OPTIMISING SOMATIC EMBRYOS FORMATION IN *COFFEA ARABICA* CULTIVAR RUIRU 11 USING TEMPORARY IMMERSION SYSTEMS IN KENYA

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Optimising Somatic Embryos Formation in *Coffea arabica* Cultivar Ruiru 11 Using Temporary Immersion Systems In Kenya

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DECLARATION

This thesis is my original work and it has not been presented for award of a degree in any other University.

Signature ……. Date: ……

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

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DEDICATION

I dedicate this manuscript and all the work leading up to its completion to my family. To dad and mum, your support, financially, emotionally and spiritually have been my rock through this challenging venture. To my brothers, Charles and Kevin, your unrelenting prayers and reassuring words encouraged me to continue when I felt like giving up.

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LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	Benzyl amino purine
CBD	Coffee Berry Disease
CLR	Coffee Leaf Rust
CRI	Coffee Research Institute
DSE	Direct Somatic Embryogenesis
GDP	Gross Domestic Product
HFSE	High-frequency somatic embryogenesis
IBA	Indole-butyric acid
ICA	International Coffee Agreement
ISE	Indirect Somatic Embryogenesis
KIN	Kinetin
PGR	Plant Growth Regulator
RITA®	Recipient for Automated Temporary Immersion.
TIS	Temporary Immersion Systems
VP	Vegetative Propagation

ABSTRACT

Coffea arabica, F1 hybrid variety, Ruiru 11 is a highly sought-after crop in Kenya due to its alleviated resistance to Coffee Berry Disease and Coffee Leaf Rust coupled with high yield capacity and good cup quality. Access to the variety's planting materials is limited due to challenges with difficulty in propagation using conventional methods of seed and vegetative propagation; and somatic embryogenesis is regarded as a suitable alternative propagation method. Temporary Immersion Systems are automated systems where cultures are submerged in liquid nutrient media using negative pressure for a specified immersion time then released. Temporary Immersion Systems are ideal for embryo induction and embryo maturation for *C. arabica* hybrids. Therefore, the current study aimed to establish an induction protocol in F1 composite hybrid Ruiru 11. The current study investigated the effects of genotype and plant growth regulators, auxins and cytokinins, on induction of embryogenic callus and embryo proliferation and maturation in two composite genotypes of *C. arabica* L. F1 hybrid variety Ruiru 11 -Code 71 and Code 93. Leaf explants from the F1 hybrid were cultured on half-strength Murashige and Skoog (MS) media supplemented with varied concentrations and combinations of plant growth regulators. Callus formation was evaluated weekly until the 60th day. Genotypic effects were assessed based on the difference on callus induction rates, callus proliferation and embryo induction and proliferation. The genotypes tested showed highest callus induction rates at 88% (Code 71) and 100% (Code 93) with respect to the formation of embryogenic calli. Highest fresh weight was obtained at 0.973 ± 0.011 g in Code 71 and 0.649 ± 0.03 g in Code 93 in MS media supplemented with 2,4-D + BAP (0.53 + 0.11 µM). Highest number of embryo induced was obtained from inoculum callus density 0.005 g/cm^3 which generated 94.222 (Code 93) and 76.333 (Code 71) after two months in Temporary Immersion Systems (1-L RITA®) immersed for 3 minutes every 6 hours. As for callus proliferation in solid media (conventional method), Code 71 induced highest embryos at 15.11 in 84 days and in Code 93 at 56.333 after 91 days in 2,4-D at 0.33 µM and 0.44 µM respectively. Optimization achieved in Temporary Immersion Systems compared to conventional process based on time efficiency, embryo yield and resource use. TIS induced highest embryos at 94.222 after 170 days with elimination of gelrite which reduces cost of embryo production against conventional method induced highest embryos (56.333) after 162 days using solid media supplemented with gelrite. The study opens new prospects for Coffee Research Institute to adopt Temporary immersion systems for improved propagation of Ruiru 11 *Coffee arabica* hybrids for mass production to cater for increasing demand for coffee seedlings.

CHAPTER ONE

INTRODUCTION

1.1 Background of Study

The agricultural sector is a major contributor to Kenya's economic growth accounting for 25.4% (indirectly) and 27% (directly) of the country's Gross Domestic Product (ICO, 2019; Kenya Coffee Platform, 2018). The sector concurrently employs 80% of the rural workforce and is responsible for 65% of the total export earnings (ICO, 2019). The coffee sub-sector is among the contributors to the growth of Kenya's GDP through foreign exchange, income generation, employment opportunities, and food security. The sub-sector accounts for 5.5% of the country's total exports, with a share of 0.22% of the GDP equivalent to Kshs. Twenty-three billion per annum (ICO, 2019). The production capacity has consequently decreased from 120,000 metric tons in the 1980s to 40 000 metric tons in 2018 (Monroy *et al.*,; 2013, ICO 2019). The decline in coffee production results from decreased competitive global prices, poor management of the sub-sector and inadequate value addition policies. In addition, poor production practices such as inadequate control of coffee diseases in the country results in lower yields (Monroy *et al.,* 2013). Despite the decrease in production, coffee remains a major cash crop in the country, employing about 800,000 small-holder farmers (ICO, 2019).

To revamp the coffee subs-sector, the National Government put in place a coffee revitalisation program under the National Task Force on the coffee sub-sector. The program seeks to revive the sub-sector through the Vision 2030 platform's support under the agricultural pillar. Under the program, the emphasis has been put on increasing acreage of coffee alongside improved management strategies for small-scale farmers. Primarily, the approach targets the increase in production by adopting elite coffee hybrid varieties that are disease resistant and high yielding crop varieties Ruiru 11 (Agwanda *et al*., 1997). The sector is yet to meet the objectives' threshold, with production recorded between 40,000 and 50,000 metric tons since 2013 (ICO, 2019). Part of the slow growth in coffee hectarage, productivity, and overall production is due to inadequate planting materials. The underlying factor is that

the production of planting materials of the superior varieties is inadequate to meet the growing demand for the elite coffee varieties.

1.1.1 Propagation of Coffee Cultivar in Kenya

For a long time, access to planting materials has largely depended on conventional propagation techniques that rely on hand pollination and vegetative propagation (VP) to produce seed and seedlings, respectively. With production primarily undertaken at the Coffee Research Institute (Ruiru, Kenya), the Institute is unable to meet the current demand due to several reasons. The seed and seedling production methods used are inept. They are characterized by limitations such as dependency on weather factors for growth, risks of spreading pests and diseases through VP, labor intensiveness, and high cost of production. Conventional methods are complex; thus, prove insufficient on return on investment. The adoption of improved technology through tissue culture has the potential to enhance production capacity through direct somatic embryogenesis. The method provides the best avenue for the propagation of Ruiru 11, which has otherwise proved difficult to propagate via traditional methods due to the risk of loss of genetic fidelity. The ability to meet the demand for seedlings of the hybrid is still insufficient. The Institute is only able to propagate a maximum of 0.1 million seedlings per annum against a demand of one million. The proposed adoption of automated technology, Bioreactor Systems, the Temporary Immersion Systems, with high throughput, presents a viable option to improve the elite variety's production capacity.

1.1.2 Bioreactor Technology

Bioreactor, an automated system, is a powerful technology that improves production capacities in coffee tissue culture laboratories. A bioreactor provides a biological culture environment that sustains the growth and development of tissues and cells. Various bioreactor technologies exist based on the objective of use, desired products, and mode of operation. These include liquid-phase bioreactors, gas-phase bioreactors, temporary immersion systems and hybrid bioreactors. The limitations to the bioreactors is due to presence of hyperhydricity, mechanical agitation, malformations and loss of material as a result of asphyxia consistent with low oxygen levels and excess water in culture media (Georgiev *et al*., 2014). The system

has been shown to significantly improve coffee plants production in tissue culture using 1-L RITA® (Watt, 2012; Krishnan, 2012; Georgiev *et al.,* 2014; Etienne *et al*., 2018). The improved production is associated with increased nutrient intake via liquid media and reduced soma-clonal variation rates (Etienne *et al*., 2018).

In coffee tissue culture, Temporary Immersion Systems (TIS) with specificity to 1-LRITA® have gained prominence in relation to somatic embryogenesis (Ahloowalia *et al*., 2004; Watt, 2012). In *C. arabica*, for example, Sondhal (1992) reported production of 45,000 embryos using 5-L stirred bioreactors. Etienne and Berthouly (2002) on the other hand produced 15,000-50,000 in 1-L RITA® and de Feria *et al*., (2003) produced 70,000 in a 2-L bioreactor, while Ducos *et al*., (2007) reported on increased embryo production ranging 200,000-400,000 in Robusta in mechanically agitated bioreactors.

Success in utilizing Temporary Immersion Systems is determined by a number of factors including species of plant, culture parameters, and genotype of the plant. Scientists at the Coffee Research Institute (CRI), Kenya, adopted the technology with urgency with the need to meet the increasing demand for coffee planting materials. However, the realization of TIS's potential proved difficult given that the available protocols of solid-based media direct somatic embryogenesis were not fully adaptable to TIS techniques when integrating the elite coffee hybrids. To fully realize the potential of the TIS, optimization of TIS protocols in somatic embryogenesis specific to F1 Hybrid, Ruiru 11 is required to improve efficiency in adopting the technology in Kenya. The specific factors on culture parameters, namely plant growth regulator types and concentration levels and cell densities, are necessary for optimum embryo production. The current research was designed with the above in mind.

1.2 Statement of the Problem

Since 1950, coffee has maintained a central role in Kenya's economy, despite the volatile international prices and declining production. It is among the leading foreign exchange earner for the Kenyan economy and is ranked third after tea and horticulture in the agricultural sector. The Kenya Vision 2030 recognizes the importance of the coffee sector as part of the broader national initiative of employment creation, food and nutrition security, poverty

reduction, industrial transformation, and foreign exchange earnings. The key to the sector's growth is the increased sustainable production of coffee, which can be achieved by adopting high performing disease-resistant hybrid varieties. In line with this, several interventions by county government units, in addition to promotion and marketing efforts of the Coffee Directorate, is the dissemination of elite planting material which has led to an increase in the production area at 30% in the year 2015/2016 (Kenya Coffee Platform, 2018). The increased impetus to enhance coffee production in the country catalyzed the increased demand for elite hybrid planting material, which has subsequently outstripped the supply. This is largely due to the use of inefficient propagation methods such as manual pollination and vegetative propagation. Overcoming the perennial shortage of planting materials of the improved varieties require the adoption of propagation technologies with high planting material throughput and are both technically and economically efficient. Tissue culture technique offers these traits and is currently being deployed for mass propagation of Ruiru 11 seedlings at CRI. However, the Institute still relies on direct somatic embryogenesis in solid media whose efficiency is limited to 0.1 million seedlings annually against the annual demand of one million seedlings. The modern automated Temporary Immersion Systems is a powerful alternative to the direct embryogenesis system and combines high prolificacy with safety against somaclonal variation. Hence, it is a superior tool in optimizing the techniques of tissue culture for the mass production of Arabica coffee hybrid planting materials. It possesses inherent cost-effectiveness and is amenable to varying seedling production levels with minimum adjustments in labour, laboratory space, and associated logistical factors. However, the advantages associated with TIS have not been realized at CRI due to a number of protocol-based limitations that need to be refined to achieve the desired level of production efficiencies. *C. arabica* is recalcitrant in nature and proves a challenge towards adoption in tissue culture and specifically in the use of Temporary Immersion Systems. Extensive literature in *C. arabica* suggests the use of Temporary Immersion Systems for induction of embryo although through progressive introduction of the technology in somatic embryo formation specific to the coffee species. The current project proposal is informed by this need and aims to improve the efficiency of TIS using the RITA® bioreactors.

1.3 Justification

Traditional tissue culture is characterized by several constraints that limit the successful mass propagation of hybrid coffee varieties. Modern tissue culture methods offer improved alternatives and efficient techniques in the mass propagation of planting material. The automation of micro-propagation is a recent technique that is more precise and efficient. However, the gradual adoption of this technology is due to limited or lack of precise protocols. Hence, the specificity of the Temporary Immersion Systems necessitates optimization of the control parameters and protocol to successfully micro-propagate planting material in mass. The study aimed at determining optimal plant growth regulator levels for the induction of calli from leaf cultures. Combinations of plant growth hormones were used based on differential levels that include benzyl-amino purine (BAP), IBA, Kinetin, and 2,4-Dichlorophenoxyacetic acid. Standard referencing involved the use of only one plant growth regulator. Studies in *Coffea arabica* have relied on the use of one plant growth regulator but, different response rates in callus induction and embryo induction and proliferation have been observed. Assessment on Ruiru 11 was necessary as it is a hybrid and its response would be expected to be unique. Analysis of embryo induction rates in 1-L RITA® was assessed to determine potential use of the TIS for coffee production.

1.4 Objectives

1.4.1 General Objective

The main objective of the study was to determine the optimization of somatic embryo formation in *C. arabica* Ruiru 11 using temporary immersion systems in Kenya.

1.4.2 Specific Objectives

The three objectives are:

1. To investigate the effects of different plant growth regulator combinations concentrations on induction of callus in *C. arabica* hybrid Ruiru 11 in solid Murashige and Skoog Media.

2. To determine the effects of plant growth regulators combination concentrations on callus proliferation in *C. arabica* hybrids Ruiru 11.

3. To determine the effect of inoculum callus densities in somatic embryo induction Ruiru 11 coffee hybrids in a Temporary Immersion System, 1-L RITA®.

1.5 Null Hypotheses

1. The effect of plant growth regulator concentrations has no effect on callus induction in Ruiru 11 F1 hybrid.

2. Plant growth regulators have no effect on callus proliferation in Ruiru 11.

3. Optimal callus densities have no effect on embryo induction of Ruiru 11 F1 hybrids in Temporary immersion systems.

1.6 Limitation

The study only considered two Ruiru 11 F1 hybrid *cv*. Code 71 and Code 93. The results may limit the adoption of the Temporary Immersion System for all codes of Ruiru 11.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Coffee is among the most important agriculturally traded commodities and substantially contributes to the livelihoods of about 25 million smallholder famers in more than 60 countries globally (ICO, 2015). The smallholder farmers are facing considerable difficulties due to the unstable and unpredictable coffee prices reported to have a direct impact on capacity to produce coffee. The dramatic decline of coffee prices estimates to a 100-year low since the 1970s which has substantially affected more than 100 million individuals who rely on the coffee industry directly (Monroy *et al*., 2013). The coffee price volatility remains a constant threat as a result of shifting paradigms from climate change, structural changes in coffee markets, and difficulties at country-levels to address the plight of the smallholder farmers to mitigate the industry's challenges (Kenya Coffee Platform, 2018). In Africa coffee-producing countries, coffee exports constitute a sizeable share of export earnings and contribute to the sustainable development goals including generation of income, creation of employment, food security, and alleviating poverty (Watt, 2012). The significance of the crop cannot be undervalued and addressing the challenges in the coffee industry is necessary to stabilize the future of coffee and the livelihoods of the smallholder farmers and individuals who depend on the crop.

Efforts to revitalize the coffee industry are ongoing with the objective being to improve coffee supply. The goal is for stakeholders and national governments to address the unique challenges in each coffee producing country. The goal has witnessed an increase in coffee reserve which has had a direct impact on the positive balance of coffee prices. Coffee production is increasing with records indicating that production increased by 2.9% between 2014 and 2019 from 150.848 million bags to 167.193 million bags (ICO, 2019). Campaigns in Brazil, a leading coffee producer accounting for about 50% of world coffee production, have resulted in increased coffee supply perpetuated by increased mitigation efforts to improve income generation, reduce poverty levels, and increase adoption of disease-resistance coffee

varieties (Etienne *et al*., 2018). African coffee-producing countries are also taking up the challenge and Uganda, Côte d'Ivoire and Ethiopia accounted for the largest coffee exports at 77.1% of Africa's production and 8% of global contribution between 2014 and 2019 (ICO, 2019). With the increase in coffee productivity, coffee prices have stabilized presenting a unique opportunity for countries such as Kenya to position itself competitively in improving its coffee production which will significantly improve export earnings and enhance sustainable development goals.

2.2 Economic Performance of Coffee in Kenya

Kenya's coffee sector experiences unique problems that require specific solutions. Coffee production in Kenya has witnessed a dynamic performance between the 1960s to early 2000. Production of the commodity saw tremendous growth between 1963 and 1988 with production increasing at an average rate of 6.6% per annum (ICO, 2015). Production subsequently declined between 1989 to early 2000 by 62% with a decline rate of 5.5 % p.a. (ICO, 2015). The production capacities consequently decreased as well, with coffee exports falling from 1.5 million 60Kg bags 1970 to 800, 000 bags by 2000 (ICO, 2015). Until 1980s, coffee was the leading foreign exchange earner before being overtaken by tea and horticulture (ICO, 2015).

