Direct Somatic Embryogenesis of Selected Commercial *Coffea Arabica* L. Varieties in Kenya

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

Sign……………………………………………Date…………….

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

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DEDICATION

To Mr Wahome, thank you for instilling in me the culture of hard work and love for scientific research. May the almighty God rest your soul in eternal peace.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICE

LIST OF ABBREVIATIONS AND ACRONYMS

BAP   Benzyl amino purine

IAA   Indole acetic acid

IBA   Indole butyric acid

NAA   Naphthalene acetic acid

2, 4-D   2, 4-Dichlorophenoxyacetic acid

TDZ   Thidiazuron

DNA   Deoxyribonucleic acid

SSR   Simple sequence repeats

RAPD   Random amplified polymorphic DNA

PGR   Plant Growth Regulators

CRI   Coffee Research Institute

ICO   International Coffee Organization

ICRAF   International Centre for Research in Agroforestry

DSE   Direct somatic embryogenesis

ISE   Indirect somatic embryogenesis

ABSTRACT

Breeding programs in Kenya have produced disease tolerant *Coffea arabica* varieties such as Ruiru 11 and Batian. Subsequently demand has surpassed supply. Existing propagation methods do not provide enough planting materials, hence the need to develop alternative methods of coffee propagation. The objective of this study was to develop an efficient *In vitro* regeneration protocol for coffee varieties Batian, Ruiru 11 and SL28 commercially grown in Kenya. Sterilization was carried out using different concentrations of Jik® (3.85 v/v sodium hypochlorite) for varied exposure times. A single step sterilization procedure was established using 28% for 25minutes and is recommended for further work. The effects of various auxins and cytokinins on the different *In vitro* regeneration stages of coffee were evaluated. The results showed significant differences among the different cytokinin and auxin levels for regeneration of the evaluated varieties. The best cytokinin level for induction of somatic embryogenesis was found to be 13.3µM Benzyl amino purine giving the highest mean number of somatic embryos across all selected varieties. Germination of somatic embryos was achieved on hormone free (control) Murashige and Skoog media and there were no significant differences amongst the evaluated levels of Benzyl amino purine. 2.5 µM NAA was best in induction of a well-developed root system for all the evaluated varieties with a mean length of 1.71±0.09mm for SL28 variety. The findings of this research work open an otherwise inadequately explored area for maximizing *In vitro* plantlet production in coffee that needs to be fully exploited.

CHAPTER ONE

INTRODUCTION

1.1   Background of the study

Coffee is the second most traded commodity in monetary value in the world after crude oil (Carneiro, 1999). It contributes to foreign exchange earnings, family incomes, food security and employment in the cultivation, processing as well as trade sections in coffee growing countries (Carneiro, 1999). Although over 100 coffee species exist, only two *Coffea arabica* L. (arabica coffee) and *Coffea canephora* P. (Robusta coffee) are commercially cultivated. The two varieties account for about 70% and 28% percent respectively of the world coffee cultivation and consumption (Cesar *et al*., 2006). *Coffea arabica* L. is native to the Ethiopian highlands while *Coffea canephora* is indigenous to the evergreen forests of central and West Africa (Carneiro, 1999). Kenya produces the most favored *Coffea arabica* L. variety (Carneiro, 1999; Jeremy *et al*., 2005; Cesar *et al*., 2006; Coffee Research Foundation strategic plan, 2010).

Plant breeding is an important way of sustaining coffee production and it involves conventional breeding methods of plant selection, progeny evaluation and backcross. This technique has been used to introduce traits, such as disease resistance, high yield and vigorous plant growth, to *C. arabica* varieties from related species (Carneiro, 1999). Despite the tremendous contribution of classical breeding to coffee improvement, this approach is tedious, slow and expensive given the limitations of selection of superior genotypes due to the many crossing cycles of progeny evaluation and genetic barriers as a result of chromosome number for instance diploid vs. tetraploid (Carneiro, 1999; Cesar *et al*., 2006). For instance, the release of a new coffee variety is estimated to require a minimum 24 years of continued breeding and the resulting seed are usually insufficient to satisfy the needs of coffee growers (Carneiro, 1999; Jeremy *et al*., 2005; Cesar *et al*., 2006; Coffee Research Foundation strategic plan, 2010).

1.2   Problem statement

Kenyan coffee is highly rated in the world market and contributes significantly to the country's economic growth. Hence, it can be assumed that existing cultivars in the market are highly productive. However, there are demands for improvements related to resistance to diseases, pests and tolerance to abiotic stresses such as drought. Existing varieties were improved via conventional breeding programs. Conventional breeding is a long, intricate process and breeders have turned to *in vitro* culture techniques and genetic engineering to shorten time taken to release improved varieties. In Kenya, much remains to be done to establish mass *in vitro* propagation protocols for commercial varieties that are of importance in conventional breeding programs. Existing systems involve mass micropropagation of superior hybrid genotypes obtained via conventional breeding programs (Kahia, 1999). The existing mass propagation methods involve indirect somatic embryogenesis (ISE). The time taken to regenerate coffee plantlets using direct somatic embryogenesis (DSE) is normally shorter compared to 12-13 months taken for ISE is (Ducos *et al.,* 2010; Etienne *et al.,* 2010). This prolonged stay in a plant growth regulator (PGR) supplemented media increases the chances of somaclonal variation in ISE. The protocol developed in this study ensures rapid production of true to type coffee plantlets.

1.3   Justification

One of the constraints to coffee production in Kenya is infection by diseases. Coffee Berry Disease (CBD) that is caused by *Colletotrichum kahawae* and Coffee leaf rust (CLR), caused by *Hemileia vastatrix* (Gichuru *et al*., 2012). Coffee breeding programmes in the country have recently resulted into the release of the improved varieties such as Ruiru 11 and Batian. These new varieties are resistant to both CBD and CLR (Gichimu and Omondi, 2010). The release of these varieties coincided with favourable coffee prices that resulted in overwhelming demand for planting materials. As a rapid multiplication method for planting materials, tissue culture offers a feasible alternative to supplement the conventional propagation methods to meet the high demand for planting materials. Therefore it is essential that efficient *in vitro* culture protocols be established for all commercial cultivars by assessing different biological,

chemical and physical factors on regeneration. Once established, these techniques will extensively advance coffee improvement programs. Biotechnology can further supplement efforts of coffee breeders with additional tools that can overcome the limitations of classical breeding especially for mass *in vitro* propagation of seedlings, genetic improvement and germplasm conservation (Carneiro, 1999). The susceptibility of *C. arabica* to pests, diseases and abiotic stress coupled with its low genetic diversity, can be overcome by introduction of genes from other coffee germplasm and other plant species through genetic transformation (Carneiro, 1999; Cesar *et al*., 2006).Biotechnology will be useful in circumventing genetic barriers and accelerating release of varieties with superior traits through mass propagation of seedlings to satisfy farmer demand for planting material, thus increase productivity. Coffee improvement can benefit from these approaches through developing *In vitro* culture protocols for selected varieties of importance to breeding programs (Jeremy *et al*., 2005; Cesar *et al*., 2006; Coffee Research Foundation strategic plan, 2010).

1.4 Objectives

1.4.1 General objective

To develop direct somatic embryogenesis protocol for selected commercial *Coffea arabica* varieties in Kenya.

1.4.2 Specific objectives

1. To determine the optimum sterilization technique for leaf explants.
2. To evaluate the effects of various cytokinin concentrations on the induction and maturation of somatic embryos.
3. To assess the effects of various auxin concentrations on the induction of roots.

1.5 Hypotheses

1. Different hypochlorite solutions have no effect on surface sterilization of leaf explants

2. Various cytokinin concentrations have no effect on the induction and maturation of somatic embryos

3. Various auxin concentrations have no effect on the induction of roots.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin and distribution

The coffee tree, scientifically known as *Coffea arabica*, is native to Abyssinia and Ethiopia, but grows well in Java, Sumatra, and other islands of the Dutch East Indies; in India, Arabia, equatorial Africa, the islands of the Pacific, in Mexico, Central and South America and the West Indies. The plant belongs to the large sub-kingdom of plants known scientifically as angiosperms, or Angiospermae, family Rubiaceae, Genus *coffea* and species *C. arabica* which means that the plant reproduces by seeds (Indu, 2004).

Coffea arabica is a shrub with evergreen leaves, and reaches a height of 14 to 20feet when fully grown. The shrub produces dimorphic branches, i.e., branches of two forms, known as uprights and laterals. When young, the plants have a main stem, the upright, from which side shoots or the primary laterals develop. The laterals may send out other laterals, known as secondary laterals, but no lateral can ever produce an upright. The laterals are produced in pairs and are opposite, the pairs being borne in whorls around the stem. The laterals are produced only while the joint of the upright, to which they are attached, is young; and if they are broken off at that point, the upright has no power to reproduce them. The upright can produce new uprights also; but if an upright is cut off, the laterals at that position tend to thicken up. This is very desirable, as the laterals

produce the flowers, which seldom appear on the uprights. This fact is utilized in pruning the coffee tree, the uprights being cut back, the laterals then becoming more productive. Farmers generally keep their trees at a height of about six feet (http://www.web-books.com/Classics/ON/B0/B701/20MB701.html).

2.2 Coffee production

Coffee is an important product in the world economy. It is one of the most traded commodities, second in value only to oil and a huge contributor of foreign exchange to developing countries. Its cultivation, processing, trading, transportation and marketing provide employment for millions in coffee growing countries (Leroy *et al.,* 2000). The coffee market is dominated by a few large producing countries. As of 2006, the top three producers in terms of export volume were Brazil 30%, Vietnam 15% and Colombia 12% (ICO, 2008). Brazil is the largest arabica coffee producer in the world, while Vietnam, a relatively new entrant is now the world's largest robusta coffee producer (ICO, 2008). Kenya produces arabica coffee (categorized among "Columbian mild's"- high quality coffees) and accounts for around 1% of the global coffee production, and 2% of the value of global exports. Germany accounts for about 35% of total exports out of Kenya, followed by Sweden, UK, USA, Netherlands, and Belgium among others (Kegode, 2005).

World coffee consumption has been increasing at a steady growth rate of 1.6% over the 1993-2003 periods, with total consumption at 6.8 million metric tons in 2003 (World Resource Institute, 2005). Countries such as the United States, Brazil, Russia, Poland and Ethiopia are among the largest coffee consuming countries in the world (World Resource Institute, 2005; Mintel, 2007). Demand for coffee in Kenya is low, Kenya has a tea rather than a coffee drinking culture thus consumption is low in comparison to other coffee producing countries; while Ethiopians consume 50% of the coffee they produce, Kenyans consume only 1% (World Resource Institute, 2005). Industry players are nurturing the development of a coffee drinking culture by opening retail shops

throughout the country (Condliffe *et al.,* 2008). Coffee was the leading export crop from independence to 1988. Between 1975 and 1986, it contributed over 40% of the total Kenyan exports value. After the 1978/88 production peak, the international prices fell sharply in 1989 and by 1992 coffee contributed less than 9% of the total export value. Coffee earned about Kshs. 107 billion which is about 10% of the Agricultural share of GDP between 1987-88 and 1997-98 (Kegode, 2005). In 2003/04 coffee ranked fourth among the country's top foreign exchange earners after tourism, tea and horticulture and accounted for 15% of the exports a decade ago. Coffee production has declined over the past decade leaving over 600,000 households on the brink of poverty. The crop remains a key economic venture for millions of households and is therefore fundamental to wealth creation strategies (Condliffe *et al.,* 2008). Currently, Kenya coffee prices are on the rise following a rise in the global prices of arabica coffee and specialty markets, which Kenyan coffee happens to fall (Economic review of agriculture, 2010).

