbwazirume developed normal roots and were weaned onto soil. Though the embryo to plant conversion rate was low, these embryogenic callus responses were within the range and in some instances higher than those reported even in other genotypes using the scalp method. For instance Xu *et al.* (2005) reported a 10% embryogenic response for Williams and 0.1 to 6.2% for other Cavendish-type bananas.

Though embryogenic response from male flower buds is often influenced by genotype and the development stage of the buds (Youssef *et al.*, 2010), the immature male flower bud cultures of most of the same cultivars (Mpologoma, Mbwazirume, Nakabululu, Nakinyika and Nfuuka) on callus induction medium supplemented with TDZ and 4-CPPU showed 4.6 - 22.2% embryogenic response especially on the medium with 10 μ M TDZ+10 μ M CPPU combination. These embryogenic callus responses were also comparable and in some instances higher than those reported even in other genotypes using the immature male flower method. For example Escalant *et al.* (1994) reported a range of 0-7% embryogenic response in different banana and plantain genotypes. Navarro *et al.* (1997) reported 2 - 6% embryogenic callus response for Grand Naine (AAA) while Strosse *et al.* (2003) reported a

mean percentage of ideal embryogenic callus of 8% for the same cultivar. Recently Ghosh *et al.* (2009) established embryogenic cell suspension culture (ECS) of Cavendish banana cultivar Robusta (AAA) using immature male flowers and achieved percentage embryogenic callus response of 10.3. This implies application of TDZ and 4-CPPU overcomes recalcitrance in AAA-EA banana cultivars and makes them responsive to somatic embryogenesis to levels comparable to that of other banana genotypes such Cavendish.

When cultivars Mpologoma and Nakinyika were cultured on the same medium containing 10 μ M TDZ+10 μ M 4-CPPU but the MS salts substituted with the other salt formulations, their cultures recorded 11.4% and 8.3% embryogenic response respectively on Gamborg B5 salt mixture which was almost twice their response on pure MS medium. The outstandingly higher performance of both cultivars on Gamborg B5 basal salt mixture than on other salt mixtures including on MS medium one of the most commonly used media for plant tissue culture suggest that TDZ and CPPU interacts with salt components and interacts more favorable with Gamborg B5 salts for induction of embryogenesis.

From the scalp method the embryogenic callus response of the different cultivars varied from 2.5 – 20% on medium with equal proportions of 9 – 13 μ M TDZ and 4-CPPU. The range of embryogenic callus response for the same cultivars on almost the same proportions of TDZ and 4-CPPU using immature male flower method was 4.6 - 22.2%. Though there were differences in response of some of the cultivars using the two methods the range for the two methods was similar. This is consistent with observation by Strosse *et al.* (2006) that both methods could yield similar results.

Considering all the 3 experiments and not contradicting the discussions in chapter 4, the trend in the regeneration ability of the AAA-EA banana cultivars used in this study appears to be linked to either their seed fertility or suckering ability in the field. For instance among the cultivars that responded fairly well, cv. Nakabululu belongs to Nakabululu clone set while cultivars Nfuuka and Nakinyika belong to Nfuuka clone set. These two clone sets were reported to be more female fertile than cultivars in Musakala and Nakitembe clone sets (Ssebuliba *et al.*, 2006). The ability of cv. Mbwarzirume to regenerate plantlets with normal

roots yet it belongs to Nakitembe clone set which is less seed fertile suggests that cultivar regeneration ability may also be associated with its suckering ability in the field. According to Karamura and Pickersgill (1999), cultivars in Nakitembe and Nakabulu clone sets have higher suckering ability than those from other clone sets. This further means that cultivar Mpologoma performed poorly because it belongs to Musakala clone set which is less female fertile (Ssebuliba *et al.*, 2002) and also produces fewer suckers in the field (Karamura and Pickersgill, 1999). However this observation in banana requires further research to elucidate.

CHAPTER EIGHT

CONCLUSIONS AND RECOMMENDATION

8.1 Conclusions

In vitro proliferation of AAA-EA banana on the widely adopted MS medium modified for banana multiplication varied from 3-4 new shoots in each subculture cycle and between 57 and 169 recoverable shoots from one starting shoot-tip explant in 18 weeks. The results suggest that the number of shoot buds produced by AAA-EA banana in MS medium is cultivar dependent however; the number of recoverable shoots is influenced by the cultivar's shoot growth characteristics and is independent of its proliferation ability. Since the multiplication rate of recoverable shoot depended on the cultivars' shoot growth characteristics, the number of recoverable shoots from one shoot-tip explant of each cultivar in a given time could be predicted using a regression model.