There are several reasons for the declined performance with primary issue linked to increased incidences of coffee berry disease and coffee leaf rust, two main diseases that affect *C. arabica* varieties. Coffee farmers in Kenya still cultivate traditional varieties which are highly susceptible to the two diseases consequently, affecting yield/cost of the varieties. Concurrently, world coffee prices plummeted resulting in reduced income to the Kenyan farmers. Increased temperatures and changing weather patterns affect the growth conditions ideal for coffee (Lubabali *et al.*, 2014). For instance, increasing temperature are suitable for increased incidences of coffee leaf rust whereas decreased temperatures, particularly the night temperatures coupled with increased humidity results in high incidence and severity of CBD in the coffee growing regions. In addition, the unpredictable weather patterns primarily with rainfall trends have been inconsistent leading to reduced capacity for growth and ability to

flower in coffee (Kenya Coffee Platform, 2018). As a result, this affects the fruiting potential of the coffee plants reducing production capacities. Currently, coffee is cultivated on about 170, 000 Ha with more than 700, 000 families and about 3, 200 coffee estates (ICO, 2015).

The importance of coffee to Kenya's economy cannot be emphasized enough with more than 1.4 million Kenyans relying directly or indirectly to sustain their livelihoods. Under the Vision 2030 framework, efforts to revitalize the sector including restructuring of institutions managing the sector, amendment of coffee policies and government intervention regulations and improved coffee management by small-scale farmers (Kenya Coffee Platform, 2018). To boost productivity, the national government plans on expanding coffee farming to non-traditional regions (Rift Valley, Nyanza and Western Kenya) where coffee varieties exist and to train farmers in the traditional coffee areas on improved production practices to facilitate improved productivity and quality of coffee (ICO, 2015). As part of this drive, farmers are encouraged to cultivate new varieties (Ruiru 11) which have resistance to CBD and CLR coupled with high yield potential per tree compared to the traditional varieties with low-yield and susceptibility to coffee diseases (Gichimu *et al*., 2014). Demand for planting materials of the variety have subsequently increased and significantly outstripped the supply. As a result, there is a need to integrate novel models for propagating adequate quantities of the elite planting material

2.3 Ruiru 11 F1 Hybrid

In response to the continuing threat to coffee due to increased incidence of CBD and CLR, the Coffee Research Foundation (currently Coffee Research Institute), initiated a breeding program in 1972. The program's objective was to develop varieties resistant to coffee berry disease and coffee leaf rust. The program was successful leading to the release of Ruiru 11 hybrid cultivar (Opile & Agwanda, 1993). Ruiru 11 is a compact, high yielding variety combining resistance to coffee berry disease and coffee leaf rust. The variety has a compact growth making it amenable to high density planting (2000-3000 tree/ha) compared to the traditional varieties whose planting density is 1300 tree/ha. Ruiru 11 thrives in altitudes higher than 1000m.The cultivar combines resistance to Coffee Berry Disease and Coffee Leaf

Rust with high yields (Opile & Agwanda, 1993; Kathurima *et al*., 2010; Gichimu *et al*., 2014). The variety owes its name, Ruiru from the location it was developed at the Coffee Research Station, Ruiru.

The variety is a complex F1 hybrid derived from the crossing of Catimor progenies (mother) and pollen from multiple crosses (Kathurima *et al*., 2010; Gichimu *et al*., 2014). Introduction of resistance genes to the susceptible varieties included crossing donor varieties followed by back-crossing to SL28 and SL34 to restore desirable traits including yields and quality (Gichimu *et al*., 2014). Traditional varieties SL 34, SL 28 and non-commercial progenitors Rume Sudan and Hibrido de Timor are among the donor plants. The wide-spread adoption of resistant F1 variety Ruiru 11 was encouraged to minimize input costs on fungicides with improved yield for coffee farmers.

2.4 Propagation of Coffee at Coffee Research Institute

Currently, propagation of coffee in Kenya *in vitro* relies on direct somatic embryogenesis. Direct somatic embryogenesis in solid media is regarded as a traditional micro-propagation process. The technique is labor intensive and requires periodic transfer of cultures due to exhaustion of nutrients and the size of the container (Watt, 2012).

The use of direct somatic embryogenesis in Kenya has its roots in the studies in the 1980s (Owuor, 1987). Nodal and leaf explants from field grown *C. arabusta* seedlings were cultured *in vitro* in MS basal media and resulted in embryo development. Since then, studies to improve on propagation capacities of the coffee varieties have been achieved (Kahia *et al*., 1999, Alumiro, 2015; Kahia *et al*., 2016). Leaves from green-house grown coffee varieties Ruiru 11 have been propagated *in vitro* through direct somatic embryogenesis. Alumiro (2015) reported successful propagation of at most 10 embryos per leaf explant. As a result, the laboratory is able to produce at most 100,000 plantlets against a demand of 1M per annum. Despite the progress, the production capacity of the tissue culture laboratory has not been able to meet the growing demand for coffee planting materials due to the increased demand for the variety resulting from the push to boost coffee production in Kenya (Vision, 2007). Strategies to overcome the limitations associated with direct somatic embryogenesis are therefore under

investigations, one of which is the incorporation of automated tissue culture systems through the use of Bioreactors. According to a review by Ahloowalia *et al*., (2004) and Georgiev *et al*., (2014) bioreactors are regarded as powerful tissue culture technology that has the potential to boost production of plants in large-scale within a limited period of time. Additionally, extensive reviews on bioreactors in Coffee production have reported significant improvement in mass production (Ducos *et al*., 2007; Etienne & Berthouly, 2005; Etienne *et al*., 2018; Watt, 2012).

The adoption of liquid-based automated systems are linked to improved uniform culture conditions, reduced handling times for plant cultures, improved sterilization of culture vessels and reduced transfer times (Etienne & Berthouly, 2005). Bioreactor technology is specialized for intensive culture of plant through control of nutritional and physical growth factor for large-scale production (Georgiev *et al*., 2014).

2.5 Tissue Culture

Direct somatic embryogenesis continues to be an ascribed tissue culture method at the Coffee Research Institute, Ruiru, Kenya. Direct somatic embryogenesis involves a short period of 6 months including the induction of embryos, maturation and development of embryos, rooting, and acclimatization (Hapsoro *et al*., 2020; Ibrahim *et al*., 2013). The tissue culture technique primary limitation is low productivity output currently producing 0.1M per annum coffee seedlings against a demand of 1M per annum. Another challenge to the technique is the use of traditional tissue culture vessels that are limited in size and space, and culture media volume capacity which limits it's potential to optimizing increased mass production of coffee seedlings to counter increasing demand (de Almeida, 2020; Maciel *et al*., 2016; Yoas & Johannes, 2021). Indirect somatic embryogenesis requires the induction of callus from donor explants, embryo induction through callus proliferations, embryo maturation and development, rooting, and acclimatization (Ardivani, 2015). The tissue culture technique proves advantageous due to the increase in the quantity of embryos in tissue culture which could be the solution to meeting the growing demand for coffee seedlings (Etienne *et al*., 2018). Adoption of indirect somatic embryogenesis is inevitable however, using traditional

culture vessels is not the ideal tissue culture tool to realize the potential to improved mass propagation. A proposed adoption of Temporary Immersion Systems is necessary although, its adoption requires specific protocol for propagation of *Coffea arabica* F1 hybrids. Temporary Immersion Systems such as RITA® is an automated system that consists of two parts: the upper compartment is the culture chamber containing the plant cultures, and the lower compartment is the liquid-media storage compartment. The TIS uses negative pressure forcing the liquid up to the plant tissue culture under an allocated immersion time (in minutes) and then released back to the lower compartment (Georgiev *et al*., 2014). TIS is a powerful alternative to traditional culture vessels as it offers improved gaseous exchange, reduced hyperhydricity, increased nutrient uptake, low mechanical stress to reduce physiological disorders, and reduced handling of cultures due to concerns on soma-clonal variations (Georgiev *et al*., 2014). TIS require specific protocols for successful propagation dependent on the species and hybrid characterization of the plant. In *Coffea arabica*, indirect somatic embryogenesis requires induction of callus in solid MS media with consequential embryo induction in TIS systems (Ducos *et al*., 2007). The reason is due to the sensitivity in liquid-media adoption by coffee which is prone to production of phenolic compounds which alter the culture environment affecting growth and development of plant tissues (Ardiyani, 2015). Further, the adoption of the automated-system in coffee tissue culture is difficult due to *Coffee arabica* recalcitrance characteristics. Thus, specific protocol is necessary for Ruiru 11 F1 hybrids, Code 71 and Code 93 independently to determine ideal culture parameters of plant growth regulators for callus induction and optimal cell densities for embryo induction.

2.6 Somatic Embryogenesis

2.6.1 Somatic Embryogenesis of Coffee

The first successful production of coffee planting materials *in vitro* was reported by Stratisky (1970) through internodes of *C. canephora* (Ducos *et al*., 2007). Since then, successive studies have reported on mass production of coffee *in vitro* through exploitation of tissue culture techniques through micro-propagation. Micro-propagation is the growth of vegetative parts of a plant such as bud or nodes from donor plants and cultured on sterile media under controlled conditions to produce true-to-type plants. The commonly used micro-propagation

methods in coffee plant regeneration include: organogenesis, somatic embryogenesis, androgenesis and protoplast cultures. Organogenesis involves the *in vitro* culture and maintenance of an excised organ (part of whole) to regenerate an entire plant. Organogenesis is a preferred method for preservation of structure and functions of the plant where growth under *in vitro* conditions retains the physiological features. There are two forms of organogenesis, direct organogenesis and indirect organogenesis. Indirect organogenesis involves an intervening callus stage but, is not a preferred method as it increases possibility of genetic instability in *in vitro* propagation (Muniswamy *et al*., 2015). On the other hand, direct organogenesis is often used since it improves on the stabilizing influence on the meristematic tissues in micro-propagation. In most cases, coffee organogenesis is derived from auxiliary and apical buds, and nodal cultures. Several studies have reported using organogenesis to regenerate superior coffee genotypes. The technique, however, records a low multiplication rate yielding a high cost per unit in production (Muniswamy *et al*., 2015).

The technique is the *in vitro* development of haploid plants from pollen through a series of cell division and differentiation. Androgenesis has received little attention in coffee propagation with efforts directed towards novel techniques including somatic embryogenesis due to the genetic fidelity specifications. Initial attempts to produce haploid plants from anther cultures have been reported by Krishnan *et al.,* (2010) and consequently, studies on generating callus from anthers have been done as a means to understanding the difference in developmental stages in anther cultures.

Protoplast is the culture of isolated protoplasts defined as naked plant cells surrounded by plasma membranes which possess the potential to regenerate into plants. Numerous authors have reported on isolation of protoplasts in coffee leaves, calli, somatic embryos and embryogenic suspensions (Santana-Buzzy *et al*., 2007). Protoplasts cultures offer opportunities in genetic improvement of coffee such as genetic transformations, cell cloning and development of coffee hybrids with superior traits.

Somatic embryogenesis is the growth and multiplication of somatic cells and tissues resulting in the growth and development of somatic embryos. Extensive studies have defined somatic

embryogenesis as a suitable regeneration process for coffee plantlets. Various explants have been tested for regeneration including leaves, nodal explants, stems, and embryos (Muniswamy *et al*., 2015). With respect to coffee, somatic embryogenesis is ideal for rapid multiplication of *C. canephora* genotypes and also used to shorten the breeding cycle of *C. arabica* through micro-propagation of hybrids (Campos *et al.*, 2017). Often, the technique is preferred for propagation of *C. arabica* species particularly where F1 Hybrids are involved to produce true-to-type plantlets (Etienne *et al*., 2018). Reports on comparison between somatic embryogenesis and zygotic embryos elucidate on a similar development path in growth and development of embryos (Etienne *et al.*, 2013).

2.6.2 Direct Somatic Embryogenesis

Somatic embryogenesis can undergo two differential pathways depending on the exogenously supplemented plant growth regulators. Direct somatic embryogenesis refers to the growth and development of somatic embryos from explants under minimal proliferation while, indirect refers to the extensive proliferation of somatic embryos through an intervening callus stage, extensive proliferation, (Etienne *et al*., 2018). Callus is a wound tissue composed of highly vacuolated, unorganized cells; with the ability to differentiate into tissues, organs and even embryos (Jayarama *et al*., 2014). Knowledge on the cell and tissue morphological events in coffee species has made it possible to regenerate coffee in tissue culture on a large-scale level as well as improve on the techniques of regeneration to minimize costs. According to Campos *et al*., (2017), direct somatic embryogenesis is regarded as low frequency whereas; indirect somatic embryogenesis is defined as high frequency. In low frequency, somatic embryos are obtained faster (approximately 70 days) but, in a small number maximum of 10 embryos (Alumiro, 2015; Campos *et al.*, 2017). On the other hand, in higher frequency, the process of obtaining desired products is longer but, mostly preferred for mass propagation. Indirect somatic embryogenesis is a complex process comprising of: (i) induction of callus from callus media, (ii) callus proliferation and growth (iii) callus differentiation into embryo (Campos *et al*., 2017). The process takes longer (usually 6-9 months) but, the number of embryos produced per gram of callus is higher compared to direct somatic embryogenesis. The present study implemented indirect somatic embryogenesis focusing on the influence of plant growth

regulators and optimal callus densities on induction of embryos in Temporary Immersion Systems.

2.6.3 Indirect Somatic Embryogenesis and Plant Growth Regulators

Indirect somatic embryogenesis is considered to be an ideal tissue culture process in the large-scale propagation of somatic embryos of coffee species. The somatic embryo induction and regeneration in coffee is unique unlike that of other plant species. Studies agree that there are several steps required that include primary callus induction, embryogenic callus establishment, somatic embryo development, plant regeneration of somatic embryos and acclimatization (Hapsoro *et al*., 2020; Ibrahim *et al*., 2013). Each stage has to be considered independent in growth and development to improve on cell viability and embryogenic potency downstream. The *in vitro* embryogenesis through indirect induction and regeneration is well documented (Ducos *et al*., 2007, Etienne *et al*., 2013; Ahmed *et al.*, 2013). Plant growth regulators auxins and cytokinins are ideal for induction of calli in coffee species. Auxins 2,4-Dichlorophenocyacetic acid (2,4-D), Naphthaleneacetic acid (NAA) and Indole-Butyric acid (IBA) and cytokinins Benzyladenine (BA), Kinetin, Thidiazuron (TDZ), 2-isopentenyl adenine (2-iP) are commonly used in coffee somatic embryogenesis (Gatica-Arias, Arrieta-Espinoza & Espinoza, 2008; Ardiyani 2015; Bartos *et al*., 2018; Yoas & Johannes, 2021; de Almeida, 2020; Maciel *et al*., 2016). According to Campos *et al.*, (2017) somatic embryogenesis is different in *C. arabica* genotypes with some exhibiting complete recalcitrance whereas, some can induce callus to generate embryos. Yaos and Johannes (2021) reported ideal concentration of 2,4-D + KIN combinations for induction of callus after two months. Hapsoro *et al.*, (2020) reported induction of calli on Robusta coffee after four weeks in 1 BAP and Ardiyani (2015) reported induction of calli in *Coffea Liberica* at 2,4-D 1.80 mg/L after 3 months. The effects of plant growth regulators type and concentration levels have been investigated for years in *Coffea arabica* species. The consensus is that concentration levels and combinations have different effects including induction of polyploidy and soma-clonal variation (Leva *et al*., 2012). Recent studies on effects of plant growth regulators on somatic embryogenesis coffee species explored the effects of auxin and cytokinin combinations and reported improved propagation quantities and qualities (Nic-Can

et al., 2015; Ebrahim *et al*., 2007; Rezende *et al*., 2008; Aga & Khillare, 2017). Limited studies have been done to determine the effects of auxin and cytokinin combinations on induction of calli in leaf *Coffee arabica* explants based on inverse combinations (increased auxin concentrations combined with reduced cytokinin combinations). Investigation at these levels will be ideal to determine whether plant growth regulators and differences in genotypes have significant influence on embryogenesis in *Coffee arabica.*

Further, plant growth regulators and differences in genotypes are reported to have direct and significant influence on the development of embryogenic and non-embryogenic calli in coffee explants (Ardiyani, 2015; Ogita *et al*., 2020; Ibrahim *et al.,* 2013). Induction of embryogenic calli is preferred against non-embryogenic calli as reported in morphological characteristics of cell viability in coffee plant calli. Padua *et al*., (2014) reported that embryogenic *Coffee arabica* calli had higher cell viability compared to non-embryogenic calli using scanning electron microscopy. In the study, cells identified as friable (embryogenic) showed dense cytoplasm, nuclei with prominent nucleoli, large amyloplasts and absence of vacuoles whereas, non-embryogenic calli showed increased intercellular spaces, absence of other cytoplasmic organelles, and autophagic vacuole occupying cytoplasmic spaces (Padua *et al*., 2014).