2.3 Coffee in Kenya

Coffee seedlings were introduced to Kenya by French and Scottish missionaries, and grown in Nairobi at the St. Austin mission in Kikuyu. The Scottish missionaries cultivated arabica coffee at the missions in Bura and Kibwezi in 1893. The success of these seedlings led to cultivation of coffee to other areas such as Kiambu, Ruiru and Thika and continued spreading to other regions of the country where ecological conditions favor cultivation of the crop (Jeremy *et al.,* 2005).

In 1932, the colonial government enacted the coffee industry ordinance and established the Coffee Board of Kenya (CBK) in January of 1933. The role of CBK was regulatory (policy enactment, licensing, and inspectorate) in addition to promoting the consumption of Kenyan coffee both locally and internationally. In 1944, the government acquired the

jacaranda estate at Ruiru and converted it into a research station under the control of the coffee planters. This became the origin of the current Coffee Research Foundation (CRF) (Kegode, 2005).

2.3.1 Ecological requirements of Arabica Coffee in Kenya

Kenya's coffee is grown at an altitude of 1000 to 2000m (4,500-6,800ft). The optimal temperature range lies between 15 and 20°C (maximum day temperatures should not exceed 30ºC and night temperatures should not fall below 15°C). Low or wide daily temperature variation may result in distortion, yellowing and cracking of leaves and tips, a condition known as "Hot and Cold" or crinkle leaf. It should be grown in areas which receive rainfall of not less than 1000mm per year for East of Rift Valley and 1145mm for West of Rift Valley. The soils should be deep, rich, volcanic soils with free draining of up to a depth of at least 1.5m and 3m in drier areas, fertile, slightly acidic (pH range 4.4-5.4) and preferably on level or gently sloped sites (Mwangi, 1983; Jeremy *et al.,* 2005; Coffee Research Foundation- Coffee Growers Hand book, 2010).
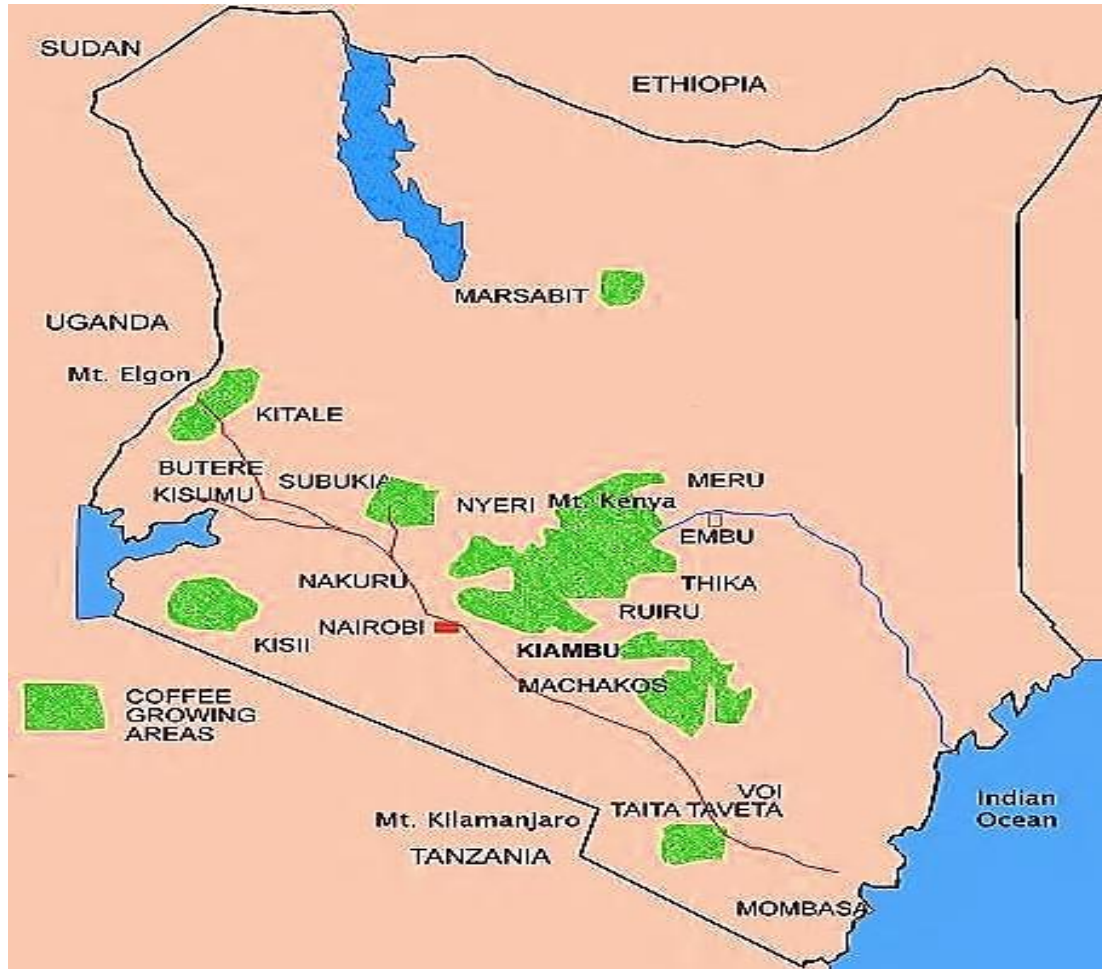
Figure 2.1: Coffee growing regions in Kenya

(Source; Anonymous, 2011)

2.3.2   Commercial coffee varieties in Kenya

Ninety percent of Kenya's coffee comes from the Scot laboratory (SL) variety series root stock, which are the main progenitors of yield and quality characters in breeding programs. The main commercially grown varieties in Kenya include; SL28, It was selected at NARL (National Agricultural Research Laboratories-formerly Scot laboratory) - Kabete, as a single selection from a drought resistant tree from Tanzania. It has a bold copper leaf-tip and green to bronze shoot tips (Omondi, 2007).

SL34 came from a single selection of French mission coffee trees at Loresho estate in Kabete. It has a bronze shoot tip with few green tip strains. The SLs' produce high yields of high quality coffee in a range of climates and altitudes. Coffee variety K7 it has narrow, copper tip leafs was selected from one of two French mission trees on Legetet estate-Muhoroni. It has good bean liquor and quality. Ruiru 11 (F_1 hybrid) with broad and bronze to dark green tip leaf, was released in 1985; the prefix "Ruiru" designates the location where it was bred. Batian (Tall variety) was released in 2010 and compares closely to SL28 variety. It has a bronze leaf tip and berry size larger than SL28. Ruiru 11 and Batian have quality attributes similar to the traditional varieties. They are resistance to coffee leaf rust and coffee berry disease in addition to early ripening and high yields in a range of agro-ecological zones (Omondi, 2007; Gichimu & Omondi, 2009).

Other varieties used in breeding programs are Blue Mountain, French Mission, Bourbon, Rume Sudan, Catimor, Pretoria and Hibrido De Timor as sources of genes of resistance to Coffee Berry Disease. Catimor and K7 are additionally used as sources of resistance to leaf rust (Opile & Agwanda, 1994; Jeremy *et al*., 2005; Coffee Research Foundation-Coffee Growers Handbook, 2010).

2.4 Existing methods of coffee propagation

2.4.1   Seed propagation

Seed propagation is currently the main mode of propagation. The viability of seeds is short lived and it is advisable to plant the seeds within two months of harvesting unless properly stored. Older seeds take longer to germinate and could lose viability. The seeds

are germinated in propagators and transferred to the field after 6-12 months (Coffee Research Foundation-Coffee Growers Handbook, 2010).

2.4.2 Clonal propagation/ Cuttings

Vegetative propagation is a method of producing planting materials using plant vegetative parts instead of seeds. The planting materials are genetically identical to the mother plant. In Kenya, vegetative propagation in coffee is done predominantly on the disease resistant cultivar Ruiru 11 since propagation by seed is a challenge. Stages in vegetative propagation include establishment of clonal garden where selected mother plants are established in the field, construction of a propagator, rooting of cuttings. This materials can be used for grafting and top-working (Coffee Research Foundation-Coffee Growers Handbook, 2010).

Grafting is the technique by which tissue from one plant are inserted into those of another so that the two sets of vascular tissue may join together. It is also simply defined as the successful healing of the union between the scion and rootstock. Materials required for any kind of grafting include scions and rootstocks. In coffee, the cleft type of grafting is used, a V-shaped cleft is made on the rootstock and the point-ended scion is inserted and tied with polythene to allow the graft union to heal (Coffee Research Foundation-Coffee Growers Handbook, 2010).

Top working is converting already established mature trees of traditional Arabica varieties into elite varieties such as Ruiru 11 and Batian through grafting. This method is cheaper and much faster compared to up-rooting and replanting (Coffee Research Foundation-Coffee Growers Handbook, 2010).

2.4.3 Tissue culture

Plant tissue culture technique is used as a term for protoplast, cell, tissue and organ culture. It's based on a phenomenon of plants called "totipotency" where any living plant cell can potentially give rise to a whole plant. Theoretically, it is possible to

regenerate any plant *In vitro*. Tissue culture is used in procedures for manipulation in artificial media setup with the intention of identifying, preserving and propagating as well as modifying plants to improve their use for mankind. The success of regeneration in a laboratory requires previous knowledge of several factors such as culture media, type and concentration of hormones as well as knowledge of the regenerative aptitude of the various primary explants. The technique involves separation of cells, tissues or organs of a plant part called "explant" and growing them aseptically on defined liquid or solid nutrient medium under controlled conditions of temperature and light. The process is divided into stages namely; initiation of explants, subculture of explants for proliferation, shooting, rooting, and hardening (Ahloowalia *et al.*, 2002; Harisha, 2007 ; Jean, 2009).