Scalps of AAA-EA bananas were achieved on over 50% of the shoot-tip cultures within 4 subculture cycles and embryogenic callus was directly formed on 2.5 – 20% of the scalp cultures on the auxin-free scalp induction medium with TDZ and 4-CPPU depending on cultivar. Most of cultivars showed better responses on the medium supplemented with TDZ and 4-CPPU at equal proportions of 9 - 13 μ M. Cultivars Nakinyika, Nakabululu, Mbwarzirume and Nfuuka developed regenerable embryogenic callus and recorded plant regeneration through direct embryogenesis. This implies that media supplemented with TDZ and 4-CPPU combination reduce the time taken to induce scalps and increase the percentage of embryogenic callus induced from scalps of EA-banana cultivars.

The callus induction medium supplemented with TDZ and 4-CPPU induced various levels of embryogenic response from immature male flower buds of AAA-EA banana cultivars Mbwarzirume, Mpologoma, Nakabululu, Nakinyika and Nfuuka. Most of the cultivars showed embryogenic response on the medium with 10 μ M TDZ+10 μ M CPPU ranging from 4.6 - 22.2%. Embryogenic response on Gamborg B5 salt mixture substituted on the callus induction medium supplemented with 10 μ M TDZ+10 μ M CPPU was higher than on other salt

formulation. The results thus suggest that the percentage of embryogenic callus induced by TDZ and 4-CPPU on immature male flower buds of AAA-EA cultivars is influenced by basal salt mixture and in the presence of these two growth regulators, Gamborg B5 salt formulation is more appropriate for callus induction followed by White and MS salts depending on cultivar.

Previously auxins particularly 2,4-D was used in MS medium in combination with purine-based cytokinins such as BA, BAP and Zeatin for induction of embryogenesis in AAA-EA banana. Besides the long time required especially for generating scalps there has been low embryogenic response on the existing protocols. It has also been rare for more than two cultivars to show embryogenic response on a same treatment. In this study, using urea-type cytokinins, TDZ and 4-CPPU, ideal scalps were achieved within 4 subculture cycles. In both the scalp and immature male flower methods, most of the studied cultivars showed embryogenic responses on the same treatment which in some instances were higher than those reported before in AAA-EA banana cultivars and even other banana genotypes. Earlier plant regeneration at lower concentrations of combined TDZ and CPPU could reduce occurrence of somaclonal variation. This implies that application and optimization of combined TDZ and 4-CPPU especially in Gamborg B5 salt formulation for routine induction of embryogenic callus and development of embryogenic cell suspensions, respectively, will improve plant regeneration and consequently propagation and genetic improvement of AAA-EA banana cultivars.

8.2 Recommendations

The study demonstrated the potential of *in vitro* approach for production of banana planting materials. Depending on the AAA-EA banana cultivar's shoot culture growth characteristics, producers of tissue culture plants through shoot-tip culture can project the number of recoverable shoots from one shoot-tip explant in a given time using a regression model. The model for instance for cv. Mpologoma is $Y = e^{[0.261(x-1) - 0.00386(x-1)^2 + 1.39]}$; where Y = number of recoverable shoots, e = 2.718282 and x = time in weeks. This prediction model can give a fair estimate up to the 4th - 6th subculture cycle corresponding to 4.5 - 6.5 months. Care must be taken when predicting beyond this period as changes may occur in proliferation or growth

pattern depending on cultivar. The model may be applied to other cultivars that have similar shoot culture growth characteristics to that of AAA-EA bananas. As the ability of a cultivar to produce recoverable shoots is influenced by the number and morphology or growth characteristics of its shoot culture, further studies are recommended on how to improve both proliferation and efficiency to produce recoverable shoots of AAA-EA banana cultivars.