2.6.4 Bioreactor Technology: Temporary Immersion Systems

Bioreactor technology was developed with the intention of commercializing plant micro-propagation. These include liquid-phase bioreactors, gas-phase bioreactors, temporary immersion systems and hybrid bioreactors.

Temporary Immersion Systems are ideal for coffee species because they are simple to operate, designed specifically for coffee propagation, low mechanical stress, and improved mechanism of action for improved nutrient absorption and gaseous exchange which are limitations of other bioreactor technologies (Georgiev *et al*., 2014). The technology involves the automation of the culture process with temporary immersion of cultures in liquid medium under predetermined immersion cycle (Persson, 2012). The immersion period is often limited within a few minutes where the plant cultures are provided with enough exposure time for

gaseous exchange under longer periods. The prominent bioreactor technology is the temporary immersion systems which offer augmented advantages against traditional bioreactor technology that involved continuous immersion of plant cultures in liquid media. Advantages include reduced asphyxia, reduced hyperhydricity, and improved propagation of plant cultures.

Efficiency in TIS is dependent on (i) volume of culture vessel (ii) immersion regimes and (iii) volume of liquid media (Etienne & Berthouly, 2002; Watt, 2012). Immersion times dictate the level of nutrients uptake and reduced hyperhydricity. Immersion times vary with the type of species, *in vitro* technique and type of temporary immersion systems (Ducos *et al*., 2007; Etienne &Berthouly, 2002). Shorter immersion periods in *C. arabica* (1 min every 12 h) is optimal for embryo production. For example, the volume of liquid media should be optimal in order to limit media renewal in TIS. According to Etienne and Berthouly, (2002) higher liquid volume decrease proliferation rates and is less efficient whereas, lower volumes are not ideal for optimum absorption of nutrients. RITA ® is a standardized culture container capacity at 1-L and contains 0.2 L liquid media (Ducos *et al*., 2007). As such, the volume of containers of TIS provides more headspace greater than conventional culture vessels. Additionally, TIS provides for aeration through bubble aeration (Georgiev *et al*., 2014). Bubble aeration encourages growth with limited immersion of plants. Therefore, TIS provide the most effective culture system for large scale propagation of plants.

Temporary Immersion Systems (TIS), as the name implies, involves the immersion of plant cultures in defined immersion times in liquid media. Bioreactor systems that involve temporary immersion have various characteristics that make it suitable for use as semi-automated immersion (Watt, 2012). TIS comprises of two compartments: one compartment contains liquid media and the other compartment plant cultures. TIS avoid continuous immersion of plant cultures, improve adequate gaseous exchange, facilitates sufficient mixing, limited shear levels and reduced contamination (Etienne & Berthouly, 2002; Georgiev *et al*., 2014; Watt, 2012). A number of temporary immersion systems are in use for micro-propagation and vary from Twin-flasks, hybrid ebb-and-flow, ebb-and-flow systems,

RITA®, and Temporary Immersion Root System to Stirred Tanks (Afreen, 2008; Georgiev *et al.*, 2014). The novel approaches have improved successful plant regeneration and derived plant products. However, the design and the micro-environment deployed rely on the desired end product (such as shoot multiplication, somatic embryos, root induction) and objective of operation in question for example secondary metabolite accumulation while, RITA® are ideal for embryogenic formation.

TIS vessels are constructed either using glass or plastic material allowing for light permeation to the cultured plants, and allow ease in sterilization and cleaning. TIS-based bioreactors employ periodic immersion of cultures through determined cycles, where the liquid media is placed in contact with the plant cultures, after which the media is drained to the bottom media chamber thereby exposing cultures to prolonged duration of gaseous environment. The reduced period of contact between the plant cultures and the liquid media reduce incidences of hyperhydricity (Georgiev *et al*., 2014; Watt, 2012). This is in addition to reduced physiological abnormalities associated with soma clonal variation due to the limited handling times of plant cultures. Similarly, TIS enhance the absorption of oxygen to the cultured cells unlike in fully submerged cultures in liquid-phase bioreactors thereby reducing morphological abnormalities due to asphyxia. Enhanced modifications, in some of the TIS provide head-space options for the optimization of gas exchange and exposure more so, on carbon (IV) oxide. The build-up of gases such as Carbon (IV) oxide facilitates physiological processes such as photosynthesis, growth and development in cell and tissue morphology (Georgiev *et al.*, 2014).

2.6.5 Temporary Immersion System: RITA®

The RITA® (Recipient`a Immersion Temporaire Automatique) are systems developed for intense *in vitro* propagation. The system consists of an autoclavable vessel (1-L) with two compartments. The two compartments are separated using a micro-sieve that has a mesh and a plastic pipe. The upper part of the vessel is a culture chamber and the bottom part is for liquid-media storage. The vessel is closed with a wide lid coupled with two perpendicular external ports at the top. The ports are secured using membrane filters where the central port

is connected to the automated pumping system. The automated system contains a timer clock and three-way solenoid valve which regulates immersion times and immersion frequencies (for instance, 3 min every 12 hours). The RITA® TIS is a simple system that is reliable in operation, and has compact space for apparatus accommodation. Overpressure is applied to the lower compartment pushing the liquid medium to the upper vessel (Ducos *et al.,* 2007; Georgiev *et al*., 2014). During the immersion period, the air is bubbled through the medium and agitates the tissues gently and renews the head space atmosphere inside the culture vessel.

2.7 Effect of Cell Densities in Temporary Immersion Systems

In Temporary Immersion Bioreactors, cell densities play a vital role in proliferation to somatic embryos. Initial cell densities determination is crucial to the survival rate and proliferation of calli into embryos (Ahloowalia *et al*., 2004; Aitken-Christie *et al.,* 1995). Previous studies have determined that low cell densities are optimal for embryo development whereas, higher inoculation densities pose a detrimental effect on embryogenesis. Particular studies on cell densities, indicated that 1-5 g/L Pack Cell Volume promoted embryogenic regeneration in liquid or semi-solid medium (Etienne & Berthouly, 2002). Subsequent submission of embryogenic cells in medium resulted in an increase in multiplication and biomass increase. IT is the same effect that cell densities coupled with renewal of medium has on embryogenesis. Low cell densities submitted to fresh media have low or no proliferation capabilities, inclining to have detrimental effects on somatic embryogenesis. Also, renewal of media on cell densities higher than 0.5 g/L, embryogenesis was inhibited (Watt, 2012). It is these observations that indicate that coffee strongly depends on cellular densities especially initial cell densities to determine success of embryogenesis since high cell densities inhibit somatic embryogenesis (Georgiev *et al*., 2014).

Hence, determination of optimum cell densities coupled with optimum PGR combinations will be based on determined standard densities ranging from 0.5, 1, 2, 3, 4 and 5 g/L Pack Cell Volume. The differential weights will be placed in different culture vessels congruent to their subjected concentration combinations of PGRs.

Tissue culture is the multiplication and manipulation of cells and tissues to regenerate whole plants under aseptic and controlled conditions of growth. Tissue culture is an efficient propagation method that overtakes traditional propagation techniques (vegetative propagation) in production of planting materials (Watt, 2012). According to Owuor (1976), the *in vitro* propagation of coffee offered great potential in improving limitations witnessed in propagation *in vivo* at the research institution. Tissue culture offers rapid multiplication over a shorter period. It overcomes the challenges associated with conventional techniques which often lead to loss of genetic fidelity when dealing with coffee varieties such as Ruiru 11 F1 hybrid (Kenya Coffee Platform, 2018). The pitfalls associated with conventional methods make them inefficient in meeting the increasing demand for coffee planting materials. Therefore, tissue culture is an ideal propagation technique for large-scale multiplication of the elite F1 hybrid maintains clonal genetic fidelity of the regenerants (Muniswamy *et al*., 2015).

The improved adoption of the RITA ® has proven ideal for large-scale commercial production of coffee. Etienne and Berthouly (1995) reported successful propagation of *C. arabica* hybrid embryos at 15,000 – 50,000 which was an improvement from Sondhal (1992) 45,000 embryos in *C. arabica cv. Catuai.* De Feria *et al*., (2003) obtained 70,000 in *C. arabica cv. Catimor* while Ducos *et al*., (2007) obtained 200,000 – 400, 000 embryos of Robusta coffee. Studies comparing conventional micro-propagation with automated systems reported improved embryo production. Gatica-Arias *et al*., (2008) reported improved embryo production in RITA ® 1-L compared to semi-solid multiplication in *C. arabica*, Ibraheem *et al*., (2013) in date palms (Phoenix dactylifera L), Gutiérrez *et al.,* (2016) in giant bamboo (Guadua angustifolia) and Lyam *et al*., (2012) in pineapples.

Despite the successful reports on improved embryogenesis, efficient adoption of TIS, RITA ® is still to be achieved at the Coffee Research Institution, Kenya due to lack of precise protocol for propagation of Ruiru 11 F1 hybrid. According to Gatica-Arias *et al*., (2008), numerous protocols have been described on plant regeneration of coffee in bioreactor technology. However, specific conditions and protocols developed for a particular genotype may not necessarily be reproducible for others (Gatica-Arias *et al.,* 2008 Los Santos-Briones *et al*.,

2006; Molina *et al.,* 2002;). Additionally, tissue culture regeneration protocols have been shown to have differentiated effects on different coffee plants based on genotype, explant source, culture age and type and concentration of plant growth regulators.

Indirect Somatic Embryogenesis (ISE) is ideal for large-scale propagation of embryogenic calli and somatic embryos in Temporary Immersion Systems (Ducos *et al*., 2007; Gatica-Arias *et al*., 2008). ISE in coffee consists of callus induction and proliferation, embryo development, germination and conversion of plants (Gatica-Arias *et al*., 2008). Previous studies on coffee ISE in liquid-based TIS report on systematic induction of callus in solid media and embryo formation in Liquid media (Ahmed *et al.*, 2013; Etienne and Berthouly, 2005; Etienne *et al*., 2018; Maciel *et al.*, 2016). Leaf explants are suitable for coffee callus induction due to high frequency somatic embryogenesis as reported by Los Santos-Briones *et al.,* (2006). High frequency somatic embryogenesis is ideal for embryo formation and large-scale production of embryos. Los Santos Briones *et al.,* (2006) further report that protocols on regeneration via TIS is genotypic specific and requires determination of regeneration protocol specific to coffee hybrids. Therefore, it is necessary to conduct investigation on indirect somatic embryogenesis culture parameters for induction of callus and embryo formation on Ruiru 11 in order to establish ideal protocol specific to the genotype in TIS, RITA ®.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Research Design

The research was carried out at the Tissue Culture Laboratory at the Coffee Research Institute, Ruiru, Kenya. The research study was done on completely randomized design. Plant leaves from the third pair of each mother plant were cultured in solid-Murashige and Skoog media in a dark growth room at 25 ± 2 º C for induction of callus. The second objective analyzed the induction of embryos in ½-strength Murashige and Skoog media supplemented with different plant growth regulators in a dark growth room maintained at 25 ± 2 º C. The induced calli was also inoculated in ½-strength Murashige and Skoog liquid media in Temporary Immersion Systems, RITA® to induce embryos in a dark growth room maintained at 25 ± 2 º C. Temporary Immersion system is a novel technology that can be used to propagate Ruiru 11 using liquid-based media compared to solid-based media in induction of embryos in Murashige and Skoog ½ strength media. Contrary, the Coffee Research Institute currently uses solid-based direct somatic embryogenesis which has proven inadequate in meeting current demand for coffee seedlings from the commercial laboratory.

3.2 Plant Materials

C. arabica F1 hybrid Ruiru 11 Code 93 and Code 71 (Table 3.1) were used in this study. The donor plants were obtained from the field-grown as seedlings for nine months. They were transferred to the greenhouse at the Tissue Culture Laboratory site and acclimatized for six-months prior to use. The total number of donor plants was 80 for the research study. The third pair of leaves were then excised from the healthy and disease-free mother plants and placed in a beaker containing tap water and transferred to the tissue culture laboratory for sterilization. The explants were surface sterilized using diluted detergent. Further sterilization was done under sterile laminar flow hood, using 20% commercial bleach (JIK), which contains 3.85% (w/v) sodium hypochlorite for 15 minutes and rinsed three times with sterile distilled water (Alumiro, 2015). The leaf explants were dissected 1 cm x 1 cm prior to inoculation in Murashige and Skoog media. The cultures were maintained in dark growth rooms maintained at 25 ± 2 º C.

Table 3.1: Pedigree on Code 71 and Code 93 C. arabica Ruiru 11.

Coffee Hybrid	Pedigree
Code 71	SL28 x [(N39 x HT) x (SL28 x RS)] x Catimor
Code 93	SL28 x [(SL34 x RS) x HT)] x Catimor

Key: RS = Rume Sudan, HT = Hibrido de Timor (Gichimu *et al*., 2014)

3.3 MS Media Preparation and Establishment of Cultures

The ½ -strength solid Murashige and Skoog (1962) culture media was prepared using 10ml each from Stock A and B, 5ml each from Stock C, D, E, F and G (Appendix I) and 30 grams of sucrose dissolved in 1L distilled water in a beaker and placed on magnetic stirrer so as to mix the ingredients until the ingredients were completely dissolved. Subsequently, 100mg of Myo-inositol and 100mg of l-cysteine were added into the mixture, and the final volume adjusted to 1000ml. The solution's pH was adjusted to 5.7 (using 1 N NaOH or 0.1 M Hydrochloric Acid (HCL) and autoclaved at 121ºC for 15 minutes. Culture media was used within 12 hours after autoclave with remaining media stored for later use in a cold room at four ºC. Each treatment comprised of twenty culture glass-bottles and each bottle contained three leaf squares which contained 60 as the total sample size per treatment. The research experiment was done three times.

3.4 Callus Induction Media

Experimental treatments for the first objective were determined based on the auxins and cytokinins shown in Table 3.2 in different concentrations as indicated. The plant growth regulators were used singly and in combinations (auxins + cytokinins). Plant growth regulator combinations were IBA + BAP, IBA + KIN, 2,4-D + BAP, and 2,4-D + KIN as shown in Table 3.3. The control experiment's MS culture media was not supplemented with any plant growth regulators. Concentration levels of Plant Growth Regulators used in induction of callus on *C. arabica* Hybrid Ruiru 11 leaf explants.

Table 3.2 Concentration levels of Plant Growth Regulators used Singly in Induction of Callus on *C. arabica* Hybrid Ruiru 11 Leaf Explants

Plant Growth Regulator	Concentrations (µm)				
IBA	0.10	0.20	0.30	0.41	0.51
2,4-D	0.11	0.22	0.33	0.44	0.53
BAP	0.11	0.23	0.34	0.45	0.56
KIN	0.11	0.21	0.32	0.43	0.54
MS Media	0	0	0	0	0

Table 3.3. Concentration levels of Plant Growth Regulators used in Combinations in Induction of Callus on *C. arabica* Hybrid Ruiru 11 Leaf Explants

Plant Growth Regulator	Concentrations (µm)				
IBA + BAP	0.10 + 0.11	0.20+ 0.23	0.30+ 0.34	0.41+ 0.45	0.51+ 0.56
IBA + KIN	0.10 + 0.11	0.20+ 0.21	0.30+ 0.32	0.41+ 0.43	0.51+ 0.54
2,4-D + BAP	0.11+ 0.11	0.22+0.23	0.33+0.34	0.44+0.45	0.53+0.56
2,4-D + KIN	0.11+ 0.11	0.22+0.21	0.33+0.32	0.44+0.43	0.53+0.54
MS Media	0.00	0.00	0.00	0.00	0.00

3.5 Callus Proliferation Media

The calli induced from the first objective experiment were used in callus proliferation in solid MS media. The callus proliferation media was supplemented with auxin, 2,4-D and cytokinin BAP as shown in Table 3.4.

Table 3.4. Plant Growth Regulators used in Experiment 2 on *C. arabica* L. cv Ruiru 11 leaf explants.