In Kenya *In vitro* propagation of F1 hybrid Ruiru 11 has been done using nodal culture (Kahia, 1993) and indirect somatic embryogenesis of F1 hybrid Ruiru 11 (Kahia, 1999). Approaches such as somatic embryogenesis, embryo rescue, anther culture, somaclonal variation, protoplast culture, *In vitro* germplasm conservation and marker assisted selection along with genetic transformation have immense potential for the genetic improvement of coffee in Kenya. Micropropagation or plant tissue culture can achieve short-term and long-term objectives of mass propagation of seedlings, genetic improvement as well as *In vitro* preservation of germplasm. Plant tissue culture involves growing plant cells, tissues or organs isolated from the mother plant (explant), on artificial media *In vitro*. Explants can be of various kinds, for instance, meristem cultures, shoot tip or shoot cultures and node cultures. The choice of explant depends on the kind of culture to be initiated, the purpose and the plant species to be used. Regeneration of plant cultures *In vitro* relies on addition of plant growth regulators to the medium. Tissue culture medium is composed of a completed mixture of nutrients and growth regulators. Growth regulators can be altered according to the variety of plant, or at different stages of culture (George *et al.,* 2008).

2.4.3.1 Types of culture techniques

12

2.4.3.2 Embryo rescue

Embryo rescue assists breeders to rescue weak, immature or hybrid embryos. This could arise from crossing distant parents resulting in crop hybrids which could produce seeds that might have been aborted. Embryo abortion occurs during embryogeny and it is possible to culture this embryo and recover the hybrid plants (Carneiro, 1999).

2.4.3.3 Anther culture

Anther culture is a technique in which pollen containing anthers are excised and cultured on nutrient medium. This could spur the formation of embryoids and subsequently haploid plantlets. The ploidy level can be doubled to obtain doubled haploid plants as well as completely homozygous diploid plants. Doubled haploid plants assist breeders in genetic studies such as mapping quantitative trait loci, bulk segregant analysis, back-cross breeding and also cultivar development (Germana, 2010; Ahloowalia *et al.*, 2002; Carneiro, 1999).

2.4.3.4 Protoplast culture

Protoplast culture involves the culture of cells that have their cell walls entirely removed. Coupled with protoplast fusion, isolation as well as regeneration of protoplasts presents the basis for somatic hybridization, genetic analysis and genetic manipulation in addition to strain improvement (Yasuda *et al.,* 1994; Carneiro, 1999).

2.4.4   Somatic Embryogenesis

The initiation and development of embryos from somatic tissues without the involvement of sexual fusion is known as somatic embryogenesis (Mishra & Slater, 2012). In coffee, two methods of somatic embryogenesis have been studied, this include direct somatic embryogenesis (DSE) where embryos originate directly from the explants and indirect somatic embryogenesis (ISE) where embryos are derived through an

intervening callus phase (Kahia, 1999; Hatanaka *et al*., 1991; Carneiro and Ribeiro, 1989; Yasuda *et al*., 1985).

ISE is a two-step procedure which involves callus formation and differentiation procedure thus similar to high frequency somatic embryogenesis (HFSE).The time taken to regenerate coffee plantlets using DSE is normally shorter (nine months) than the time taken for ISE (12-13 months) (Ducos *et al.,* 2010; Etienne *et al.,* 2010). On the other hand, the later one is more prolific and yields many embryos per explants.

In coffee, induction of somatic embryogenesis and plant regeneration was first reported from the inter-nodal explants of *C. canephora* (Staristky, 1970). In *C. arabica*, calluses were successfully induced from seeds, leaves and anthers of two different cultivars, that is, Mundo Novo and Bourbon Amarelo. During the last 35 years, a number of protocols for somatic embryogenesis have been developed for various genotypes of coffee (Carneiro, 1999; Etienne *et al.,* 2006).

Various attempts have been made to reduce the time needed for embryogenesis and increase the embryogenesis frequency in coffee. These include use of Murashige and Skoog medium supplemented with growth regulators such as Benzyl amino purine (BAP), Triacontanol, silver nitrate (AgNO3), salicylic acid, thidiazuron (TDZ), and 6-(3- methyl-3-butenylamino) purine (2ip) and polyamines to induce/promote coffee somatic embryogenesis (Mishra & slater, 2012).

The utilization of cytokinins, at low levels, in tissue culture, for the induction of direct somatic embryogenesis has been reported in several plant species such as, geranium, alfalfa and water melon. Cytokinins are associated with several processes that promote somatic embryogenesis, this include association between cytokinin induced responses and endogenous plant growth regulators especially auxins. Cytokinins also promote the synthesis and accumulation of purines and are involved in cytokinin metabolism (Giridhar *et al.*, 2004). These processes have been reported to promote DSE.

Differences in gene expression at both RNA and protein levels have been observed between the embryogenic and non-embryogenic cell clusters. It was observed that the number of genes turned off in somatic cells to allow for the change from somatic to embryogenic state is higher than those genes that are turned on (Mishra & slater, 2012).

Other investigations include use of *In vitro* leaves for production of somatic embryos. Histological studies have shown that in leaf tissue of *In vitro* plants, the palisade parenchyma is composed of weakly comprised cells of different size and shape when compared to leaf tissue from greenhouse seedlings. Also, leaves from *In vitro* plants have less epicuticular wax. Therefore, this anatomic character could favor a better uptake of media components and growth regulators thus could improve the response of the leaf tissue. In addition, *in vitro* plants of coffee have higher contents of potassium, nitrogen and phosphorus, elements that play an important role in somatic embryogenesis induction (Mishra & slater, 2012).

The use of somatic embryos on an industrial scale, by inducing somatic embryos of *C. arabica* in liquid medium using bioreactors has been reported by various workers. The yield of embryos achieved was about 46,000 embryos/3L Erlenmeyer flask (after 7 weeks of culture) (Etienne *et al.,* 2006). However, to date the major obstacle associated with production of somatic embryos on a commercial scale is synchronization of embryogenesis and conversion to plantlets (Etienne *et al.,* 2006; Mishra & slater, 2012).

2.5 Somaclonal variation

Somaclonal variation or tissue culture induced variation describes occurrence of genetic variants derived from *in vitro* procedures. Such variations arise as manifestation of epigenetic influence or change in the genome of differentiating vegetative cells, chromosome alterations such as breakages and translocations (Siamak *et al*., 2010). From the available literature it is difficult to conclude the exact cause of somaclonal variation however several authors have reported factors such as plant genotype, age of donor plant, length of culture period, repeated subcultures and use of some growth

regulators (for instance, use of high levels of kinetin in medium induced variation in carrots), as possible causes (Ibrahim, 1969; Jain, 2007; Mohamed, 2007). In addition, several authors have reported the use of somaclonal variation as a method to shorten coffee breeding programs, since it provides access to new mutant forms in high yielding genotypes within a short period of 4-8 years compared to conventional breeding's 20-25 years (Larkin *et al.,* 1981; Söndahl, 1992).

2.6 Molecular markers

Molecular markers have allowed scientists to identify markers dispersed throughout the genome of any species of importance and use markers to identify genes of interest. This has improved breeding accuracy in addition to speeding up the release of new varieties. Molecular markers will advance characterization of coffee germplasm, development of markers linked to stress tolerance, identification and isolation of leaf rust resistance genes from resistant cultivars as well as isolating caffeine degradation genes for manipulation of caffeine content in beans which are potential research areas of coffee biotechnology in Kenya. Previous methods of identification of somaclonal variation only detected phenotypic variations based on visible morphological changes such as deformation or modified leaf shape and undifferentiated tissue formation. However, some genetic shifts might not manifest themselves phenotypically. The development of molecular markers for identification of genetic variance or DNA polymorphisms has advanced identification of phylogenetic origins of plants by distinguishing species to sub-species levels thus simplifying detection of tissue culture induced variations (Agarwal *et al*., 2002).

2.7 Genetic transformation

Genetic transformation will allow scientists to transform genes from unrelated species to coffee, for instance, engineering insect resistance using *Bt*-genes (*Bacillus thuringiensis*). Transformation were useful for protecting coffee plants from pests and diseases, optimizing plant growth in stressful ecological niches, improving yield in addition to development of specialty coffees such as caffeine free varieties (Jean, 2009; Harisha, 2007).

In the future, it is conceivable that coffee plantations may comprise new varieties generated from biotechnological approaches such as genetic engineering, protoplast fusion, embryo rescue, somaclonal variation or haploid culture. These new varieties can be multiplied via *In vitro* micropropagation systems, identified and selected by means of molecular markers. Thus in the 21st century, coffee in Kenya may be produced using biotechnology in combination with traditional agricultural techniques. (Harisha, 2007; Ahloowalia *et al.*, 2002; Carneiro & Ribeiro, 1989)

2.8 Coffee production constraints

Pest and disease control is the most important constraint to sustainable economic production of *C. arabica* in Kenya. The most important are coffee leaf rust (CLR) (*Hemileia vastatrix*) also known as orange leaf rust. Orange patches appear on lower surface of the leaf and cause low photosynthetic capacity, defoliation, reduced vegetative and berry growth thus affecting the crops yield. Coffee berry disease (CBD) (*Colletotricum kahawae*) is also known as green berry anthracnose. Infected berries show dark sunken pits that spread to cover the whole berry, infecting the bean thus affecting quality (Ian, 2004). Bacterial Blight of Coffee (BBC) *(Pseudomonas syringae Pv. garcae)* also referred to as Elgon/Solae dieback, attacks healthy parts of a plant leading to death of leaves, twigs, and young nodes resulting in loss of entire crop (Waller *et al.,* 2007).

Important coffee insect pests attack berry, foliar and roots. The leaf miner, giant loope and Antestia bug attack foliar and cause yield losses in the long run. The berry borer attacks berries leading to loss of quality of the beans. Root knot nematodes, especially *Meloidogyne incognita* and the root mealy bug attack roots of the crop causing failure of beans to ripen thus loss of quality. These pests and diseases can be controlled by chemical application, introduction of resistant varieties or biological and integrated pest management (IPM) strategies (Jean, 2009).

Other constraints include the vulnerability of *C. arabica* to environmental oscillations. The plant poorly adapts to abiotic stresses such as frost, poor soils and drought. These cause imbalances in the plants physiological sink leading to attack by pests and diseases. In addition, constant coffee price fluctuations in the world markets results in periodic booms and crashes for farmers. Any advances in research should cushion market volatility by improving diversification of coffee product and by- products as well as reduction of labor and production costs through new, efficient coffee management strategies. These advances should be tailored to embrace sustainability and protect the environment (Ian, 2004; Waller *et al.,* 2007; Jean, 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study was carried out at the Plant Tissue Culture laboratory of the Coffee Research Institute situated at Ruiru, (altitude 1620 m above sea level 1º06'S; 36º45'E), Kenya.

Experimental design and data analysis

Completely randomized design was used for this experiment. Each single explant was considered as an experimental unit. The experiment and each treatment were repeated three times.

Culture conditions

For induction of somatic embryo, cultures were incubated at continuous dark conditions (25±1°C). For embryo germination and rooting, the cultures were transferred to a growth room (25±1°C) and incubated at 16 hour photoperiods under cool white fluorescent light at 1000lux.