The scalp induction medium and callus induction medium supplemented with equal proportions of 9 - 13 μ M TDZ and 4-CPPU and 10 μ M TDZ+10 μ M CPPU respectively induced embryogenic responses from scalps and immature male flower buds respectively from most the studied cultivars. Embryogenic response on Gamborg B5 salt mixture substituted on the callus induction medium supplemented with 10 μ M TDZ+10 μ M CPPU was higher than on other salt formulations. Though the embryo to plant conversion rate was low, application and optimization of combined TDZ and 4-CPPU especially in Gamborg B5 salt formulation for routine induction of embryogenic callus and development of embryogenic cell suspensions respectively is worthwhile.

The overall response of the studied AAA-EA banana cultivars from different clone sets suggests a linkage between their regeneration ability and their seed fertility or suckering ability in the field. It is therefore recommended that further studies are done to elucidate whether the regeneration ability of the AAA-EA banana cultivars is linked to their seed fertility or suckering ability in the field. This will provide a vital insight to the basis for recalcitrancy in this banana group.

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APPEDICES

Appedix I

Murashige and Skoog (MS) based Banana micropropagation medium with modification by Talengera *et al.* (1994).

No.	Element/Compound	Group A (Major Elements)	Use 100 ml per litre of Medium
		Stock Dissolve in 1000 ml of DDH ₂ O	
1	HN ₄ NO ₃	Weigh 16.5gm	
2	KHO ₃	Weigh 19.0gm	
3	CaCL ₂ .2H ₂ O	Weigh 4.4gm	
4	MgSO ₄ .7H ₂ O	Weigh 3.7gm	
5	KH ₂ PO ₄	Weigh 1.7gm	
Group B (Minor Elements)			Use 5 ml per litre of Medium
		Stock Dissolve in 500 ml of DDH ₂ O	
1	H ₂ BO ₃	Weigh 0.620 gm	
2	MnSO ₄ .4H ₂ O	Weigh 2.230 gm	
3	ZnSO ₄ .4H ₂ O	Weigh 0.860 gm	
4	KI	Weigh 0.0830 gm	
5	NaMoO ₄ .2H ₂ O	Weigh 0.025 gm	
6	CuSO ₄ .5H ₂ O	Weigh 0.0025 gm	
7	CoCl ₂ .6H ₂ O	Weigh 0.0025 gm	
Group C (Vitamins Elements)			Use 5 ml per litre of Medium
		Stock Dissolve in 500 ml of DDH ₂ O	
1	Nicotinic acid	Weigh 0.050 gm	
2	Pyridoxine-HCL	Weigh 0.050 gm	
3	Thiamine-HCL	Weigh 0.010 gm	
4	Glycine	Weigh 0.02 gm	
Group D (Iron)			Use 5 ml per litre of Medium
		Stock Dissolve In100 ml of DDH ₂ O	
1	Na-EDTA	(Dissolve 0.746 gm pre-heat to dissolve with 7)	
2	FeSO ₄ .7H ₂ O	(Dissolve 0.557 gm)	
Group E (Myo-inositol)			Use 10 ml per litre of Medium
1	My-inositol	Weigh 10 gm per litre of DDH ₂ O	
Group F (Ascobic Acid)			
1	Ascobic acid	Weigh 2 gm per 500 mls DDH ₂ O	
Group H (Phytohormones)			Use 5 ml per litre

		of Medium
1	BAP	0.1 gm per 100 mls of DDH ₂ O
		Use 30 gm per litre of Medium
		Group G (Carbon source)
1	Sucrose	Weigh 30 gm in 1000 mls of medium
		Adjust PH
1	PH	Use 0.1M of HCL or 0.1M of NaOH to adjust PH to 5.8
		Gelling Agent
1	Phytigel, Gelrite or Agar	Add 2.3 gm of Phytigel or 2 gm of Gelrite or 7.0 gm of Agar, heat to dissolve, dispense to culture vessels, autoclave at 121°C for 15 min
		Key: DD = Double Distilled Water

Appendix II

The callus induction medium that was supplemented with TDZ and 4-CPPU for inducing embryogenic callus from male flowers

Compound	Concentration
MS salts	Full strength
MS vitamins	Full strength
IAA	5.7 μ M (1mg/l)
2,4-D	5.0 μ M (1.105mg/l)
Biotin	4.09 μ M (1mg/l)
NAA	5.4 μ M (1mg/l)
Ascorbic acid	20 mg/l
Sucrose	30g/l
Gel-rite	2.0g/l
PH 5.7	