Plant Growth Regulator	Concentrations (µm)				
2,4-D	0.11	0.22	0.33	0.44	0.53
BAP	0.11	0.23	0.34	0.45	0.56
2,4-D + BAP	0.11+ 0.11	0.22+0.23	0.33+0.34	0.44+0.45	0.53+0.56
MS Media	0.00	0.00	0.00	0.00	0.00

3.6 Embryo Induction Media

½ strength Murashige and Skoog (1962) liquid-media was supplemented with Thiamine (10 mg/L), pyridoxine (1 mg/L), glycine (1 mg/L), niacin (1 mg/L), Citric acid (250 mg/L), coconut water (200 mg/L), L-cysteine (10 mg/L), Myo-inositol (100 mg/L) and sucrose (20 mg/L) according to (de Rezende *et al*., 2012). The liquid media was dispensed in 1-L RITA®.

3.7 Data Collection and Analysis

Analysis of Variance (ANOVA) was assessed based on Fischer's Test using Minitab® 17.1 Software separated by Least Significant Difference (LSD) at p-value 0.05 and means expressed as mean ± SE. Photography for the experiment was performed using a digital camera, and two photographs were taken. The following are the parameters assessed:

3.7.1 Callus Induction Rates

The rate of callus induction was assessed two weeks after the inoculation of explants, and thereafter, observations were made at an interval of 7 days. The data collected was evaluated and expressed as a percentage (%) using the formula:

$$\text{Callus induction (\%)} = \frac{Total\ Number\ of\ explants\ with\ induced\ callus}{total\ number\ of\ explants\ inoculated\ for\ each\ treatment}\ X\ 100$$

3.7.2 Callus Characteristics Analysis

The callus induced in leaf explants were measured based on color, callus score and morphology. Callus morphology was characterized according to (i) friable (embryogenic) and (ii) compact (non-embryogenic). Callus characteristics were also assessed based on callus

formation viability based on five categories: NC= no callus formed; + = very poor; ++ = poor; +++ = good and ++++ = very good callus formation as per plant growth regulator treatment.

3.7.3 Callus Induction Time

The callus growth period was assessed based on the time taken to induce visible callus from leaf explants. The eye-ball technique was used for this parameter throughout the study. Induction time was recorded as the number of days taken to induce visible callus.

3.7.4 Biomass Growth Measurement

The fresh weight of callus induced was assessed under a sterile clean bench. The culture vessels containing leaf explants with induced callus were transferred to the laminar flow hood. A small percentage of calluses formed were sacrificed for analysis for this parameter at 14^{th} day and 60^{th} day after inoculation. Callus was carefully separated from the leaf explants using sterile dissecting tools and weighed using a precision scale (g). Callus was then dried in an oven at 40°C overnight, and the dry weight was measured and recorded. Growth was measured as described below for the Callus growth curve.

3.7.5 Callus Growth Curve

Growth curve analysis per experimental unit was done from the first day of inoculation (day 0). Subsequently, analyses were done at intervals of 7 days until the 70^{th} day. The growth rate was measured, according to Dung *et al*., (1981):

$$\text{average growth rate} = \frac{\text{Wf} - \text{Wi}}{\text{t}}$$

where Wf= final weight of fresh callus matter, Wi= initial weight of fresh matter, and t=cultivation period (days).

3.7.6 Callus Proliferation

Callus proliferation was assessed based on callus size: 0 = no callus growth (no change), 1 = slight callus growth; 2 = good callus growth; 3 = vigorous callus growth. The total number of embryos induced in each treatment were counted and presented as means as assessed using ANOVA in Fischer's Test.

3.7.7 Embryo Induction

For inoculum cell densities, each density concentration represented a treatment with each treatment inoculated with six inoculum calli. Densities were measured using the formula below. The cultures were maintained in liquid media ½ strength MS in RITA® with immersion times of 3 min every 6 hours and sub-cultured every 14 days to fresh media for one month then every 30 days for 2 months. The experiment was repeated three times.

$$\boldsymbol{D\ (density) = \frac{M(mass)}{V\ (volume - 200ml)}}$$

The total number of globular embryos produced were determined and presented as mean ± SE separated with LSD at P-value 0.05. Embryo induction rates were assessed based on the percentage of an embryo formed as follows:

Frequency of Embryo formation (%): $\frac{\text{No.of embryo formed}}{\text{no.of inoculated calli}} x\ 100.$

Table 3.5: Inoculum Callus Densities for *C. arabica* Ruiru 11 in 1-LRITA®.

Mass (g)	Densities (g /200 ml)
< 0.1	< 0.0005 g / cm^3
0.1	0.0005 g / cm^3
0.2	0.001 g / cm^3
0.3	0.0015 g / cm^3
0.4	0.002 g / cm^3
0.5	0.0025 g / cm^3
0.6	0.003 g / cm^3
0.7	0.0035 g / cm^3
0.8	0.004 g / cm^3
0.9	0.0045 g / cm^3
1.0	0.005 g / cm^3

3.7.8 Time Efficiency, Embryo Yield and Resource Use

The objective of the study was to analyze the efficiency of Temporary Immersion Systems compared to the conventional method used at Coffee Research Institute. The use of TIS is a novel approach at CRI as it uses liquid-based media unlike the conventional process that uses solid-based media. The parameters assessed were time efficiency – time taken from induction

of calli to embryo induction in solid-based Murashige and Skoog media and liquid-based media, TIS in days. Embryo yield was the total number of embryos induced in both systems (conventional solid-based and liquid-based) as explained in 3.7.6 and 3.7.7 and resource use, compared the cost inclusion in elimination of gelrite (solidifying agent) and the cost analysis on the embryo induction and proliferation stage in somatic embryogenesis.

CHAPTER FOUR

RESULTS

4.1 *In Vitro* Callus Induction

The results obtained from the experiment demonstrated the effect of plant growth regulators on callus induction on Ruiru 11 (Code 71 and Code 93). Induction success was also validated with data on fresh biomass weight of callus produced and callus growth in the cultures supplemented with varying concentrations of PGR. The novel knowledge being introduced in the current study was analyzing how plant regulators used singly and in combination (auxins and cytokinin) in the propagation of Ruiru 11 through indirect somatic embryogenesis and how the PGRs would affect callus induction comparing how liquid-based systems compare to solid-based systems in influencing somatic embryo production.

4.2 Effect of Plant Growth Regulators Used Singly on Callus Induction

Auxin IBA did not induce any callus even after 30-days of culture. From the results, no callus induction was observed in the control treatment where the culture media was not supplemented with plant growth regulators even after 40 days after inoculation. The results of the effect of 2.4-D used singly on the callus induction rates are shown in Table 4.1. Treatment with varying levels of concentrations of 2,4-D elicited different response patterns in Code 71 and Code 93, respectively (Table 4.1.). For Code 71, there was an increase in the frequency of callus induction with increasing 2,4-D concentration up to the concentration level of 0.33 µM, for which 88±0.058 callus induction rate was observed. Callus induction rate declined with further increase in the concentration of 2,4-D, with induction rates of 50±0.043and 45±0.029observed for 2,4-D concentration levels 0.44 µM ad 0.53 µM, respectively. Unlike Code 71, the response of Code 93 to different concentration levels of 2,4-D was averagely linear, with induction rates increasing from 75±0.020 at 2,4-D concentration of 0.11 µM to 97±0.020 at of 0.53 µM concentration. From the results obtained, it was noted that Code 71 and Code 93 differed in callus induction frequencies subject to the different 2,4-D concentrations exhibiting differences in induction frequencies.

Table 4.1: Callus Induction from Leaf Explants of *C. arabica* Code 93and Code 71

Genotype	Plant Growth Regulator	Concentration (µm)	% Callus Induction
Code 71	**2.4-D**	0.11	78±0.667[a]
		0.22	85±1.45[ab]
		0.33	88±1.47[ab]
		0.44	50±3.18[bc]
		0.53	45±2.33[bc]
Code 93	**2.4-D**	0.11	75±2.33[a]
		0.22	88±2.03[a]
		0.33	86±0.882[b]
		0.44	96±0.333[bc]
		0.53	97±0.333[bc]

Means within a column followed by different letters are significantly different at $P = 0.05$.

2,4-D was observed to induce embryogenic calli across all treatments in Code 71 and Code 93. With respect to the degree of callus formation, growth differences were observed in treatment in Code 71 and Code 93 (Table 4.2). The degree of callus formation was uniformly poor (++) in Code 71 for all 2,4-D concentration levels. The shortest duration for callus induction observed was 5 weeks for 2,4-D concentration levels 0.33, 0.44, and 0.53 µM (Table 4.2). With respect to Code 93, increased levels of 2,4-D concentration both on the degree of callus formation and the duration required to reach very good callus formation were observed. A good degree of callus formation (+++) was reached within 3 weeks at 2,4-D concentration levels of 0.33 µM, whereas very good callus formation (++++) was realized within 3 weeks using 2,4-D concentration levels 0.44 and 0.53 µM (Table 4.2). The two genotypes showed the difference in degrees of calli induction response.

Table 4.2: Callus Growth Measurements in Code 71 and Code 93

Genotype	Plant Growth Regulator	Concentration (µM)	Degree of callus formation	Duration of callus formation	Callus Morphology
Code 71	**2,4-D**	0.11	++	6 weeks	FW
		0.22	++	6 weeks	FW
		0.33	++	5 weeks	FW
		0.44	++	5 weeks	FW
		0.53	++	5 weeks	FW
Code 93	**2,4-D**	0.11	++	4 weeks	FW
		0.22	+++	4weeks	FW
		0.33	+++	3 weeks	FW
		0.44	++++	3weeks	FW
		0.53	++++	3 weeks	FW

KEY: ++ = poor, +++ = good, ++++ = very good. FW= friable white

4.3 Effects of Plant Growth Regulator 2,4-D Used Singly on Fresh Weight Biomass of Callus Induced

The effects of 2,4-D used singly on callus fresh weight biomass of induced callus are presented in Table 4.3. There was a significant difference in the mean scores of the fresh weight biomass obtained, 2,4-D at 0.33 µM recorded the highest fresh weight in Code 71 (0.490 ±0.013g FW). On the other hand, 2,4-D at 0.44 µM showed the highest fresh weight for Code 93 (1.069±0.0151g FW).

There was a significant difference in the mean scores of the fresh weight biomass obtained - Code 71 (Mean = 0.230 St Dev = 0. 175) and Code 93 (Mean = 0.650, St Dev = 0.371) at conditions t-value (-2.29) and p-value (0.071) at 95% confidence interval.

In Code 71, biomass induction increased with increased concentration levels of 2,4-D up to concentration level 0.33 µM, after which a sharp fall in biomass induction was observed at increased levels of 2,4-D (0.44 and 0. 53 µM). A similar trend was observed in Code 93 with increased biomass induction for increased 2,4-D concentration levels up to 044 µM, after which a slight fall in biomass induction was recorded. Overall, the biomass yield was higher in Code 93 than in Code 71 across all 2,4-D concentration levels indicating a significant effect due to the Codes (Figure 4.1).

Table 4.3: Fresh Weight Biomass Induced in Ruiru 11

Plant Growth Regulator	**Concentration (µM)**	**Fresh weight (g)**	
		Code 71	**Code 93**
2,4-D	0.11	0.178 ± 0.026^{c}	0.190 ± 0.01^{d}
	0.22	0.320 ± 0.006^{b}	0.394 ± 0.008^{c}
	0.33	0.490 ± 0.013^{c}	0.636 ± 0.025^{b}
	0.44	0.0962 ± 0.011^{d}	1.069 ± 0.151^{a}
	0.53	0.0672 ± 0.002^{d}	0.961 ± 0.02^{a}

Means within a column followed by different letters are significantly different at $P = 0.05$ based on Fischer's Test ANOVA analysis.

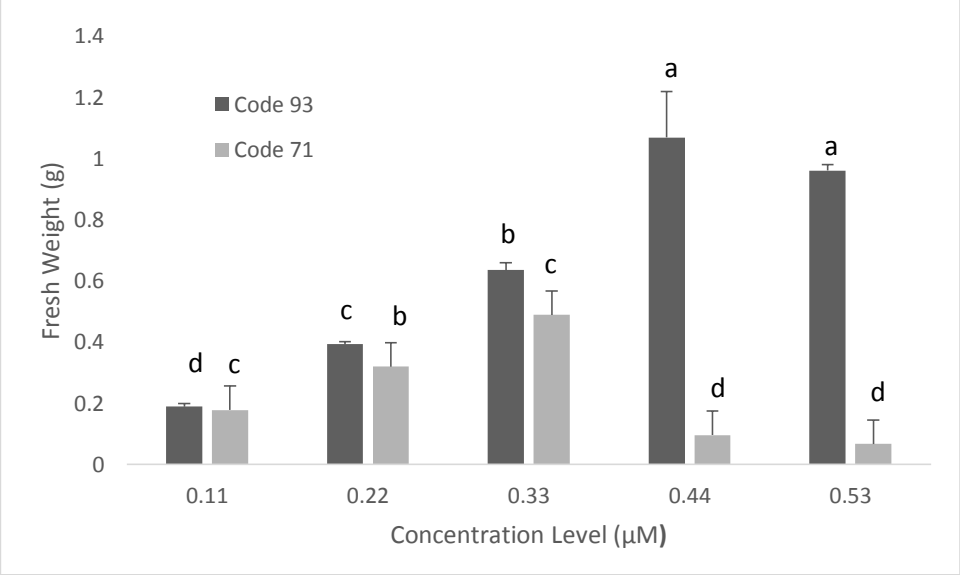

Figure 4.1. Fresh Weight Biomass Induced in Ruiru 11.

4.4 Plant Growth Regulators Combinations Effect on Callus Induction

The effects of auxins and cytokinins used in combination are presented in this section. Table 4.4. shows the effects of 2,4-D+BAP on callus proliferation. In this experiment, Code 93, showed the highest level of callus induction rate of 100±0.00compared to code 71, which showed a callus induction rate of 88%. Unlike in the treatment with 2,4-D used singly, the maximum induction rate (88±0.050) was observed at a higher concentration of 2,4-D in the 2,4-D + BAP (0.53 + 0.11 µM) mixture. A similar rate for a single treatment with 2,4-D was reached at 0.33 µM concentration level. The combination of 2,4-D + KIN (0.53 + 0.11 µM) elicited the highest callus induction rate at 76±0.019. linear response in Code 71 to increasing levels of 2,4-D in the treatment mixture, with induction rates ranging from 53±0.043at 0.11 + 0.54 µM (2,4-D + KIN) to 76±0.019for 0.53 + 0.11 µM (2,4-D + KIN). The combination of 2,4-D + BAP elicited the highest induction rate where it elicited 100±0.00response in the cultures of Code 93 and also showed 88±0.050response for cultures of code 71. Peak induction rate of 100±0.00was also observed on cultures supplemented with concentrations 0.44 + 0.23 (2,4-D + BAP) and 0.53 + 0.11 (2,4-D + BAP) in Code 93. The lowest induction rates were observed in the combination 2,4-D + KIN (0.53 + 0.11), which showed lower induction rates of 76±0.019when compared to 2,4-D + BAP. Compared to single treatment

with 2,4-D, whose lowest rate was 75±0.020and, highest rate was 97±0.020.Overall, Auxin-cytokinin combinations showed better induction responses in both Codes studied compared to plant growth regulators used singly (Table 4.4). Differences in callus induction responses between Code 71 and Code 93 were further observed under the various concentration auxin-cytokinin concentrations, with Code 93 registering superior performance.

Table 4.4: Callus Induction from Leaf Explants of *C. arabica* Ruiru 11

Plant Growth Regulator	**Concentration (µM)**	**% Callus Induction**	
		Code 71	**Code 93**
2,4-D + BAP	0.11 + 0.56	68±0.33^{a}	75±0.32^{a}
	0.22 + 0.45	80±0.84^{ab}	80±0.92^{a}
	0.33 + 0.34	75±0.63^{bc}	93±0.27^{b}
	0.44 + 0.23	76±0.67^{bc}	100±0.34^{b}
	0.53 + 0.11	88±0.68^{c}	100±0.29^{c}
2,4-D + KIN	0.11 + 0.54	53±0.33^{a}	50±0.21^{a}
	0.22 + 0.43	60±0.44^{b}	55±0.25^{a}
	0.33 + 0.32	68±0.48^{c}	63±0.35^{b}
	0.44 + 0.21	73±0.62^{c}	73±0.43^{c}
	0.53 + 0.11	76±0.91^{d}	76±0.45^{c}

Means within a column followed by different letters are significantly different at *P = 0.05* based on Fischer's Test ANOVA analysis.