Data analysis

The differences between the means were compared using Tukey's stundentized tests. The results are expressed as a mean ±SE. Analysis of variance was performed using SAS statistical software version 9.1 and means were separated by LSD. Percent (%) cultures was calculated as (total number of explants with embryos/ total number of cultured explants*100)

3.2 Preparation of Murashige and Skoog stocks

A stock solution is a solution that will be diluted to a lower concentration for actual use. The use of stock solutions reduces the number of repetitive operations involved in media preparations. Also, concentrated solutions of some materials are more stable thus can be stored for longer periods compared to dilute solutions. The stock solutions were categorized as stock A, B, C, D, E, F and G (Appendix 1). Culture bottles (300ml and 400ml) were used as glassware for somatic embryo induction, maturation and rooting. The culture bottles were closed using screw type plastic caps.

Macro nutrients

The required amounts of compound were weighted and topped-up with distilled water. In this experiment, the stocks were prepared at 10X the concentration of the final medium. These stocks were stored in the refrigerator at 4°C.

Micro-nutrients

The required amounts of compound were weighted and topped-up with distilled water. In this experiment, the stocks were prepared at 100X the concentration of the final medium. These stocks were stored in the refrigerator at 4°C. Iron stocks were prepared

and stored separately from other micronutrients in an amber storage bottle to prevent photodecomposition.

3.2.1 Preparation and preservation of plant growth regulator stock solutions

Plant growth regulators were weighted and stocks (100X) prepared using appropriate solvents. The stocks were clearly labeled and stored in the refrigerator at $8^{o}C$ to avoid deterioration by light and temperature. The following methods were used in the preparation of various plant growth regulators for this study.

a) Benzyl amino purine (BAP)

To 56.32mg of BAP a few drops of 1N potassium hydroxide (KOH) were added and 5-10ml of double distilled water was also added. The solution was well shaken and made up to the final volume of 250ml and stored.

b) Thidiazuron (TDZ)

To 55.05mg of TDZ, a few drops of 1N KOH were added and 5-10ml of double distilled water was also added. The solution was well shaken and made up to the final volume of 250ml and stored.

c) Naphthalene Acetic Acid (NAA)

To 46.55mg of NAA 2-5ml of absolute ethanol was used and a few drops of warm double distilled water till the solution become milky white. Then again, a few drops of absolute ethanol were added and gently heated until the whitish color faded away. The procedure was repeated until the solution did not turn milky on addition of warm double distilled water. At this stage, the volume was made up to 250ml and stored.

d) Indole butyric acid (IBA)

To 50.81mg of IBA, a few drops of 1N KOH were added and 5-10ml of double distilled water was also added. The solution was well shaken and made up to the final volume of 250ml and stored.

3.2.2 Murashige and Skoog media preparation

The MS media were used for all the experiments. Media were prepared by dissolving the organic and inorganic components as stock solutions in distilled water. The solutions were stirred until they dissolved and made up to final volume (Appendix 2). The pH was adjusted to 5.8 using 1 N NaOH or 0.1 M HCL before agar was added and media heated to dissolve the agar and dispensed in 10 ml aliquots into culture bottles. The media was autoclaved at 1.06 kg cm^{-2} and 121^{0}C for 15 min. The culture vessels were capped with lids then placed in trays and autoclaved. All media was autoclaved within 12 hours of preparation and freshly autoclaved media was used. However, when it was not possible to use the media immediately it was stored in a cold room at 4°C for no longer than two weeks before use. Inoculation was carried out in a sterile laminar airflow hood.

3.2.3   Sterilization of lamina flow hood

Before any culture processes the lamina flow hood was switched on and swabbed down with 70% ethanol using cotton wool or sterile towel and kept running for about 15 minutes before the work in the lamina flow hood began. All the plant materials were dissected on a sterile piece of paper. The lamina flow was frequently swabbed down with 70% alcohol and sterile pieces of paper were used between each transfer in order to ensure that sterile surfaces were used for each culture. Hands were sprayed with 70% ethanol at suitable intervals while working for protracted periods. Personal hygienic precautions were observed by wearing a clean lab coat and latex hand gloves while carrying out experiments in the lamina flow. All tools were placed in an aluminum foil and sterilized in an autoclave for 15 minutes at 121ºC. During their use in the lamina flow hood, tools were dipped in 70% ethanol followed by heat sterilization in steribead® sterilizer maintained at 250°C.

3.3 Determining a surface sterilization technique for selected coffee varieties

3.3.1   Selection, collection and sterilization of explants

To optimize an efficient *in vitro* propagation protocol for surface sterilization of selected *C. arabica* varieties. Third pair of leaves were excised using a scapel from mother plants of selected *C. arabica* commercial varieties (SL28, Ruiru 11, and Batian) grown a greenhouse at Ruiru and placed in a beaker containing tap water and taken to the

laboratory for cleaning. They were washed under running tap water with detergent teepol® and rinsed five times. The explants were transferred to the lamina flow lamina flow hood, immersed in 70% (v/v) ethanol for 30 seconds and rinsed twice with sterile distilled water. This was followed by surface sterilization using different JIK® (3.85v/v sodium hypochlorite) concentrations and exposure time interval as follows: 20%, 25%, 26%, 27%, 28%, 29% and 30% each for 25minutes. They were rinsed 3-4 times in sterile distilled water before inoculation on hormone free Murashige and Skoog, 1962 medium.

3.3.2 Inoculation of explants on Murashige and Skoog media

The sterilized explants were trimmed suitably to remove sterilizing agent affected parts. This involved aseptically removing the entire mid rib of the leaf and the leaf tips. The resulting leaf strips were cut into small squares of about 1 square centimeter. These were inoculated in MS media (Appendix 2) and maintained in culture rooms at continuous dark conditions. Contamination data were collected after 5 days. Each explant was scored as 'contaminated' or 'not contaminated' using the eyeball determination method and an average was obtained for the explants in each treatment.

3.4 Evaluation of different cytokinin concentrations on different regeneration stages

3.4.1 Preparation of Medias supplemented with different cytokinin concentrations

720 explants were cultured on half strength MS media supplemented with selected hormones was used for initiation of somatic embryos. Cytokinins were evaluated separately, Benzyl amino purine (BAP) at 4.4, 8.8, 13.3, 17.7, 22.0 µM and Thidiazuron (TDZ) at 4.5, 9.0, 13.6, 18.2, 22.7 µM. 450 explants were cultured full strength MS media supplemented with varied concentrations of BAP at 2.2, 4.4, 8.8, 13.3, 17.7µMwas used for somatic embryo maturation/germination.

3.5 Evaluation of different auxin concentrations on root induction

3.5.1 Preparation of Medias with different auxin concentrations

Half strength MS media (appendix 2) was supplemented with auxin Indole butyric acid (IBA) at 4.9, 9.8, 19.7µM and Naphthalene acetic acid (NAA) at 2.5, 5.3, 10.7 µM and 100 inositol and with 2 % sucrose was used for rooting. The pH was adjusted to 5.8 using 1N NaOH or 0.1 M HCL before agar was added and media heated to dissolve the agar and dispensed in 25 ml aliquots into culture bottles. The media was autoclaved at 1.06 kg cm^{-2} and 121^{0}C for 15 min. Subculture was carried out in a sterile laminar airflow hood.

3.5.2 Culture of mature somatic embryos

The mature somatic embryos were excised from hormone free development media and transferred to half strength MS media containing auxins for root induction. Cultures were transferred to a growth room (25±1°C) and incubated at 16 hour photoperiods under cool white fluorescent light at 1000lux.

3.6 Acclimatization of rooted plants and transfer to soil in pots

The regenerated plantlets were isolated from the culture vessels and the roots gently cleaned with running tap water to remove agar. The plantlets were then taken to the green house where they were treated with 2% fungicide (Redomil for 20 minutes). They were then placed in weaning pots containing soil, sand and manure mixture (3:2:1); sawdust (Acacia) soil and manure mixture (3:2:1); sawdust (*Grevillea robusta*), soil and manure mixture (3:2:1); Coffee husks, soil and manure mixture (3:2:1). The cover of the weaning pot had two holes which were kept closed for the first two weeks and eventually fully opened after one month. Each single explant was considered as an experimental unit.

RESULTS

4.1 Surface sterilization of explants

The use of low concentration sterilant (15%) resulted in up to 90% contamination of the explants (Table 4.1). The percentages 29 and 30% bleach for 25 minutes gave the higher percentage of clean plants. However these high percentages led to scotching of the leaves. Subsequently, 28% for 25 minutes was found to be (Table 4.1).

Table 4.1: Surface sterilization of explants

Treatment	Bleach concentration (v/v)	Time of exposure (minutes)	Percentage of clean plants
Single sterilization	15	25	8 ± 3.12^e
	20	25	13.16 ± 3.32^e
	25	25	50.02 ± 3.06^d
	26	25	58.56 ± 2.11^{cd}
	27	25	67.78 ± 4.17^{bc}
	28	25	79.55 ± 1.15^{ab}
	29	25	85.45 ± 4.73^a
	30	25	93.45 ± 3.93^a

Values represent % means± SE. Means within a column followed by different letters are significantly different at $P = 0.05$.LSD=8.9630.

4.2 Regeneration of Batian Variety

The media supplemented with 13.3μM BAP gave the highest % Embryogenic cultures of 58.33 as well as the highest number 6.06±1.18 of embryos per explant. Increasing the concentration of BAP to 22.0μM resulted in no induction of embryos (4.2).

Table 4.2: Effect of BAP on induction of somatic embryos in Batian variety

Concentration (μM)	% Embryogenic cultures	Mean No. of embryos per explant.
4.4	16.67	1.93±0.86[b]
8.8	14.58	1.75±0.79[b]
13.3	58.33	6.06±1.18[a]
17.7	25.00	1.11±0.37[b]
22.0	0.00	0.0±0.0[b]
P value		<.0001

Values represent means± SE. Means within a column followed by different letters are significantly different at $P = 0.05$. LSD=3.9518.

Medium supplemented with 9.0µM TDZ gave the highest % Embryogenic cultures of 33.33 as well as the highest mean 2.06±0.63 of embryos per explant. No embryos were formed when the concentration was increased to 22.7µM (Table 4.3).

Table 4.3: Effect of TDZ on induction of somatic embryos in Batian variety

Concentration (µM)	% Embryogenic cultures	Mean No. of embryos per explant.
4.5	0.00	0.0 ± 0.0^b
9.0	33.33	2.06 ± 0.63^a
13.6	18.75	1.00 ± 0.45^{ab}
18.2	16.67	0.82 ± 0.44^b
22.7	0.00	0.0 ± 0.0^b
P value		<.0001

Values represent means± SE. Means within a column followed by different letters are significantly different at $P = 0.05$. LSD=4.84945.

The 1 square centimeter leaf discs were cultured on embryo induction media (Plate 4.1A). Somatic embryogenesis started with development of embryo structures on the edge of the leaf explants after 10 days of culture. Embryo maturation appeared not to be well synchronized as different developmental stages were observed at the same time (Plate 4.1B).

Plate 4.1: Induction of somatic embryos of Batian variety. A. 1 square cm leaf discs. B. Different stages of embryo maturation observed at the same time (120 days after culture).