The effects of IBA + BAP on callus proliferation are presented in Table 4.5. Response to treatment with various concentrations of IBA + BAP was similar for both Code 71 and Code 93. IBA + BAP (0.41 + 0.23 µM) obtained the highest induction rates in Code 71 of 75±0.048and a similar trend was observed for cultures of Code 93 with the highest induction rates of 75±0.048 (Table 4.5). Increased levels of IBA combined with decreasing levels of BAP showed a linear increase in induction rates in both Code 71 and Code 93, up to concentration level IBA + BAP (0.41 + 0.23 µM), after which, reduction in induction rates

was observed in both Codes at IBA + BAP (0.51 + 0.11 µM). Response patterns similar to those observed for treatments with IBA + BAP were recorded in Code 71 and Code 93 when subjected to treatment with varying concentrations of IBA + KIN. Induction rates ranged from 61±0.024to 81±0.047in Code 71 and 61±0.028to 80±0.045for Code 93. The combination, on the other hand, IBA + KIN (0.51 + 0.11 µM) induced the highest callus induction rate of 81±0.047in Code 71 and 80±0.045in Code 93.

Table 4.5: Callus Induction Rates from Leaf Explants of *C. arabica* Ruiru 11

Plant Growth Regulator	Concentration (µM)	% Callus Induction	
		Code 71	**Code 93**
IBA +BAP	0.10 + 0.56	65±0.018^{a}	60± 0.018^{bc}
	0.20 + 0.45	68±0.027^{a}	64±0.028^{b}
	0.30 + 0.34	69±0.011^{ab}	68±0.015^{b}
	0.41 + 0.23	75±0.012^{b}	75±0.012^{a}
	0.51 + 0.11	71±0.011^{b}	70±0.011^{a}
IBA + KIN	0.10 + 0.54	61±0.013^{bc}	61±0.028^{bc}
	0.20 + 0.43	67±0.012^{b}	68±0.030^{b}
	0.30 + 0.32	60±0.011^{bc}	58±0.028^{c}
	0.41 + 0.21	75±0.03^{ab}	73±0.030^{ab}
	0.51 + 0.11	81±0.06^{a}	80±0.025^{a}

Means within a column followed by different letters are significantly different at *P = 0.05* based on Fischer's Test ANOVA analysis.

4.5 Fresh Weight Biomass of Induced Calli in Leaf Explants

The effect of PGR on total callus biomass was assessed under single PGR treatment and under treatments with varying PGR combinations.

4.5.1 Effect of Plant Growth Regulators on Fresh Weight Biomass

Treatments with Auxin 2,4-D recorded the highest fresh weight at 0.33 µM in Code 71 at 0.490 ±0.013g FW and at 0.44 µM in Code 93 at 1.069±0.0151g FW (Table 4.3). An

independent t-test was conducted to compare the mean difference in both codes. There was a significant difference in the mean scores of the fresh weight biomass obtained - Code 71 (Mean = 0.230 St Dev = 0. 175) and Code 93 (Mean = 0.650, St Dev = 0.371) at conditions t-value (-2.29) and p-value (0.071) at 95% confidence interval.

In Code 71, biomass induction increased with increased concentration levels of 2,4-D up to concentration level 0.33 µM, after which a sharp fall in biomass induction was observed at increased levels of 2,4-D (0.44 and 0. 53 µM). A similar trend was observed in Code 93 with increased biomass induction for increased 2,4-D concentration levels up to 044 µM, after which a slight fall in biomass induction was recorded. Overall, the biomass yield was higher in Code 93 than in Code 71 across all 2,4-D concentration levels indicating a significant effect due to the Codes (Figure 4.1.).

4.5.2 Fresh Weight Biomass in PGR Combinations

The effects of plant growth regulators used in combination are presented in table 4.6. The PGR combinations 2,4-D and BAP progressively increased the fresh weight biomass in both Code 71 and Code 93 as the levels of 2,4-D in the combination increased, and that of BAP decreased. The PGR combination 2,4-D + BAP (0.53 + 0.11 µM) produced highest fresh weight biomass in both codes with yield levels 0.649±0.026g (Code 71) and 0.348±0.017g (Code 93) (Table 4.6).

On the other hand, the effect of the combinations of 2,4-D + KIN (0.33 + 0.32 µM) yielded fresh weight biomass of 0.238±0.016g in cultures of code 71 (Table 4.6.). In Code 93, 2,4-D + KIN (0.11 + 0.54 µM) recorded highest fresh weight biomass of 0.156±0.041g. This was lower than any of the biomass yields attained through treatments involving 2,4-D + BAP, further demonstrating the inferiority of 2.4-D + KIN combinations in biomass production. There was a difference in the induction of biomass recorded within the treatments. In Code 71, 2,4- D (0.11, 0.22 and 0.53 µM) combined with KIN (0.54, 0.43 and 0.11 µM) induced lower fresh biomass weights significantly whereas, 2,4-D (0.33 and 0.44 µM) combined with KIN (0.21 and 0.32 µM) induced significantly higher fresh weights. With respect to Code 93,

induction of fresh biomass weights was lowest at 2,4-D + KIN (0.33 + 0.32 µM) compared to other combination levels.

Table 4.6: Fresh Weight Biomass Induced in Code 71 and Code 93.

Plant Growth Regulator	Concentration (µM)	Fresh weight (g)	
		Code 71	**Code 93**
2,4-D + BAP	0.11 + 0.56	0.237±0.017^{c}	0.273±0.006^{b}
	0.22 + 0.45	0.294±0.023^{b}	0.291±0.022^{ab}
	0.33 + 0.34	0.298±0.011^{b}	0.274±0.033^{b}
	0.44 + 0.23	0.593±0.03^{a}	0.341±0.011^{a}
	0.53 + 0.11	0.649±0.026^{a}	0.348±0.017^{b}
2.4-D + KIN	0.11 + 0.54	0.088±0.002^{bc}	0.156±0.041^{a}
	0.22 + 0.43	0.080±0.052^{a}	0.142±0.033^{ab}
	0.33 + 0.32	0.238±0.016^{a}	0.106±0.028^{c}
	0.44 + 0.21	0.200±0.002^{bc}	0.125±0.030^{a}
	0.53 + 0.11	0.037±0.013^{b}	0.088±0.009^{d}

Means within a column followed by different letters are significantly different at *P = 0.05* based on Fischer's Test ANOVA Analysis.

Table 4.7 shows the effects of IBA +BAP PGRs in combination. IBA +BAP (0.41 + 0.23µM) showed highest fresh weight induction of 0.304±0.011g for cultures of code 93 and 71. For Code 71 where significant within-treatment differences were observed, increasing levels of IBA concentration coupled with reducing levels of BAP progressively increased fresh biomass weight yield. The effect of varying the concentration levels of IBA and BAP on fresh biomass weight yield in Code 93 was inconsistent across the treatments.

IBA + KIN (0.41 + 0.21 µM) elicited the highest fresh weight (0.342±0.045g) observed in cultures of Code 71. On the other hand, IBA + KIN (0.41 + 0.21 µM) showed the highest fresh weight biomass (0.341±0.044g) in cultures of Code 93 (Table 4.7).

Table 4.7: Fresh Weight Biomass Induced in *C. arabica* Ruiru 11.

Plant Growth Regulator	Concentration (µM)	Fresh Weight (g)	Fresh Weight (g)
		Code 71	Code 93
IBA + BAP	0.10 + 0.56	0.124 ± 0.022^{d}	0.280 ± 0.015^{ab}
	0.20 + 0.45	0.183 ± 0.028^{c}	0.302 ± 0.011^{a}
	0.30 + 0.34	0.258 ± 0.012^{ab}	0.279 ± 0.011^{ab}
	0.41 + 0.23	0.304 ± 0.011^{a}	0.304 ± 0.011^{a}
	0.51 + 0.11	0.249 ± 0.012^{b}	0.249 ± 0.012^{b}
IBA + KIN	0.10 + 0.54	0.123 ± 0.022^{c}	0.273 ± 0.015^{b}
	0.20 + 0.43	0.190 ± 0.02^{b}	0.291 ± 0.013^{ab}
	0.30 + 0.32	0.236 ± 0.018^{b}	0.274 ± 0.015^{b}
	0.41 + 0.21	0.342 ± 0.045^{a}	0.341 ± 0.044^{a}
	0.51 + 0.11	0.248 ± 0.048^{b}	0.248 ± 0.009^{b}

Means within a column followed by different letters are significantly different at $P = 0.05$ based on Fischer's Test ANOVA analysis.

4.6 Callus Growth Measurement

Preliminary callus structures were observed at the cut edges of the leaf squares after 7 days of culture, and subsequent proliferation was observed over a period of 8 weeks (Plate 1 a and b). White callus morphology was recorded across all treatments in Code 71 and Code 93. The color of the resultant calli did not affect proliferation rates across all treatments.

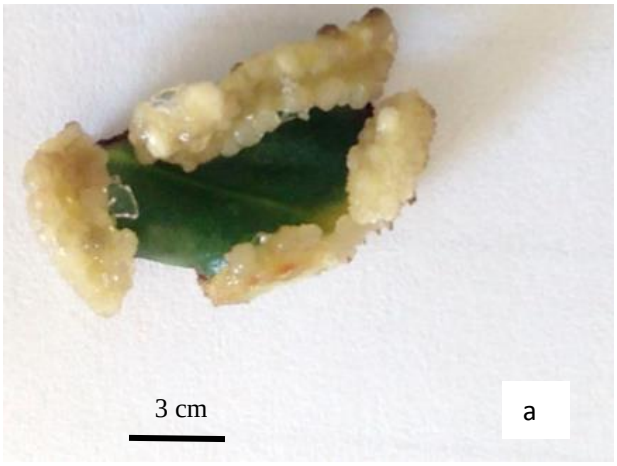

Plate 1: (a*)* Callus induced in leaf explants after 4 weeks in culture

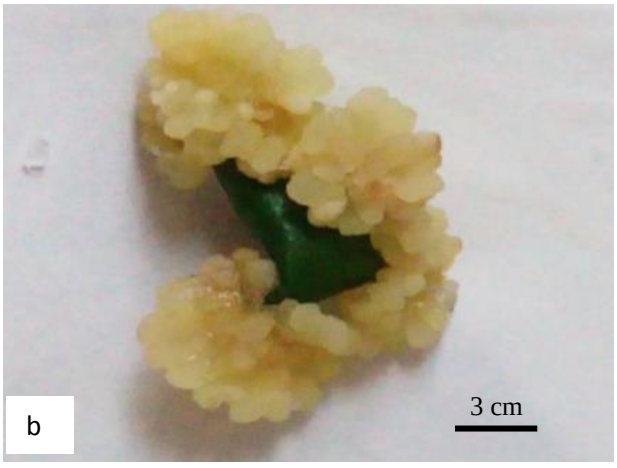

Plate 1(b): Callus growth after 7weeks in culture.

4.7 Effects of Plant Growth Regulators on Callus Characteristics

The introduction of BAP as a plant growth regulator had an impact on both the degree of callus formation and the time taken to induce callus formation the duration to realize the callus (Table 4.8). For Code 71, the combination of 2,4-D + BAP improved the degree of callus formation from poor (++) observed under treatment with 2,4-D alone to good callus formation from concentrations 0.11 + 0.56, 0.22 + 0.45. Very good callus formation was observed at concentrations 0.44 + 0.23 and 0.53 + 0.11 µM (Table 4.8). The positive impact

was also observed regarding the durations required to attain the various degrees of callus formation, with the longest duration of 5 weeks was observed for concentration 0.11 + 0.56 and 0.22 + 0.45 µM, whereas a shorter duration of 4 weeks was observed for concentration 0.33 + 0.34, 0.11 + 0.56 and 0.22 + 0.45 µM compared to the use of 2,4-D singly (Table 4.8). The combination reduced the duration by 1 week at all the concentration levels. With respect to Code 93, there was no advantage in incorporating BAP except at concentration level 0.11 + 0.56 µM (2,4-D + BAP), where the degree of callus formation improved from poor (++) to good (+++).

Table 4.8: Callus Growth Measurements in Code 71 and Code 93

Genotype	Plant Growth Regulator	Concentration (µM)	Degree of callus formation	Duration of callus formation	Callus Morphology
Code 71	2,4-D + BAP	0.11 + 0.56	+++	5weeks	FW
		0.22 + 0.45	+++	5 weeks	FW
		0.33 + 0.34	+++	4 weeks	FW
		0.44 + 0.23	++++	4 weeks	FW
		0.53 + 0.11	++++	4 weeks	FW
Code 93	2,4-D + BAP	0.11 + 0.56	+++	4 weeks	FW
		0.22 + 0.45	+++	4weeks	FW
		0.33 + 0.34	+++	3 weeks	FW
		0.44 + 0.23	++++	3weeks	FW
		0.53 + 0.11	++++	3 weeks	FW

KEY: +++ = good, ++++ = very good. FW= friable white.

Except for callus morphology, the combination 2,4-D + KIN had the same effect as 2,4-D + BAP on Code 71 with respect to degrees of callus formation and duration taken to form the

calli (Table 4.9) Concentration levels 0.11 + 0.54 and 0.22 + 0.43 µM resulted in formation of white compact calli. Similarly, 2,4-D + KIN at concentration level 0.11 + 0.54 and 0.22 + 0.43 µM in Code 93 gave rise to white compact calli.

In Code 71, 2,4-D + KIN (0.11 + 0.54 µM) induced callus in 6 weeks while increasing combination levels of 2,4-D + KIN induced in 5 weeks. In Code 93, lower concentrations of 2,4-D (0.11 and 0.22 µM) combined with KIN (0.54 and 0.43 µM) were induced in 5 weeks while increased levels of 2,4-D (0.33, 0.44, and 0.53 µM) combined with BAP (0.32, 0.21, and 0.11 µM) induced in 4 weeks.

Table 4.9: Callus Growth Measurements in Code 71 and Code 93.

Genotype	Plant Growth Regulator	Concentration (µM)	Degree of callus formation	Duration of callus formation	Callus Morphology
Code 71	2,4-D + KIN	0.11 + 0.54	+++	5weeks	CW
		0.22 + 0.43	+++	5weeks	CW
		0.33 + 0.32	+++	4 weeks	FW
		0.44 + 0.21	++++	4 weeks	FW
		0.53 + 0.11	++++	4 weeks	FW
Code 93	2,4-D + KIN	0.11 + 0.54	+++	4 weeks	CW
		0.22 + 0.43	+++	4weeks	CW
		0.33 + 0.32	+++	3 weeks	FW
		0.44 + 0.21	++++	3weeks	FW
		0.53 + 0.11	++++	3 weeks	FW

Key: +++ = good, ++++ = very good. FW= friable white, CW – compact-white.

The current study indicated that the plant growth regulator combinations IBA + BAP and IBA + KIN were not suitable in the production of embryogenic calli. The duration of callus production increased for all the treatments (Table 4.10). Non-embryogenic calli was obtained

in all the treatments supplemented with IBA + BAP and IBA + KIN in Code 71 and Code 93. IBA + BAP in Code 71 and Code 93 induced poor callus (++) across all treatments (Table 4.10). On the other hand, IBA + KIN obtained similar results in Code 71 and Code 93 with lower levels of IBA (0.10 and 0.22 µM) combined with KIN (0.43 and 0.54 µM) induced poor callus (++). Increased levels of IBA (0.30, 0.41 and 0.51 µM) combined with KIN (0.32, 0.21 and 0.11 µM) induced good calli (+++) in Code 71 and Code 93 (Table 4.10).

In Code 71, increased levels of IBA (0.41 and 0.51 µM) combined with BAP (0.11 and 0.23 0.30, 0.41 and 0.51 µM) induced callus in 5 weeks, whereas IBA combined with BAP above concentrations 0.30 + 0.10 µM induced callus in 6 weeks. In Code 93, lower concentration levels of IBA combined with BAP (0.10+ 0.56 µM and 0.20 + 0.45 µM) were induced in 5 weeks whereas increased levels of IBA +BAP induced calli in 4 weeks (Table 4.10).

In treatments supplemented with IBA +KIN, lower levels of IBA combined with KIN (0.10 + 0.54 µM and 0.20 + 0.43 µM) induced calli in 6 weeks whereas, increased levels of IBA combined with KIN induced in the formation of calli in 5 weeks in Code 71 and Code 93 (Table 4.10).

Table 4.10: Callus Growth Measurements in Code 71 and Code 93.

Plant Growth Regulator	Genotype	Concentration (µM)	Degree of callus formation	Duration of callus formation	Callus Morphology
IBA + BAP	Code 71	0.10 + 0.56	++	6weeks	CW
		0.20 + 0.45	++	6 weeks	CW
		0.30 + 0.34	++	6 weeks	CW
		0.41 + 0.23	++	5weeks	CW
		0.51 + 0.11	++	5 weeks	CW
	Code 93	0.10 + 0.56	++	5weeks	CW
		0.20 + 0.45	++	5 weeks	CW
		0.30 + 0.34	++	4 weeks	CW
		0.41 + 0.23	++	4 weeks	CW
		0.51 + 0.11	++	4 weeks	CW
IBA + KIN	Code 71	0.10 + 0.54	++	6 weeks	CW
		0.20 + 0.43	++	6weeks	CW
		0.30 + 0.32	+++	5 weeks	CW
		0.41 + 0.21	+++	5weeks	CW
		0.51 + 0.11	+++	5 weeks	CW
	Code 93	0.10 + 0.54	++	6weeks	CW
		0.20 + 0.43	++	6 weeks	CW
		0.30 + 0.32	+++	5 weeks	CW
		0.41 + 0.21	+++	5weeks	CW
		0.51 + 0.11	+++	5 weeks	CW

Key: ++ = poor, +++ = good, ++++ = very good. CW – compact-white.

4.8 Callus Growth Curve

The growth curve of the embryogenic calli were investigated for the various PGR Combinations (Figure 4.2). From the results, all the PGR by genotype combinations displayed three growth phases, namely, log phase, exponential phase and linear phase. Deceleration phase was inconspicuous in all the combinations studied. The log phase lasted for 14 days after culture inoculation in all the combinations. This was followed by an exponential phase which continued up to the 42nd day post culture inoculation. The linear phase was observed between the 42nd day to the 70th day which marked the end of the experiment. Code 93 displayed a higher growth rate during the exponential phase, attaining higher fresh biomass weight at the onset of linear phase when compared with Code 71. In this study, the 42nd day was ideal day for transfer to new media to promote callus growth and proliferation. This is because there was no gain in fresh weight biomass achieved by increasing the number of culture days. Besides, browning was also observed in cultures which indicate prolonged exposure to media culture. The results infer that assessment of callus growth is necessary to determine point of transfer to new culture media (Plate 2).

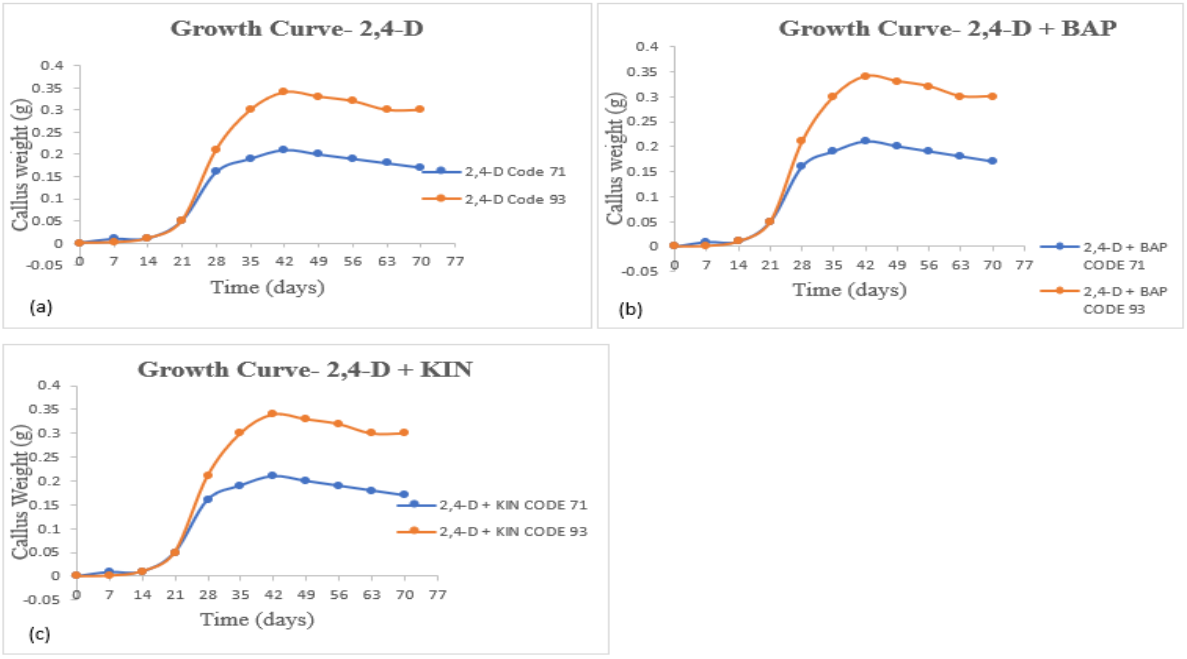

Figure 4.2: Growth curves of calli of Ruiru 11 cv.

Growth curves of calli of Ruiru 11 cv. Code 71 and Code 93 on different plant growth regulators (a) 2,4-D (b) 2,4-D + KIN and (c) 2,4-D + KIN. Growth was determined after every seven days of inoculation. Means followed by different letters are significantly different at $P = 0.05$ based on Fischer's Test ANOVA analysis.

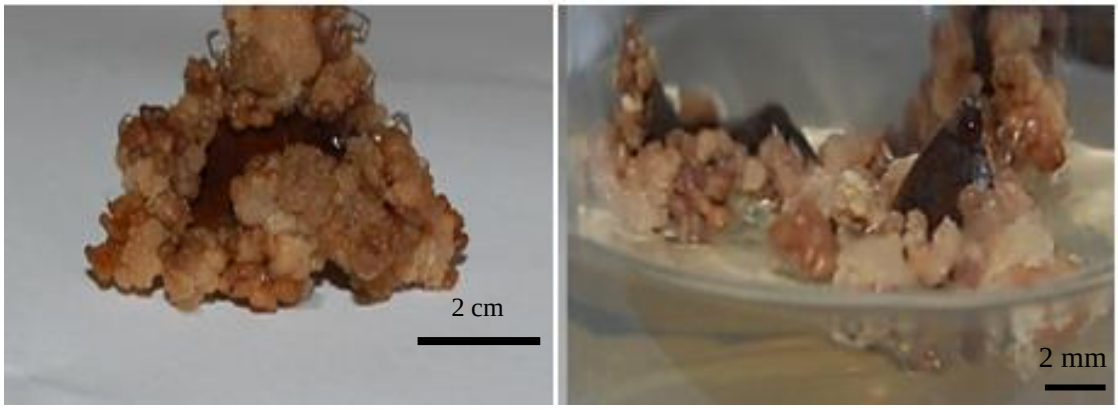

Plate 2: **Browning observations in culture media after six weeks of culture.**

4.9 Effect of Plant Growth Regulators on Callus Proliferation

Coffee Research Institute sought to adopt Temporary Immersion Systems that use liquid-based media to propagate Ruiru 11 to determine whether the technology is sufficient for the embryo induction and proliferation. The objective of the study, was to determine whether the Ruiru 11, a *C. arabica* species with a recalcitrance characteristic could adopt to the new environment and propagate embryos using TIS liquid-based system which is not currently in use at the CRI. Callus proliferation was observed in Code 71 when treated with 2,4-D + BAP (0.53 + 0.11 µM) with the same level of response realized in Code 93, 2,4-D (0.44 µM). Combination of 2,4-D + BAP further improved callus growth in Code 93, achieving vigorous callus growth with 2,4-D + BAP (0.53 + 0.11) treatment.

Callus growth was observed after 84 days (Code 71) and 91 days (Code 93) in treatment supplemented with 2,4-D at 0.33 µM and 0.44 µM respectively (Table 4.11). Code 71 showed slight response in callus proliferation with total somatic embryos derived being 15.111± 0.0009when treated with 2,4-D (0.33 µM). The response was lower with treatment of 2,4-D + BAP (0.53 + 0.11 µM) at 5.611±0.0015. Performance of Code 93 was slightly better in treatment with 2,4-D (0.44 µM) at 56.333±0.001. Treatment with combined PGRs with 2,4-D + BAP (0. 53 + 0.11 µM) produced 11.555±0.0020.

Table 4.11: Results on Callus Proliferation in *C. arabica* Ruiru 11 Leaf Explants.

Genotype	Plant growth regulator	Concentration (µM)	Time taken (days)	Mean No. of Embryos formed
Code 71	2,4-D	0.33	84	15.111± 4.315 [bc]
	2,4-D + BAP	0.53 + 0.11	77	5.611±3.889 [c]
Code 93	2,4-D	0.44	91	56.333±6.703 [a]
	2,4-D + BAP	0.53 + 0.11	82	11.555±9.309 [bc]

Means within a column followed by different letters are significantly different at *P = 0.05* based on Fischer's Test ANOVA analysis.

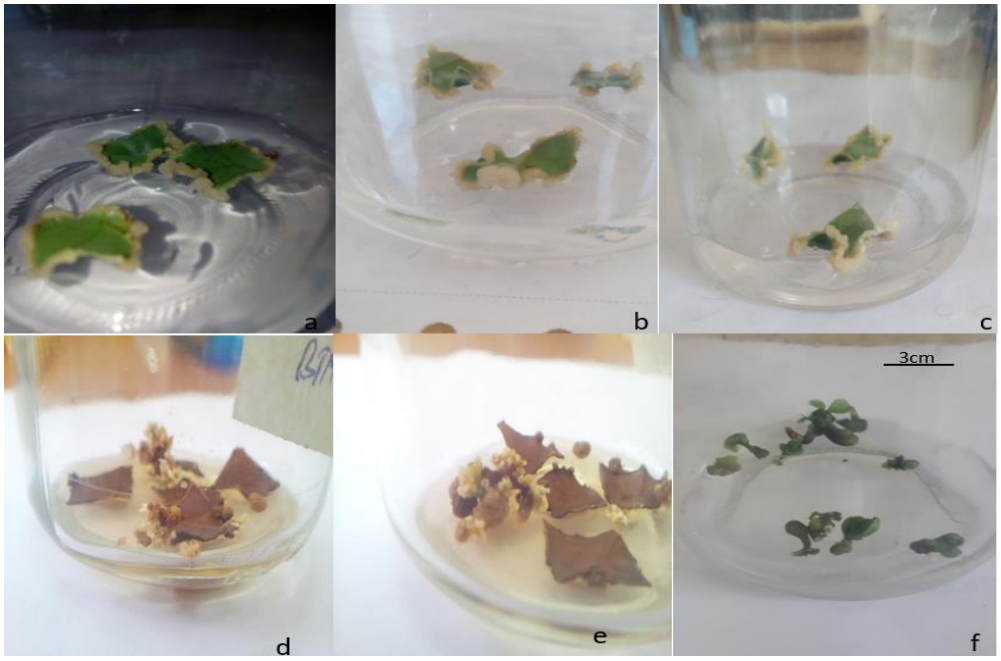

Plate 3*:* Callus Proliferation in vitro in ½ strength MS Media for C. arabica Ruiru 11. Callus Proliferation in vitro in ½ strength MS Media for C. arabica Ruiru 11 assessed in 90 days. (a) inoculated leaf squares in ½ strength MS media after 7 days of culture inoculation in MS media. (b) is the initiation of callus in leaf explants after 14 days (c) callus induction in leaf explants Ruiru 11 after 21 days (d) induced callus as obtained from MS media after 40 days with globular somatic embryos, (e) somatic embryo formation after 90 days with late-torpedo stage (f) somatic embryo cotyledonary stage showing development of two leaves in solid-MS media.

4.10 Optimal Inoculation Densities for Embryo

Results for the effect of cell densities on mean number of embryos produced are presented in Table 4.12. and Figure 4.3and Figure 4.4.

Highest embryo production was observed after 2 months in 0.0005 g/cm^3 in Code 93 at 94.222±0.0067and in Code 71 at 76.333± 0.0089 (Table 4.12). The least number of embryos were observed at density 0. 0025 g/ cm^3 where Code 71 produced 1.611 embryos. Higher densities produced no embryos in this Code. Similarly, Code 93 obtained the least number of embryos at a density of 0.003 gm/ cm^3 (1.389 embryos) with higher densities resulting into no

embryos produced. Code 93 recorded higher embryo induction numbers compared to Code 71 in any given embryo-inducing range of cell density (Figure 4.4).

Table 4.12: Effect of Inoculum Calli Density on Embryo Induction in Code 71 and Code 93 Ruiru 11 in RITA®.

Density (g/cm^3)	**Mean No. of Embryos (Standard Error ±)**	
	Code 71	**Code 93**
< 0.0005	0± 0^{d}	**0**± 0^{e}
0.0005	76.333± 6.703^{a}	94.222±5.679^{a}
0.001	20.111± 4.315^{bc}	35.833±5.679^{b}
0.0015	11.555± 9.309^{bc}	19.944±5.679^{bc}
0.002	5.611± 3.889^{c}	5.722±5.679^{cd}
0.0025	1.611± 1.481^{c}	3 ±5.679^{cd}
0.003	0± 0^{d}	1.389±5.679^{d}
0.0035	0± 0^{d}	**0**± 0^{e}
0.004	0± 0^{d}	**0**± 0^{e}
0.0045	0± 0^{d}	**0**± 0^{e}
0.005	0± 0^{d}	**0**± 0^{e}

Means within a column are significantly different at $P = 0.05$ based on Fischer's Test ANOVA analysis.

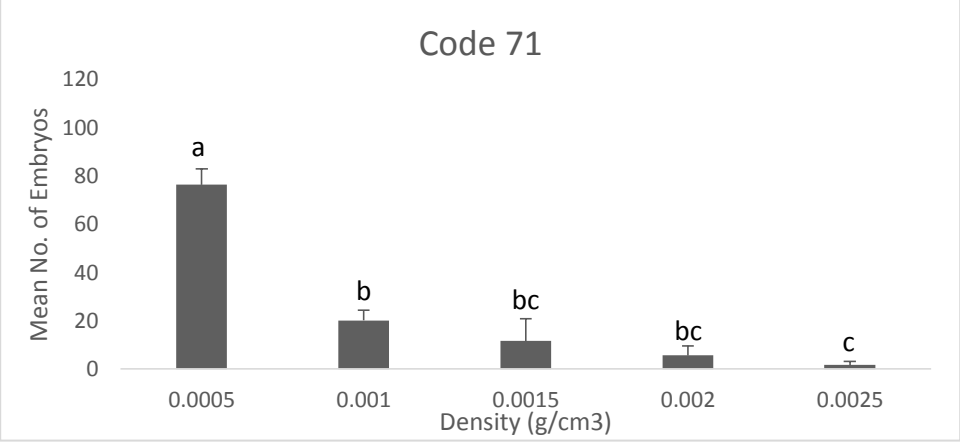

Figure 4.3: Effects of inoculum densities obtained from leaf callus cultures of C. arabica F1 hybrid, Ruiru 11.

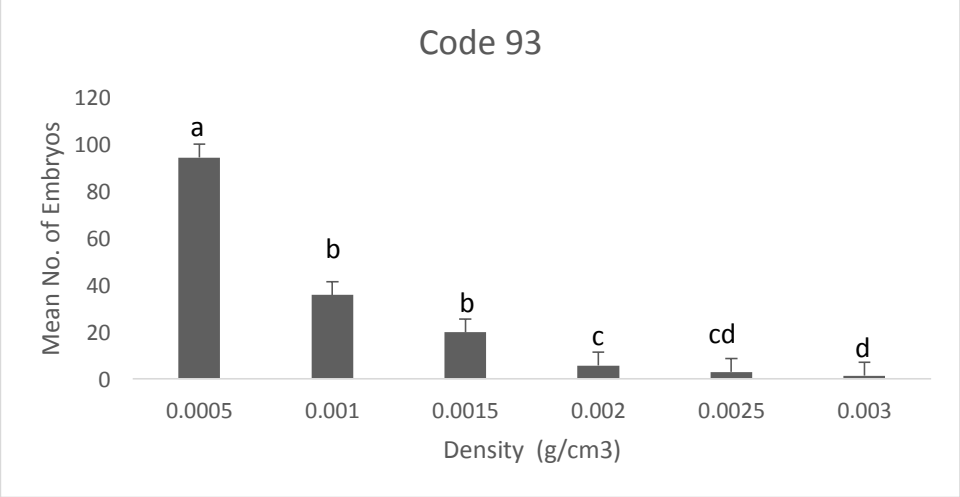

Figure 4.4: Effects of inoculum densities obtained from leaf callus cultures of C. arabica F1 hybrid, Ruiru 11.