Development of somatic embryos was influenced by the level of BAP used. The results did not reveal significant differences among the concentrations evaluated. However, hormone free media (control) was the best with an average length of 0.27mm (Figure 4.1).
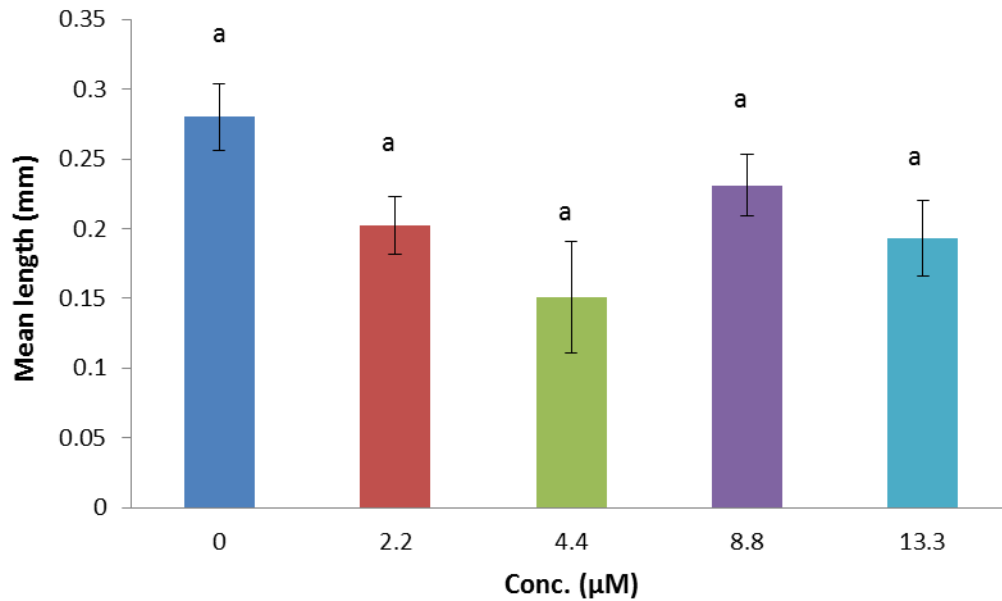
Figure 4.1: Effect of BAP on average shoots length of Batian

Means with the same letter are not significantly different at p<0.05

On germination media, the somatic embryos increased in size and synthesized photosynthetic pigments (Plate 4.2A). Somatic embryos were considered mature as soon as the shoot (epicotyl) formed and elongated with presence of a radicle structure which is a precursor to root induction (Plate 4.2B). Asynchrony was evident, subsequently, after data collection the cultures were maintained for additional two weeks to attain uniform maturation and increase number of responding embryos prior to root induction.
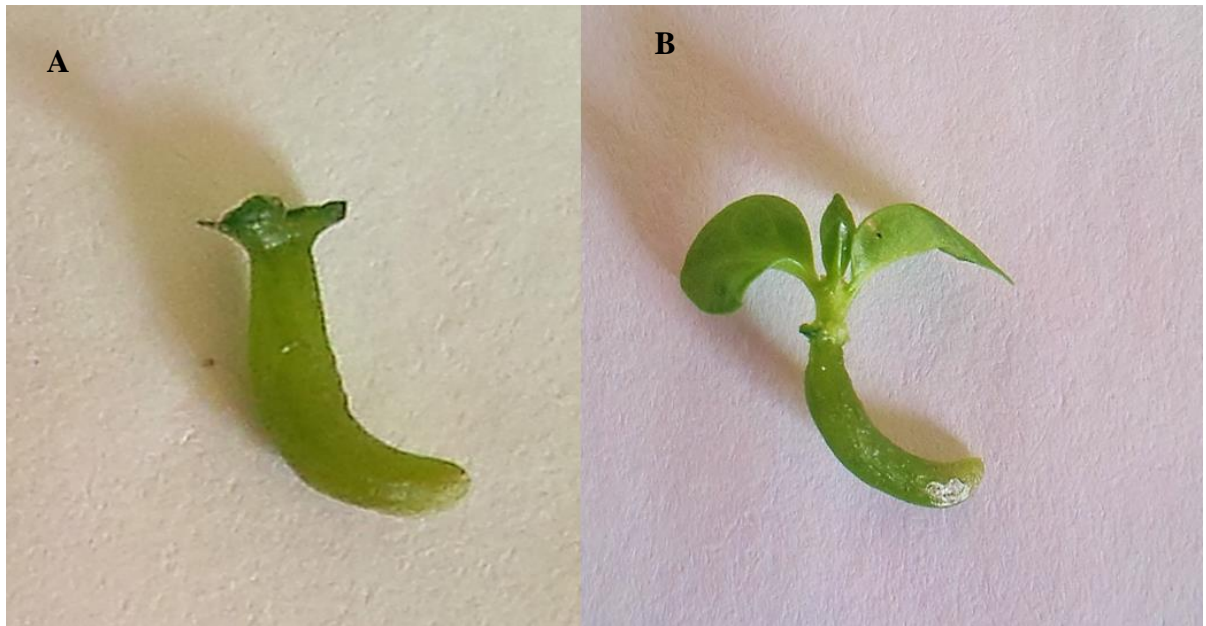
Plate 4.2: Somatic embryos germinating. A. Maturing somatic embryo with photosynthetic pigments. B. Matured somatic embryo ready for induction of rooting system.

Medium supplemented with NAA 2.5µM gave the higher mean length 1.02±0.14 of roots and highest percentage root induction of 66.67 than medium supplemented with 10.7µM (Table 4.4). A similar trend was observed on medium supplemented with IBA where an increase in concentration resulted in low percentages of root induction (Table 4.5). Low levels of auxins induced rooting (Plate 4.3). High levels of NAA inhibited rooting in some explants and inhibited root elongation leading to desiccation of the plantlets (Plate 4.3). A similar trend was observed for plantlets cultured on medium supplemented with IBA.

Table 4.4: Effect of different NAA concentrations on rooting of Batian variety

Concentration (µM)	Mean length (mm)	% root induction

2.5	1.02 ± 0.14^{a}	66.67
5.3	0.72 ± 0.18^{ab}	53.84
10.7	0.53 ± 0.11^{b}	50
P value	<.0001	

Values represent means± SE. Means within a column followed by different letters are significantly different at $P = 0.05$. LSD=0.4038.

Table 4.5: Effect of different IBA concentrations on rooting of Batian variety

Concentration (µM)	Mean length (mm)	% root induction
4.9	1.05 ± 0.09^{ab}	52.38
9.8	1.22 ± 0.09^{a}	68.62
19.7	0.85 ± 0.09^{b}	36.11
P value	<.0001	

Values represent means± SE. Means within a column followed by different letters are significantly different at $P = 0.05$. LSD=0.3446.
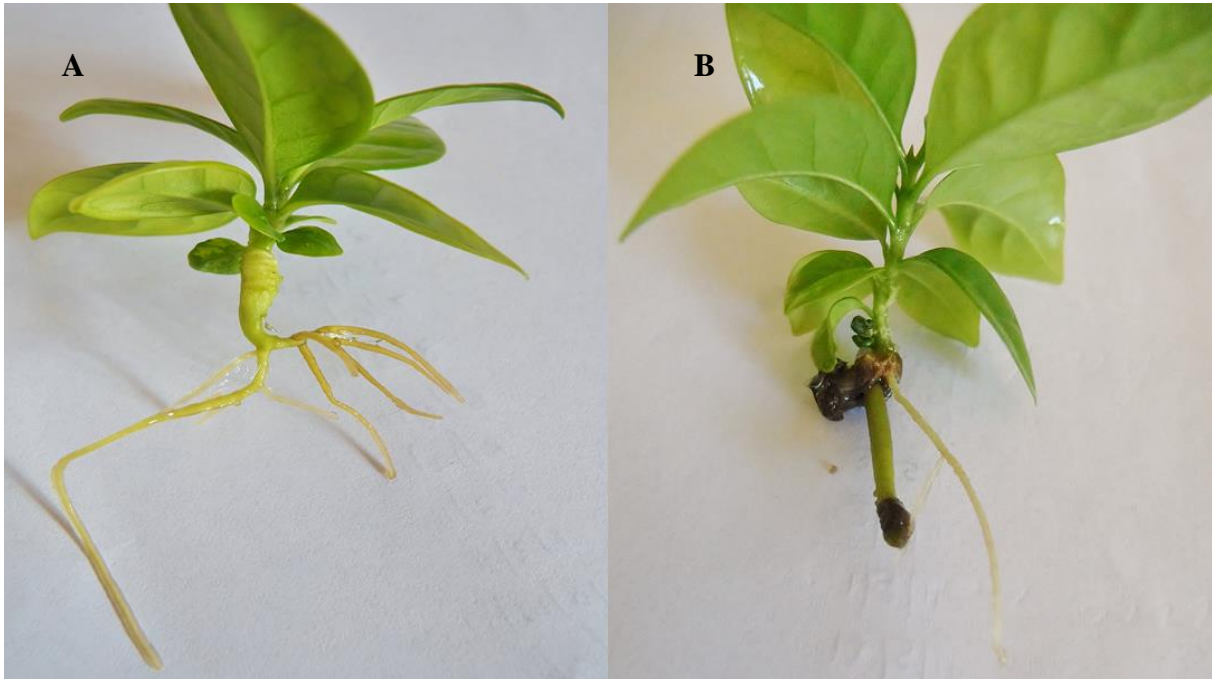
Plate 4.3: Root induction of Batian. A. Rooting of plantlet in NAA 2.5µM. B. Inhibited root elongation in IBA 19.7µM.

4.3 Regeneration of SL28 variety

The media supplemented with BAP 13.3µM gave the highest % embryogenic cultures of 66.67 as well as the highest number 7.43±4.56 of embryos per explant. No embryos were formed when the concentration was increased to 22.0µM (Table 4.6). Medium supplemented with TDZ 4.5µM gave the highest % embryogenic cultures of 29.17 as well as the highest mean 1.93±0.76 of embryos per explant. However, increasing the concentration from 9.0 to 22.7µM resulted in a fluctuation of the number of somatic embryos formed (Table 4.7).

Table 4.6: Effect of BAP on induction of somatic embryos in SL28 variety

Concentration (µM)	% Embryogenic cultures	Mean No. of embryos per explant.
4.4	2.08	0.06 ± 0.62^{b}
8.8	39.58	2.12 ± 0.53^{b}
13.3	66.67	7.43 ± 1.14^{a}
17.7	22.92	1.29 ± 0.45^{b}
22.0	0.00	0.0 ± 0.0^{b}
P value		<.0001

Values represent means± SE. Means within a column followed by different letters are significantly different at $P = 0.05$. LSD=3.9581.

Table 4.7: Effect of TDZ on induction of somatic embryos in SL28 variety

Concentration (µM)	% Embryogenic cultures	Mean No. of embryos per explant.
4.5	29.17	1.93 ± 0.76^a
9.0	18.75	1.18 ± 0.59^a
13.6	0.00	0.0 ± 0.0^a
18.2	10.42	0.52 ± 0.36^a
22.7	22.92	1.56 ± 0.55^a
P value		<.0001

Values represent means± SE. Means within a column followed by different letters are significantly different at $P = 0.05$. LSD=4.84945.