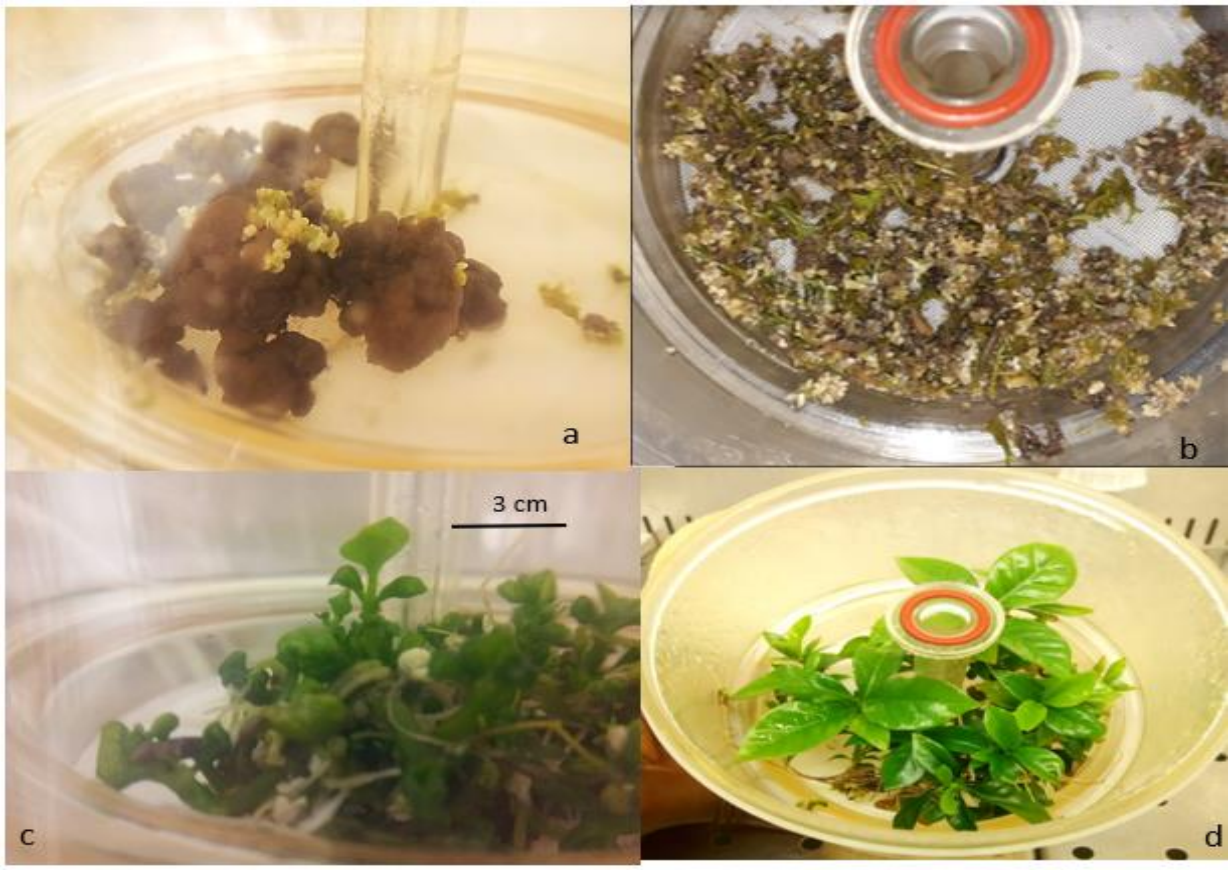

Plate 4: Embryo Induction and Maturation in 1-L RITA® after 120 days of calli inoculation**.** Embryo Induction and Maturation in 1-L RITA® after 120 days of calli inoculation**. (a**) Inoculated calli in 1-L RITA® showing induced somatic embryos with globular stage development, (b) Somatic embryos at torpedo and cotyledonary stages of growth and development in 1-L RITA®, (c) Cotyledonary somatic embryos and (d) Plantlets formed in 1-L RITA®.

4.11 Efficiency of Temporary Immersion Systems Compared to Solid-based Systems

4.11.1 Embryo Yield Capacity TIS Compared to Solid-Based Media

ISE in 1-L RITA® resulted in higher yield capacity compared to ISE in solid-MS Media. Embryo production in 1-L RITA® Code 71 induced a mean number of 76.333 and Code 93 induced at 94.222 in culture media supplemented with inoculum density 0.0005 g/cm$^{3.}$ (Table 4.9.). ISE in solid-MS media induced 15.111mean number of embryos in 2,4-D (0.33µM) in

Code 71 and 56.333in 2,4-D (0.33µM) in Code 93. The results show that ISE in 1-L RITA® had the highest embryo formation compared to ISE in solid-MS Media. 1-L RITA® hold more embryos compared to conventional glass-bottles.

4.11.2 Time Efficiency Comparison Between TIS and Solid-Based Media

Indirect SE using Temporary Immersion Systems, 1-LRITA® obtained embryo induction and formation after 170 days with 2,4-D (0.33 and 0.53 µM); in 2,4-D + BAP (0.53 + 0.11 µM), Code 71 induced embryos in 152 days and Code 93 in 162 days. ISE in solid-MS media induced embryos after 84 days in Code 71 in 2,4-D (0.33µM) and 90 days in 2,4-D (0.44µM) in Code 93. The results indicate that the somatic embryo formation period was shortest in indirect somatic embryogenesis in solid-MS media compared to indirect somatic embryogenesis in 1-L RITA®. The period accounts for the induction of callus period and embryo formation in 1-L RITA® and solid-MS media.

4.11.3 Resource-Use Comparison Between TIS and Solid-Based Media

Comparative analysis between Temporary Immersion Systems and solid-based technology was assessed. TIS utilizes liquid-based media whereas, solid-based utilizes gelrite- a solidifying agent. The cost of 1kg gelrite according to market value is Ksh. 20, 746.97 (Biochemie, 2021). Elimination of gelrite from the production process reduces the cost of production in TIS liquid-based media compared to solid-based methods leading to reduced resource input. Additionally, the 1-L TIS culture vessels have less space utilization (shelf-space due to the capacity to hold more embryo) and increased yield volume capacity compared to glass-bottle culture vessels for solid-based media (Appendix III). This translates to more capacity in yield output with reduced space utilization for TIS. The handling process in TIS is reduced compared to solid-based technology. With regard to transfer of cultures due to space limitation is reduced in TIS compared to solid-based technology. The number of sub-cultures in ISE in TIS was lesser compared to solid-based media. Based on the culture times in TIS, this was predominantly influenced by formation of phenolic compounds which can create a toxic environment for the plant cultures. A total of three subcultures (conducted after three-week intervals) were done in *C. arabica* Ruiru 11 compared to ISE in solid-based MS media which was done every four weeks due to space occupation by the plant cultures. In

general, the analysis shows that liquid-media use was higher compared to solid-based media in quantity however; the cost of producing liquid-media offsets the cost of using gelrite in producing solid-based media.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

In the study, varying level of success in callus induction was observed in MS media supplemented either with Auxin 2,4-D alone or when 2,4-D and Indole-3-butyric acid (IBA) were used in combination with Cytokinins. From the results obtained, two conclusions could be arrived at. In the first instance, the callus induction in the two coffee genotypes studied depend on the concentration level of the PGR used. This is true whether the auxins are used singly or in combination with cytokinins. For the concentration levels of 2,4-D above 0%, increased level of the PGR promoted increased callus proliferation, though at a declining rate. In Code 71, a peak concentration (0.33 µM) beyond which repressions of callus induction was observed. In line with this observation, it is necessary to determine the optimal concentration level of 2,4-D when using tissue culture techniques to mass propagate Code 71 or other Codes with similar response pattern, to avoid overreaching the optimum concentration point hence retarding callus induction rate in such family of Codes. The use of PGR beyond such optimum concentration levels would also unnecessary additional cost spend on the growth regulators hence increasing the cost of resulting seedlings. Code 93 did not show response profile similar to that observed for Code 71, While Code 71 increased with increasing 2,4-D concentration between 0.11 to 0.33 µM after which there was a decline in the callus induction rate, the response of Code 93 was linear, increasing with increasing concentration of 2,4-D through the whole treatment spectrum studied. This could indicate that the optimum 2,4-D concentration level for maximum callus induction in Code 93 fell beyond the range of the experiments. Further treatment including higher concentration levels of 2,4-D may be necessary to determine the point at which optimum callus induction occurs.

Single treatment with IBA which is an auxin did not promote any embryogenic response. This could be explained based on the mode of uptake. Auxin uptake is

dependent on an influx-uptake-efflux polar transport (Michniewicz *et al.,* 2007). Unlike 2,4-D, IBA is quickly utilised within the cells hence does not accumulate in the cells because the PGR is released with an efflux carrier whereas 2,4-D is taken up by an influx carrier but, is not released by an efflux carrier making the latter accumulate within the cells. Synthetic IBA is thus a biologically active regulator whereas, 2,4-D is a non-biologically active regulator (Michniewicz *et al*., 2007). Indole-derived molecules regulate growth and developmental processes either in embryogenic and post-embryonic development through accumulation and response by cells and tissues. The inability of IBA to accumulate in the plant cells in adequate quantities therefore makes it less useful in promoting embryogenic response when used singly. This indeed conforms to Gaspar *et al*., (1996) who concluded that IBA is rapidly metabolised in plant cells and IBA used singly is thus ideal for developmental phases that require less auxin such as rooting (Varshney *et al*., 2013; Ikeuchi *et al*., 2016). 2,4-D, therefore, is presented as the best alternative auxin in vascular differentiation.

Given that cultivar Ruiru 11 is composite in nature, comprising of different individual hybrids (Codes) with different genetic background, the results tend to suggest that one tissue culture protocol may not suffice in optimizing callus induction to levels necessary to undertake mass propagation of the variety. It is therefore proposed that the respective constituents of the variety be profiled with respect to their embryogenic propensities under different PGR treatments and subsequently clustered on the basis of their similarity in response to PGR treatment. Tissue culture protocols which optimise callus induction in such clusters would subsequently be developed. This will ensure that each of the constituent Codes of Ruiru 11 are represented in the desired proportions thereby ensuring the genetic constitution of the seedlings released for commercial planting by farmers maintain the requisite genetic fidelity.

Unlike auxins, the single treatment with cytokinins (BAP and KIN) did not yield any embryogenic response. The result is in line with the observation by Gapsar *et al*., (1996) who noted that cytokinins are suitable for cell division, with BAP and KIN being the most commonly used. On the other hand, the studies by Mun and Mun

(2016) and Etienne *et al*., (2018) successfully induced callus in leaf explants of *Rheum coreanum Nakai* and *Coffea canephora* respectively using KIN and BAP. This suggest that successful callus induction under cytokinin treatment is dependent on the genotype of plant species under study. *Coffea canephora* and *C. arabica* are different species within the Genus *Coffea* and the lack of response in the Arabica hybrids could be explained in the same fashion (Molina *et al*., 2002; Rezende *et al.*, 2012).

Cytokinins in combination with auxins either improved or reduced the rate of callus induction depending on the cytokinin included in the treatment. Auxin 2,4-D used in combination with Cytokinin BAP increased the rate of callus induction in both genotypes studied compared to the use of 2,4-D alone, with no point of callus repression reached (Table 4.1.2). This is in line with the conclusion by Gaspar *et al*., (1996) who observed that cytokinins are effective in callus formation when combined with auxins. The results may be explained by the fact that cell division is a joint action that requires a synergistic relation between auxins and cytokinins (Varshney *et al*., 2013; Ikeuchi *et al*., 2016) with cytokinin promoting cell division and auxins catalysing cell growth. The reason is that auxins regulate DNA replication while cytokinins exert control over events such as mitosis and cytokinesis (Durrani e*t al*., 2017). The fact that no point of callus repression was reached point to the need for experimentation with progressively increasing concentrations of 2,4-D and BAP combinations to identify the combination levels at which callus induction is optimised when studying coffee genotypes of comparable genetic origins.

The highest rate of callus induction in Code 71 of 88% achieved under 2,4-D (0.53 µM) +BAP (0.11 µM) combination was however the same level achieved using treatment with 2,4-D (0.33 µM) alone. Incorporation of cytokinin BAP in the protocol for inducing callus in this Code may therefore not be necessary within the range of treatments used. It is however a considered opinion that the optimum auxin-cytokinin concentration levels for Code 71 fell beyond the ones considered under this study and that further improvement in callus induction could be reached once such combination

is determined. Combining 2,4-D with BAP had a significant positive impact on callus induction in Code 93, attaining 100% induction rate for the combination 2,4-D (0.44) + BAP (0.23), again emphasizing the need to establish optimum PGR combinations for the different coffee genotypes under consideration.

The observed differences relating to PGR concentration and combinations suggest that for each combination, there exist optimum level of concentration at which the synergies of the combined PGRs is maximized, depending on the genotype under consideration. Similar conclusions have been reached in other studies including Gatica-Arias *et al*., (2008), Maciel *et al*., (2016) and Aga *et al*., (2017) in *C. arabica* callus induction using auxin and cytokinin combinations. In the current study, BAP combined with 2,4-D obtained highest induction rate compared to BAP combined with IBA. For instance, 2,4-D + BAP (2.5 + 0.5 mg/L) induced 88% in Code 71 and 100% in Code 93 whereas, IBA + BAP (2.0 + 1.0 mg/L) induced 75% in Code 71 and 75% in Code 93. This indicates that it is advantageous to use 2,4-D as the Auxin base instead of IBA when undertaking tissue culture in Arabica coffee. The results are however contrary to those observed by Etienne (2005), Gatica-Arias *et al*., (2008) and Menéndez-Yuffa *et al.*, (2010) who reported that the best callus induction was obtained in treatments supplemented with 2,4-D + KIN. The observed differences could be attributable to the nature of plant materials used in these studies.

For PGR to be effective, Gaspar *et al*., (1996) observed that endogenous PGR interacts with exogenous PGR by which exogenous PGR, and that the biological activity can either be equivalent to or in excess to endogenous PGR. The interaction with endogenous PGR is specific, and cell and tissue responses greatly rely on plant species, the genotype of species and explant source (Keiber & Schaller, 2018). In most cases, auxin and cytokinin interaction can either be synergistic or antagonistic, whereby, auxins inhibit cytokinin action and vice versa. In all cases in the current study, the treatments with IBA in combination with auxins did not lead to differences in response between the two Codes. The overall performance was likewise poorer that the treatments involving 2,4-D. IBA is therefore not a good candidate for mass tissue

culture propagation of Ruiru 11 hybrid. The observed results emphasise the need to determine the optimum combination and concentration levels of PGRs necessary to achieve maximum callus induction in coffee in a cost-effective manner. The second conclusion which can be made from the results is that the two genotypes under study responded differently to the treatments used in the study. The divergence in performance could be attributable to the differences in the genetic background of the two Codes studied. From the genealogy, the Codes differed with respect to the expected residual Timor (Hibrido de Timor and Catimor) genetic background, with Code 93 having a total of 62.5% Timor background and Code 71 having only 56.25%. Hibrido de Timor and its Catimor derivatives have considerable biochemical semblance to Robusta coffee *Coffea canephora* which itself is highly embryogenic. This difference in genealogy is therefore significant since it makes Code 93 more closer to Robusta, a highly embryogenic species compared to Arabica, with respect to the total genetic background than Code 71. A possible pathway through which this difference affected the rate of callus induction is through the production phenolic compounds deleterious to the process of callus formation. Arabica coffee is known to produce caffeine and chlorogenic acid under *in vitro* culture. The two metabolites have high callus repression properties in coffee (Nic-Can *et al*., 2015) and therefore the Code with closer genetic background to Arabica coffee, Code 71, could have suffered this phenomenon. This could have further been accentuated by the differences in the Catimor maternal parents through cytoplasmic effect. Catimor lines differ in their morphological expression with Catimor 86, the maternal parent in Code 71 being closer to the pure Arabica varieties in terms of traits such as organoleptic quality, an indication of their genetic proximity. In which case, the results observed could have been influence by the respective parental cytoplasm, Code 71 having closer resemblance to Arabica varieties and Code 93 being closer to the Robusta coffee.

The difference in embryogenic response to PGRs in Code 93 and Code 71 was varied based on assessed parameters in both TIS and solid-based system. From the results of the current study, 2,4-D used singly displayed increase in fresh biomass weight with

increasing concentration up to a peak point at which deceleration in biomass accumulation set. In the case of Code 71, peak biomass was observed at a concentration level of 0.33 µM after which sharp deceleration was registered. The same trend was observed for Code 93 except that the deceleration point was reached at a higher 2,4-D concentration level of 0.44 µM and the deceleration gradient much gentler as compared to Code 71. The results are quite similar to those observed for callus induction rates and may be explained in the same fashion as was done for impact on callus induction. Two conclusions could be reached based on these observations. Firstly, interaction between the exogenous PGRs and the endogenous counterparts depends on the concentration of the former and remains positive up to a point beyond which the interactions become deleterious leading to the onset of deceleration. In the current study, the point was reached at 2,4-D concentrations of 0.33 µM for Code 71 and 0.44 µM for Code 93. The second plausible conclusion is that each genotype/PGR combination has its unique point of deceleration which subsequently depends on the nature of interaction between the exogenous PGRs used and the endogenous ones. This point was reached at a lower concentration level in the case of Code 71 and was higher in Code 93. This is in conformity with the conclusion by Nic-can *et al*., (2015) that coffee somatic embryogenesis is highly dependent on the genotype of coffee, explant source, and type and concentration of plant growth regulator. Code 93 yielded higher biomass across all the treatments further attesting to the significance of genotype/PGR interaction in catalyzing embryogenic response from the test plants.