Somatic embryogenesis started with development of embryo structures on the edge of the leaf explants after 10 days of culture. Embryo maturation appeared not to be well synchronized as different developmental stages were observed at the same time (Plate 4.4). Germination of SL28 somatic embryos and elongation of the shoots was highly influenced by the level of BAP used with the treatments 2.2 and 8.8µM better than other concentrations. However, hormone free media (control) was the best with an average length of 0.32mm (Figure 4.3.2).

Plate 4.4: Prolific regeneration of somatic embryos of SL28 variety and formation of somatic embryos at different stages.
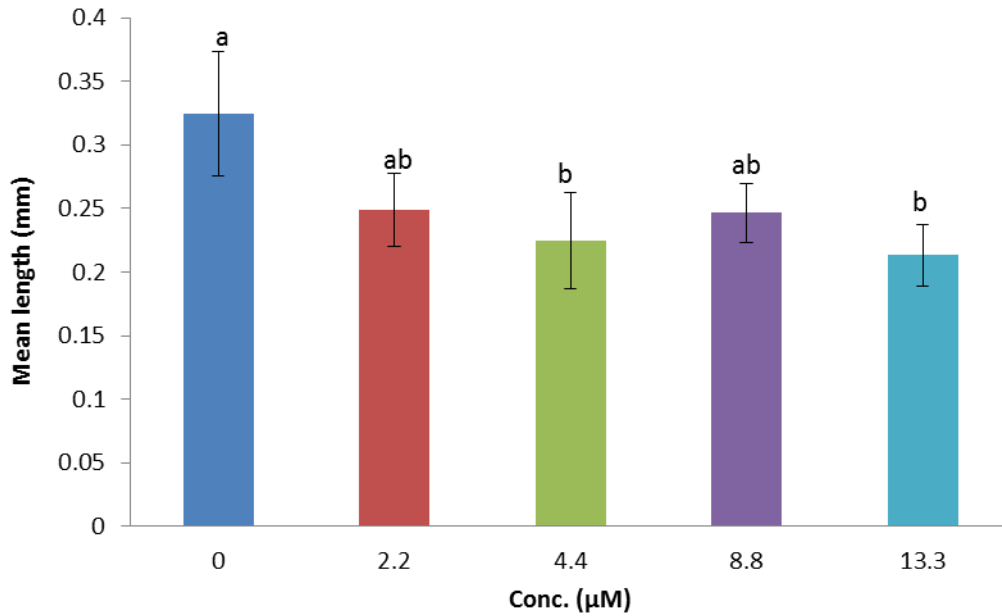
Figure 4.2: Effect of BAP on average shoot length of SL28

Means with the same letter are not significantly different at p<0.05

Germination commenced within four weeks. On germination media, the somatic embryos increased in size and synthesized photosynthetic pigments (Plate 4.5). Somatic embryos were considered mature as soon as the shoot (epicotyl) formed and elongated with presence of a radicle structure which is a precursor to root induction (Plate 4.5). Medium supplemented with NAA 5.3μM gave the higher mean length 1.71±0.17mm of roots and highest percentage root induction of 77.78 (Table 4.8). Increasing the concentration from 5.3 to 10.7μM resulted in inhibition of root elongation (Table 4.8). A similar trend was observed on medium supplemented with IBA where an increase in concentration resulted in low percentages of root induction. On the other hand, 4.9μM IBA gave the highest mean length 0.95±0.10 and the highest rooting induction percentage of 74.60 (Table 4.9).

The formation of callus at the base of the plantlet prior to formation of the root system can be caused by using high levels of auxins in culture media (Plate 4.6). This causes a

weak connection of the developed roots to the plantlet thus these plantlets have low chances of success.

Plate 4.5: Germination of somatic embryos; matured somatic embryo ready for induction of rooting system.

Table 4.8: Effects of different NAA concentrations on rooting of SL28 variety

Concentration (µM)	Mean length (mm)	% root induction
2.5	1.49 ± 0.13^b	67.90
5.3	1.71 ± 0.17^a	77.78
10.7	1.12 ± 0.22^{ab}	58.02
P value	<.0001	

Values represent means± SE. Means within a column followed by different letters are significantly different at $P = 0.05$. LSD=0.6489.

Table 4.9: Effects of different IBA concentrations on rooting of SL28 variety

Concentration (µM)	Mean length (mm)	% root induction
4.9	0.95 ± 0.10^a	74.60
9.8	0.37 ± 0.11^b	31.75
19.7	0.28 ± 0.05^b	25.40
P value	<.0001	

Values represent means± SE. Means within a column followed by different letters are significantly different at $P = 0.05$. LSD=0.3376.
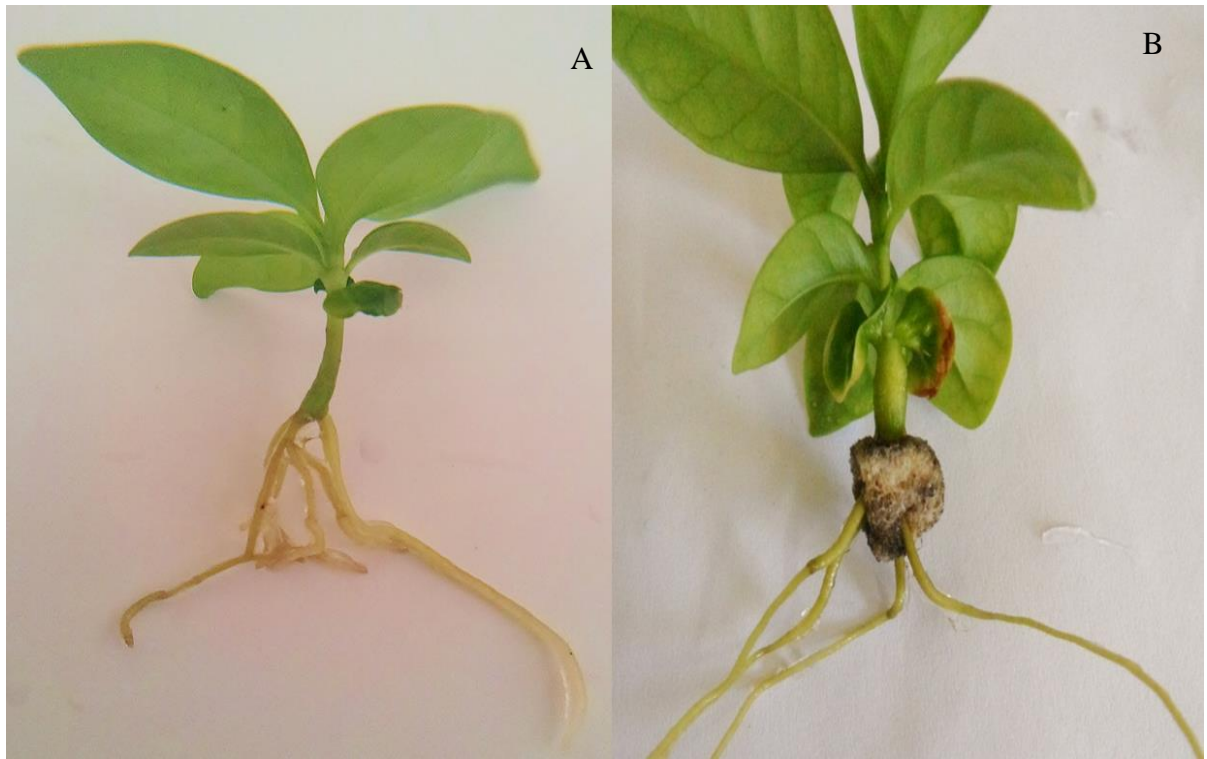
Plate 4.6: Root induction of SL28. A. Rooted plantlet 8 weeks after subculture. B. Formation of callus at the base prior to root induction in IBA 9.8μM 8 weeks after culture.

4.4 Regeneration of Ruiru 11 variety

The media supplemented with 13.3µM BAP gave the highest percentage of cultures forming embryos of 62.50 as well as the highest number (3.25±0.47mm) of embryos per explant. Increasing the concentration of BAP from 13.3 to 22.0µM and above resulted in a significant reduction of the number of embryos formed (Table 4.10). Somatic embryogenesis started with development of embryo structures on the edge of the leaf explants after 10 days of culture. Embryo maturation appeared not to be well synchronized as different developmental stages were observed at the same time (Plate 4.7). Germination commenced within four weeks. The results did not indicate a significant difference among the levels of BAP evaluated for germination of somatic embryos (Figure 4.3). On germination media, the somatic embryos increased in size and synthesized photosynthetic pigments (Plate 4.8). Somatic embryos were considered mature as soon as the shoot (epicotyl) formed and elongated with presence of a radicle structure which is a precursor to root induction.

Table 4.10: Effect of BAP on induction of somatic embryos in Ruiru 11 variety

Concentration (µM)	Percentage of cultures forming embryos	Mean No. of embryos per explant.
4.4	10.42	0.50 ± 0.25^a
8.8	14.58	0.75 ± 0.30^a
13.3	62.50	3.25 ± 0.47^a
17.7	0.0	0.0 ± 0.0^a
22.0	0.0	0.0 ± 0.0^a
P value		<.0001

Values represent means± SE. Means within a column followed by different letters are significantly different at $P = 0.05$. LSD=3.9518.

Plate 4.7: Induction of somatic embryos of Ruiru 11 variety.
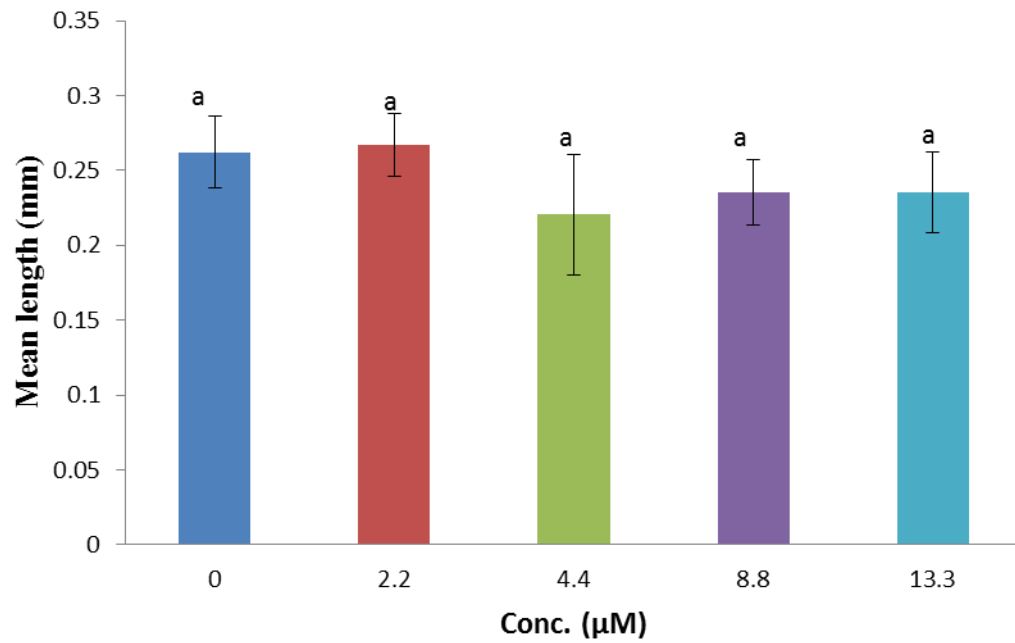
Figure 4.3: Effects of BAP on average shoot length of Ruiru 11

Means with the same letter are not significantly different at $p < 0.05$

Plate 4.8: Germination of Ruiru 11 somatic embryos. Maturation of embryos took longer than other varieties and formed a wide base.

Medium supplemented with NAA 2.5µM gave the highest mean length 0.91±0.15 of roots and highest percentage root induction of 56.79 (Table 4.11). A trend was observed on medium supplemented with IBA where an increase in concentration resulted in low percentages of root induction. On the other hand, 4.9µM IBA gave the highest mean length 0.78±0.05 and the highest rooting induction percentage of 55.56 (Table 4.12). High levels of NAA inhibited rooting in some explants and inhibited root elongation leading to desiccation of the plantlets (Plate 4.9). A similar trend was observed for plantlets cultured on medium supplemented with IBA.

Table 4.11: Effects of different NAA concentrations on rooting of Ruiru 11 variety

Concentration (μM)	Mean length (mm)	% root induction
2.5	0.91 ± 0.15^a	56.79
5.3	0.90 ± 0.11^a	48.15
10.7	0.88 ± 0.11^a	48.15
P value	<.0001	

Values represent means± SE. Means within a column followed by different letters are significantly different at $P = 0.05$. LSD=0.4647.

Table 4.12: Effects of different IBA concentrations on rooting of Ruiru 11 variety

Concentration (μM)	Mean length (mm)	% root induction
4.9	0.78 ± 0.05^a	55.56
9.8	0.52 ± 0.06^{ab}	40.00
19.7	0.44 ± 0.08^b	31.00
P value	<.0001	

Values represent means± SE. Means within a column followed by different letters are significantly different at $P = 0.05$. LSD=0.2907.
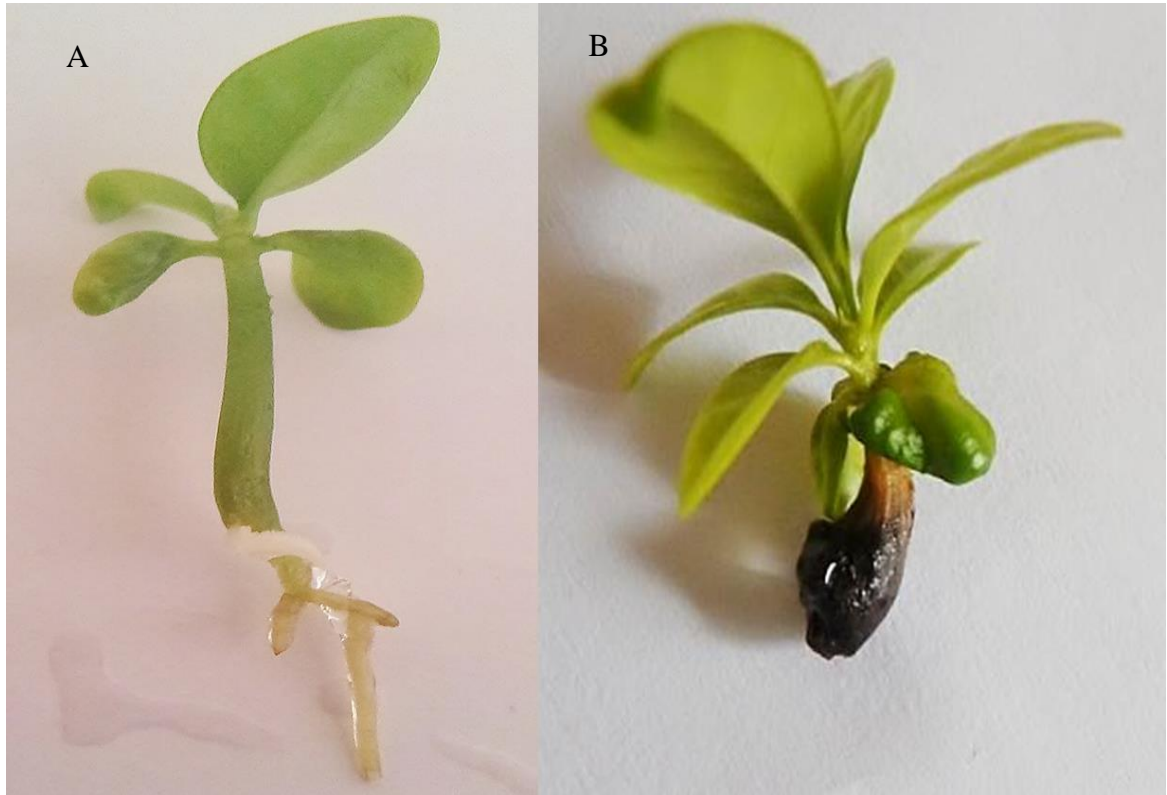
Plate 4.9: Root induction of Ruiru 11 variety. **A**. Rooted plantlet 4 weeks after culture. **B**. Desiccation of Ruiru 11 plantlet due to high levels of auxins.

4.5 Hardening

The effect of hardening mixture on mean of plantlets is shown in the Table 4.13 below. The weaning mixture of soil, sand, and manure (3:2:1) gave the highest rate for Batian, Sl28 and Ruiru 11 with 12.00±0.05, 9.22±0.02 and 8.57±0.07 respectively. Varietal differences were observed within treatments. There was significant difference on the mean number of plantlets between the hardening mixtures. *G. robusta* and manure gave the lowest mean number of plantlets across the evaluated varieties (Table 4.13). This can be attributed to the toxic characteristics (hydrogen cyanide) of *G. robusta.*

Table 4.13: Effect of hardening mixture survival of explants

Variety

Hardening mixture	Batian	SL28	Ruiru 11
Sand	12.00±0.05[a]	9.22±0.02[a]	8.57±0.07[a]
Coffee husks	8.6±0.03[b]	6.14±0.04[b]	7.2±0.043[a]
Acacia	5.56±0.07[c]	5.11±0.047[c]	5.18±0.046[b]
G. robusta	4.11±0.02[c]	4.28±0.01[c]	4.68±0.06[b]

Values represent means± SE. Means within a column followed by different letters are significantly different at $P = 0.05$. LSD=0.9267.

The cover of the weaning pot has two holes which are kept closed for the first two weeks and eventually fully opened after one month (Plate 4.10).

Plate 4.10: Weaning and hardening of plantlets. A. Weaning pots; B&C: the top showing closed holes which are opened after two weeks.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECCOMENDATION

5.0 Development of a sterilization protocol for selected explants

Plant tissue culture is a system of growing plant cells excised from the mother plant (explant) in artificial nutrient media. A critical stage of introducing plants into tissue culture is to obtain cultures free from microbial contamination. In this study, the protocol for sterilization was developed using Jik® (3.85v/v sodium hypochlorite-NaOCl), ethanol and surfactant teepol. Explants sourced from the field present a major challenge in *in vitro* cultures. This is because the surface of the plant carries a wide range of microbial contaminants (Odutayo *et al.,* 2007). Subsequently, only greenhouse grown mother plants were used in this study; however, there could be other sources of contamination. This include systemic contaminants which are not killed by surface sterilization, poor aseptic handling in the laboratory or from laboratory instruments. Even under greenhouse conditions, young leaves maybe less contaminated than mature ones. Therefore, prudent selection of explants from healthy mother plants followed by effective surface sterilization is paramount in avoiding *In vitro* culture contamination. This can be achieved using a comprehensive surface sterilization protocol using various sterilization agents before inoculation on nutrient culture media.

A common sterilization agent that has been used to disinfect many explant types is Jik® bleach. Other agents include ethanol, mercuric chloride, hydrogen peroxide, silver nitrate and bromine water. Several authors have shown that use of locally produced Jik® bleach containing 3.85% hypochlorite produced the highest reduction in bacterial and fungal contamination at time intervals between 20-45 minutes (Odutayo *et al.,* 2007). It has the capability to be an effective sterilization agent for leaves from greenhouse source as observed by Okudera and Ito, 2009 while working on *A. crasna* and *A. sinensis*. Ethanol is extremely phytotoxic hence the explant is exposed to it for only a few seconds. To enhance effectiveness in sterilization procedure, a surfactant like teepol or tween 20 is added to the protocol. In this study NaOCl, ethanol and surfactant teepol were preferred due to their safety, availability and economic merits. Also, these

sterilants have been used in many studies for wide range of explants for *In vitro* culture (Ndakidemi *et al.,* 2013).

In this study, the best results were obtained when explants were immersed in 70% (v/v) ethanol for 30 seconds followed by 28% bleach (JIK[©]) for 25 minutes. The results do not agree with those of Kahia, 1999 who found 25% for 25minutes to be effective in the sterilization of Ruiru 11 leaf explants. However several reasons such as the age of the explant used in the different studies could explain this difference. The results also showed that lower concentrations of NaOCl had poor response and also increased concentration (30% for 25minutes) caused adverse tissue damage on the leaf explants. Perhaps the poor response of lower concentrations could be attributed to the woody nature of coffee. In addition, the adverse tissue damage observed on higher concentrations is due to the fact that sterilants are toxic to the plant tissues (Colgecen *et al.,* 2011).

The results in this study confirm the need to determine the proper concentration of sterilants, duration of exposing the explants and the sequence of using these sterilants so as to minimize injury and maximize survival.

5.1 Effects of various cytokinin and auxin concentrations on regeneration of selected *C. arabica* varieties

In this study, Somatic embryogenesis started with development of an embryo-like structure on the edge of the leaf explants after 10 days of culture. The somatic embryos were formed only at the cut edges. These observations are in agreement with those of Hatanaka *et al.,* 1991 who reported that somatic embryos were formed only at the cut edges which were in contact with the media containing growth regulators. This may suggest that the cut surface (wounding) might be sites for rapid uptake of minerals and hormones which resulted in the high percentage of embryos formed. Embryo maturation appeared not to be well synchronized, as different developmental stages were observed at the same time. This observation is similar to that of Zimmerman (1993) and Zegzouti *et al.,* (2001) who observed different stages of embryogenesis in the same culture

system. Embryo development involves several main steps; the globular, heart, torpedo and cotelydonary stages (Goldberg *et al*., 1994).

BAP at 13.3µM gave the highest mean of embryos per explant and highest percentage of embryogenic cultures for Batian, SL28 and Ruiru 11. These results are in agreement with Gatica *et al.,* 2007 and Yasuda *et al.,* 1985 who found that culture mediums supplemented with BAP stimulated development of somatic embryos. In contrast to the results in this study, where an increase in the concentration resulted in the decrease of formation of somatic embryos, Yasuda *et al.,* 1985 observed that media supplemented with 17.7 to 26.4µM were more efficient in induction of somatic embryos of F1 hybrid *coffea arabica*. However, an influence of genotype may account for this contrast or other precise conditions that may not have been reproduced on the two studies.

Application of TDZ at 9.0µM produced the highest (33%) frequency of embryogenic cultures for Batian variety. Similar results have been reported by Gill and Saxena (1993) who reported 100% induction of somatic on *Nicotiana tabacum* L. leaf explants using TDZ. TDZ is chiefly used as cotton defoliant (Giridhar *et al.,* 2004) but it also acts as a growth regulator in tissue culture systems. In peanut and geranium the use of TDZ has effectively replaced the requirement of auxins and or cytokinins for inducing somatic embryogenesis (Gill and Saxena, 1993). The use of TDZ in formation of somatic embryos was achieved with low concentrations. This is in agreement with Giridhar *et al.,* 2004 who reported that low levels of TDZ stimulated DSE in *coffea*.

The time taken to regenerate coffee plantlets using DSE is normally shorter (nine months) compared to the time taken for ISE (12-13) months (Ducos *et al.,* 2010; Etienne *et al.,* 2010). It is a two-step procedure thus similar to high frequency somatic embryogenesis (HFSE).On the other hand, the later one is more prolific and yields many embryos per explants. Due to the long period the cultures stay in a PGR supplemented media, the chances of somaclonal variation in ISE are much higher (Ducos *et al.,* 2010). The protocol developed in this study ensures production of true to type plantlets.

Development commenced within four weeks and asynchrony was evident, subsequently, after data collection the cultures were maintained for additional two weeks to attain uniform maturation and increase number of responding embryos prior to root induction. The use of full strength hormone free MS medium gave higher conversion ratio compared to medium supplemented with different levels of BAP. Although germination was achieved on all media evaluated, hormone free media with a mean length of 0.32±0.03mm proved best for development of somatic embryos for SL28 variety.

The results did not reveal significant differences among the BAP concentrations evaluated in this study. On development media, the somatic embryos increased in size and synthesized photosynthetic pigments. These pigments facilitate photosynthetic activity, extra storage reserves like lipids, triglycerides, proteins and other hydrates which favor *in vitro* germination (Nasim *et al*., 2010). However, for Ruiru 11, the embryos took longer to develop and formed a wider base compared to a distinct radicle structure observed in Batian and SL28.

The successful utilization of somatic embryogenesis in tissue culture is hinged on the germination/ development of somatic embryos, formation of a well-developed root system which is a precursor to successful acclimatization and final establishment in the field. Subsequently, the establishment of an efficient rooting protocol cannot be over-emphasized. *In vitro* rooting of coffee plantlets has been achieved using medium supplemented with auxins either singly or in combination with cytokinins. In this study the effect of auxins, evaluated singly, on rooting was observed.

Auxins stimulate root initiation by activating quiescent pericycle cells to initiate division and then expansion which facilitate lateral root emergence (Fukaki and Tasaka, 2009). Root induction frequency and average root length at 2.5μM NAA was higher than other concentrations when compared to IBA at 4.9μM similar concentrations with a mean length of 1.71±0.09mm for SL28 variety. Low levels of NAA induced a well-developed root system. High levels of NAA inhibited rooting in some explants and inhibited root

elongation leading to desiccation of the plantlets. A similar trend was observed for plantlets cultured on medium supplemented with IBA.

Taiz and Zegler (2003) reported that auxins are required for root induction however root growth is inhibited at higher auxin concentration. Kollmeier et al., (2000) stated that high auxin concentration inhibits root elongation. Riov and Yang, (1989) reported that auxins intensify the rate of ethylene biosynthesis. Subsequently, it is conceivable that high concentration of NAA and IBA induced ethylene biosynthesis which is inhibitory to root elongation. Although rooting was induced at levels of 4.9µM, there was formation of callus at the base of the plantlet prior to formation of the root system. These plantlets had a poor rate of survival at the weaning stage thus not desirable.

On the other hand, NAA proved to be better than IBA in induction of rooting system across evaluated varieties except Batian. These results contrast findings by Kahia, (1999) who observed that IBA was better than IAA and NAA in rooting of Ruiru 11 microshoots. In general, results of this current study indicate that low levels of auxins are optimum for successful induction and elongation of well-developed root system in *C. arabica* variety Batian. These results are in agreement with findings reported by Nguyen *et al.,* (1999) on *In vitro* rooting of coffee.

Santana *et al.,* (2004) claims that one of the main factors influencing somatic embryogenesis in coffee is genotype. In this current study, all varieties exhibited a general tendency to form somatic embryos at 13.3µM BAP in addition a higher level of BAP significantly reduced the frequency of induction of somatic embryos. Despite these similarities of response along different varieties, there was a striking difference in regeneration of maximum number of embryos across varieties. SL28 was more competent in the regeneration of somatic embryos followed by Batian and F1 hybrid Ruiru 11. According to Roy and Banerjee (2003), such differences can be attributed not only to the genotype or plant growth regulators concentration levels, but also on interaction of plant growth regulators with endogenous regulators. Growth *in vitro* is

highly dependent on the interaction between naturally occurring endogenous regulators and the exogenous plant growth regulators added to the medium (Goebel, 1993).

Grieb *et al.,* (1997) claim that the endogenous regulators are of great importance since they regulate the process of explant differentiation *in vitro*. Bhaskaran and Smith, (1990) postulated that endogenous hormones could be the main difference between genotypes with regards to competence of regenerating somatic embryos. It is conceivable that a similar explanation holds for the difference in regeneration of maximum somatic embryos for the evaluated varieties in this current study. Another possible hypothesis is the status of the mother plant, such as age, developmental and physiological status which has been postulated to affect competence of explants in regeneration of somatic embryos.

Deo *et al.,* (2010) claims that the type and age of explants has an impact on somatic embryogenesis and highlights the observation that young, dividing and possibly less differentiated cells are more likely to be stimulated towards the embryogenic pathway than older cells. The author further states that even though a variety of explants can be utilized, the correct developmental stage of the explants is also crucial for the initiation of embryogenesis. This coupled with the physiological status of the leaf (Staristky, 1970) contributed to the incompetence for regeneration of somatic embryos in Ruiru 11 explants used in this study.

5.2 Conclusions

The present study has developed a surface sterilization protocol for *C. arabica* leaf explants in *In vitro* culture. Significant difference between the concentration levels of

NaOCl was noticed. The study revealed that immersing the leaf explants in ethanol for 30 seconds and rinsing with sterile distilled water before immersing in 70% ethanol for 30 seconds. This is followed by immersing the explants in 28% NaOCl 3.85v/v for 25 minutes to produce the best results.

The best level for induction of somatic embryogenesis was found to be 13.3 μM BAP giving the highest mean number of somatic embryos across all selected varieties. Hormone free media proved to promote germination and development of somatic embryos. Low levels of auxins promoted induction of a well-developed root system for all the selected varieties. In the present study, the selected varieties responded differently to the varied treatments used.

In general, the protocol involves culturing the leaf discs on half strength MS media supplemented with BAP 13.3 μM. The somatic embryos developed on full strength MS media without hormones. The plantlets were rooted on half strength MS media supplemented with 9.8μM IBA.

The development of an efficient *in vitro* protocol for production of somatic embryos will not only increase rates of clean planting material but can also be exploited to generate uniform clonal material for use in experimental work such as genetic transformation. Further, it allows for the development and the rapid mass multiplication of improved cultivars to meet the increasing consumer demand.

The findings of this research work open an opportunity area for maximizing *in vitro* plantlet production in coffee.

5.3 Recommendations

In general, asynchrony was observed at all stages of regeneration. Subsequently, further work needs to be done to address this challenge. In addition, asynchrony especially at the somatic embryo induction and development stage can be used as a marker to identify stable embryogenic lines which will not only be used for enhanced mass propagation but also in regeneration of transgenic lines.

Hormone free media proved to promote germination and development of somatic embryos. However further work needs to be done to induce shooting and rooting simultaneously, this could further reduce the time taken for coffee somatic embryogenesis.

There is need to validate this protocol with other coffee genotypes which can lead to production of somatic embryos. Further work needs to be done to investigate the effects of different mineral salt media formulations as well as effects of genotype and endogenous hormones on coffee somatic embryogenesis

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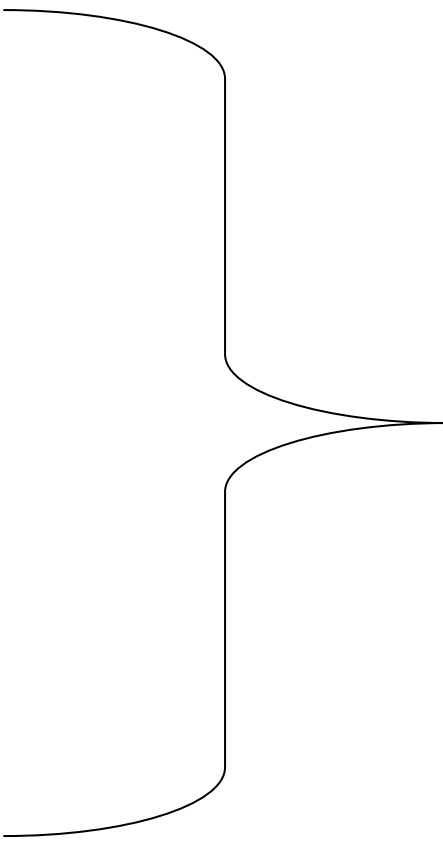
APPENDICES

Appendix 1: Murashige and Skoog stock formulations

STOCK	CONSTITUENTS	Conc. (g/l)
Stock A	NH_4NO_3	82.5
Stock B	KNO_3	95.0
Stock C	H_3BO	1.24
	KH_2PO_4	34
	KI	0.166
	Na_2MO_4	0.05
	$COcl_2.6H_2O$	0.005
Stock D	$CaCL_2.2H_2O$	88.0
Stock E	$MgSO_4.7H_2O$	74
	$MnSO_4.4H_2O$	4.46
	$ZnSO_4.7H_2O$	1.72
	$CuSO_4.5H_2O$	0.005
Stock F	Na_2EDTA	7.45
	$FeSO_4.7H_2O$	5.57
Stock G	Glycine	0.4
	Nicotinic acid	0.1
	Pyridoxine HCL	0.1
	Thiamine HCL	0.2

The constituents are dissolved in distilled water and topped-up to 1000ml and stored in a refrigerator at 4°C for two weeks.

Appendix 2: How to prepare 1000ml of media

Stock A	20ml
Stock B	20ml
Stock C	10ml
Stock D	10ml
Stock E	10ml
Stock F	10ml
Stock G	20ml
Sucrose	20g
Myo-inositol	100mg
l-cysteine	100mg
pH	5.7
Gelrite	3g

Top-up to 1000ml