The results obtained have shown a strong inclination on the influence of auxins and cytokinins and concentration levels on callogenesis. This means that both PGR choice and concentration levels are an important consideration in the optimization of protocols for somatic embryogenesis in the species. The difference in growth response is also attributed to the diversity in the genealogy of F1 hybrid Ruiru 11 Code 71 and Code 93. Molina *et al.,* (2002) observed that coffee genealogy plays a crucial role in

embryogenic capacity in genotypes which is based on the genealogy of progenies and embryogenic response is under a strong genetic control (Orians, 2000; *Molina et al.*, 2002). Fresh weight biomass is an essential factor in successful induction of resultant embryos in *in vitro* culture (Haida *et al*., 2019). In the present study, PGR combinations 2,4-D and BAP induced the highest fresh weight biomass compared to other combination treatments experimented. From a comparison aspect, PGR used in combination induced the highest fresh weight callus compared to PGR used singly. Analysis of the optimum fresh weight of the callus produced across all treatments is paramount to determining the optimum callus biomass for callus production in the genotypes (Ashokhan *et al*., 2020). The callus biomass obtained in the induction stage has a great influence on the inoculum size used for proliferation of embryos in *in vitro* culture (Ashokhan *et al*., 2020). PGR combination 2,4-D and BAP induced highest biomass (0.53 + 0.11 µM). The difference in callus fresh weight biomass suggest that biomass induction is dependent on genotype. Code 71 produced the highest fresh weight biomass compared to Code 93.

This study observed induction of white callus across all treatments of Code 71 and Code 93. The influence of color on embryogenic potential is obscure and results indicate that callus color did not affect embryogenic induction competencies across treatments in Code 71 and Code 93. Similar observations were made by Maciel *et al.*, (2016) on *C. arabica*. However, optimization of coffee somatic embryogenesis depends on assessment of embryogenic (friable) and non-embryogenic (compact) calli. Embryogenic calli is suitable due to characteristic loosely aggregated cells of low densities which have high cell viability for embryogenesis (Leva *et al.,* 2012); whereas non-embryogenic calli lack cell viability for embryogenesis. The present study observed embryogenic calli formation in treatments supplemented with 2,4-D, 2,4-D + BAP and 2,4-D + KIN. Similar results were reported by Molina *et al*., (2002), Etienne et al. (2005), Maciel *et al*., (2016) and Aga *et al*., (2017) in coffee species leaf explants; Durrani *et al*., (2017) *Solanum spp*, and Gopitha *et al*., (2010) *Saccharum*

officinarum. Interestingly, the results indicated induction of friable and compact calli in PGR combinations across treatments of Code 71 and Code 93. Jiménez (2001) confers that embryogenic and non-embryogenic competence in callus formation in similar explants from genetically identical cells and tissues respond differently to varying stimuli which could explain the varying calli morphologies induced in Code 71 and Code 93 with IBA combined with BAP and KIN inducing non-embryogenic calli.

With regard to callus score, difference in degree of callus formation was observed in this study alongside induction time. Code 71 showed poor callus formation (++) with variation in induction time of 5-6 weeks; whereas, Code 93 showed good callus induction with variation in induction time of 3-4 weeks. Improved callus formation scores were observed in auxin-cytokinins combinations across treatments of Code 71 and Code 93. The results did not indicate any relationship between callus induction response rates to callus score and induction time. However, optimized callus induction also described as high-frequency somatic embryogenesis (HFSE) depends on induction time with shortest time taken alongside highest induction rate being ideal for callus induction. Time is a critical factor in HFSE as shorter induction time are superlative to longer induction periods (Samson *et al*., 2006). Highest induction rates at 2,4-D and 2,4-D + BAP in Code 71 and Code 93 showed good calli formation in 3 and 4 weeks respectively.

The difference in embryogenic response to PGRs in Code 93 and Code 71 was varied based on assessed parameters. From the results of the current study, 2,4-D used singly displayed increase in biomass weight with increasing concentration up to a peak point at which deceleration in biomass accumulation set in. In the case of Code 71, peak biomass was observed at a concentration level of 1.5mg/L after which sharp deceleration was registered. The same trend was observed for Code 93 except that the deceleration point was reached at a higher 2,4-D concentration level of 0.44 µM and the deceleration gradient much gentler as compared to Code 71. The results are quite

similar to those observed for callus induction rates and may be explained in the same fashion as was done for impact on callus induction. Two conclusions could be reached based on these observations. Firstly, interaction between the exogenous PGRs and the endogenous counterparts depends on the concentration of the former and remains positive up to a point beyond which the interactions become deleterious leading to the onset of deceleration. In the current study, the point was reached at 2,4-D concentrations of 0.33 µM for Code 71 and 0.44 µM for Code 93.

The current results conform to these optimum inoculum ranges for embryo induction in TIS using RITA®. Secondary embryogenesis was observed in TIS using RITA®, which may explain the increase in number of embryos produced. Analysis of callus proliferation in Code 71 and Code 93 showed varied proliferation rates in PGR auxin (2,4-D) and auxin-cytokinin (2,4-D + BAP) combinations. Code 71 showed an increased proliferation rate in 2,4-D + BAP (6.5%) compared to auxin, 2,4-D (5.4%). On the other hand, Code 93 indicated an increase in proliferation rate in treatments supplemented with 2,4-D (7.7%) compared to 2,4-D + BAP (4.2%). This indicates that response with respect to the duration necessary to obtain optimum degree and rate of callus proliferation depends both on the genetic background of the test material and the PGR combination used. In the case of Code 71, combined use of auxins (2,4-D) and cytokinin (BAP) was advantageous in reducing the number of days necessary to achieve optimum degree of proliferation and in increasing the rate of callus proliferation thereby improving the efficiency in terms of time and turnover rate. Incorporation of BAP in the tissue culture media involving Code 93 was only advantageous in reducing the number of days necessary to achieve optimum proliferation and increasing the degree of callus proliferation. Rate of proliferation was however reduced. For mass propagation of composite coffee hybrids such as cultivar Ruiru 11, the results are significant and point to the need to first cluster the respective genotypes in the composite hybrid based on their response patterns. Separate protocols can then be developed for each of the clusters and the clusters propagated separately

and appropriate admixtures constituted with the desired ration of each of the cluster groups. This is to ensure that the genotypes which are otherwise shy to TC manipulations are not eroded as would be the case if all the genotypes are propagated using the same protocols. Several studies have observed callus growth regarding effects of plant growth regulators in different plant species. Similar observations were made by Maciel *et al*., (2016) in *C. arabica* and Mahajan *et al*., (2016) in *Picorrhiza kurroa*. Mun and Mun (2016) observed increased callus growth in media supplemented with 2,4-D + BAP compared to media supplemented with 2,4-D in *R. coreanum*.

Initial cell density is a critical factor in production of embryos in liquid media (Ducos *et al.,* 2007). Reports on initial cell densities indicate that low densities are ideal whereas, high inoculation densities are not ideal as they strongly inhibit embryogenesis. In the case of this study, the highest embryo induction rate was observed at explant inoculum density of 0.0005 g/cm^3 for both Code 71 (76.333 embryos) and 93 (94.222 embryos) with steep decline in the rates observed at higher inoculum densities. Inoculation densities above 0.0035 g/cm^3 were observed not to induce embryos in Code 93 and Code 71. Decreasing proliferation rate with increasing inoculum density is known in many plant species and can be attributable to increased competition for nutrients among the embryos under high density and increased exudates and phenolic compounds inhibitory to callus induction (El Boullani *et al*., 2017; Mazri, 2013). The data indicates that inoculum mass of 0.1 g (0.0005g/cm^3) is superlative for successful embryo induction in liquid media in TIS RITA®. Ducos *et al*., (2007) reported ideal inoculum density to be at 0.5 – 1.0 g in *C. canephora*, Perez *et al*., (2013) reported ideal inoculum density at 1.5 g in *Quercus suber* (cork oak) and Maciel *et al*., (2016) reported use of 200 mg in *C. arabica* to induce embryos. Secondary embryogenesis is an important factor in coffee SE under large-scale production and is characterised by repetitive embryogenesis in which secondary embryos develop from cells within the plant cultures (Landey, 2013). Therefore,

induction efficiencies in TIS using RITA® in embryo production is 763.3 in Code 71 and 943.3 per gram of callus.

5.2 Conclusion

The present study determined the optimisation of somatic embryogenesis in F1 hybrid Ruiru 11 Code 71 and Code 93. Indirect SE shows a more efficient process compared to Direct SE. The study revealed that utilization of indirect somatic embryogenesis is suitable for large-scale production of somatic embryos compared to direct somatic embryogenesis. Also, the study proves that implementation of TIS, RITA® alongside indirect somatic embryogenesis is possible and can result in increased embryo production. The first research objective and hypothesis focused on effect of plant growth regulators concentration on callus induction in Ruiru 11 F1 hybrid, and results show that PGR have an effect on callus induction. Callus induction was observed to be influenced by plant growth regulators and genotype. Optimal callus induction was observed at 2,4-D + BAP (2.5 + 0.5 mg/L) in Code 71 and Code 93 based on high embryogenic response rates according to biomass fresh weights, induction rates and embryogenic callus formation factors. The results refute the second hypothesis on effect of auxins on callus proliferation in Ruiru 11 F1 hybrid. Optimal inoculum density was obtained at 0.0005 g/cm^3 in Code 71 and Code 93- which refutes the research hypothesis that callus densities have not effect on embryo induction in Ruiru 11 F1 hybrid. Additionally, the study observed uniform somatic embryogenesis in Indirect somatic embryogenesis compared to direct somatic embryogenesis which showed non-uniform embryogenic response in experimental units in Code 71 and Code 93. The divergence in response patterns observed between Code 71 and Code 93 suggest that Ruiru 11 variety may not be treated as homogeneous when undertaking mass propagation of tissue culture seedlings for commercial purposes. Instead, the synthetic nature should be given consideration by clustering the variety into groups with similar response patterns and appropriate protocols developed to optimize mass production of the variety.

This knowledge permits for adoption of new and optimized induction protocols and regeneration protocols at CRI Tissue Culture Laboratory with the intent to improve mass production to meet current demand for coffee planting materials. The regeneration protocols may open-up opportunities for improvement of the coffee varieties in line with maximizing quality of coffee planting materials *in vitro*.

5.3 Recommendations

1. Overall, the present study was successful in achieving optimization of somatic embryogenesis via ISE using TIS. However, improvements specific to modification of the protocol is necessary to fully appreciate the advantages of TIS in coffee somatic embryogenesis.

2. Further studies need to be conducted with regard to optimization of optimal PGR concentration levels ideal for improved ISE. The present study observed critical peak points in treatments supplemented with 2,4-D and 2,4-D + BAP in Code 71 and Code 93. The observations indicate that optimal concentration levels of PGR are obtained prior to the peak. Therefore, it is recommended to address this with the objective of determining the optimum concentration levels.

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APPENDICES

Appendix I: Preparation of MS Media Stock Solutions as used in the experiments.

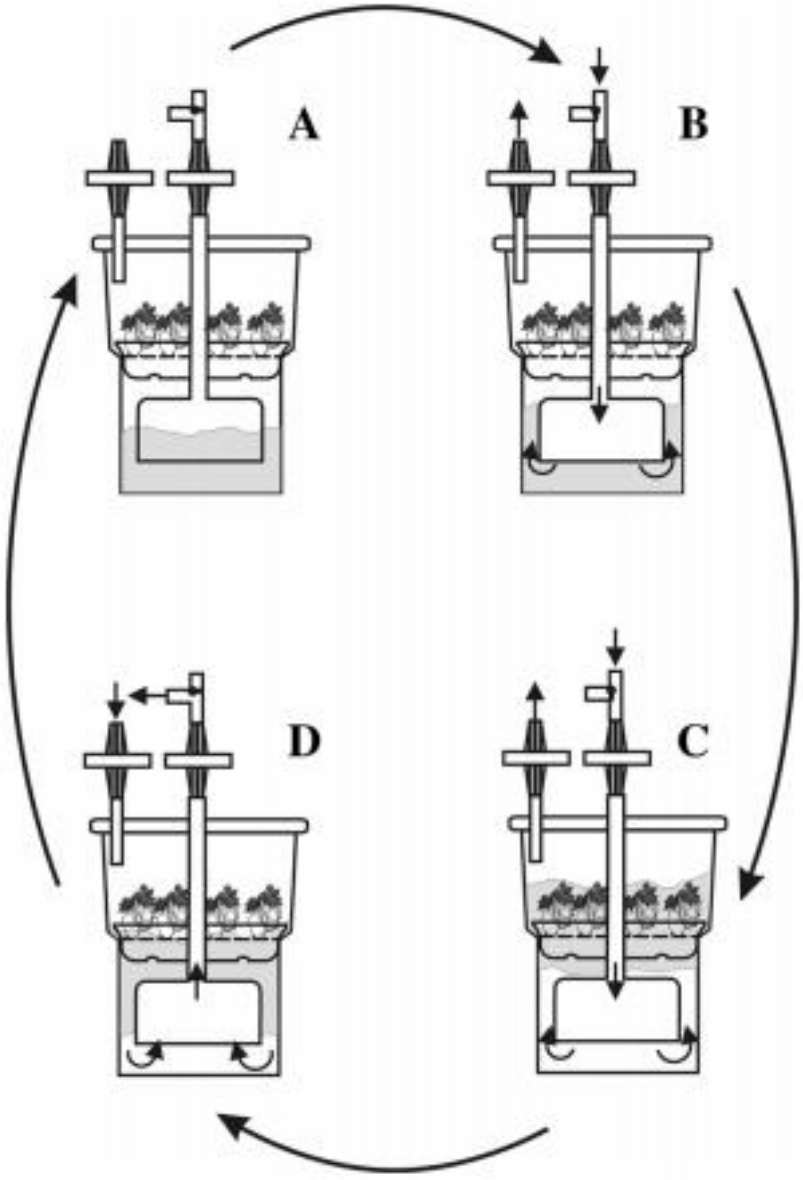
Stock Solution	Components	1x conc. In mg/L of distilled water
Stock solution A	NH_4NO_3	82.5
Stock solution B	KNO_3	95.0
Stock solution C	H_3BO_3 KH_2PO_4 KI $Na_2MoO_4.2H_2O$ $COcl_2.6H_2O$	1.24 34 0.166 0.05 0.005
Stock Solution D	$CaCL_2.2H_2O$	88.0
Stock Solution E	$MgSO_4.7H_2O$ $MnSO_4.4H_2O$ $ZnSO_4.7H_2O$ $CuSO_4.5H_2O$	74 4.46 1.72 0.005
Stock Solution F	Na_2EDTA $FeSO_4.7H_2O$	7.45 5.57

Stock Solution G	Glycine	0.4
	Nicotinic acid	0.1
	Pyridoxine-HCL	0.1
	Thiamine-HCL	0.2

Appendix II: Standard Operating Protocol

Procedure:	Tissue Culture Work
Section 1:	Personal Protective Equipment
- Laboratory coat - Gloves for handling - Proper enclosed footwear. - Hair tied or wrapped.	
Section 2: Procedure	
- The laminar flow hood light and fan was turned on for a duration of twenty minutes prior to using the equipment. - Laminar flow hood was cleaned and disinfected using cotton wool soaked in ethanol (70%) prior to use. - Every item used in the tissue culture laboratory was labelled including the name and the date. - Dissecting tools were sterilized before and after use in an autoclave for 15 minutes under 121 psi. - Prepared culture media was labelled with regards to the growth hormone and date of production and stored in a cold room at 4°C.	

Appendix III: Temporary Immersion System, 1-L RITA® Operation Process



The figure represents the technological design and operational principle of RITA®. (A) period of exposure; (B) Dislocation of liquid medium. Air pressure is applied to the bottom compartment through the central pipe. The liquid medium is moving to the upper compartment; (C) period of immersion; (D) draining out the nutrient medium. The air flow is

stopped and the medium flows back to the bottom compartment due to gravity. Source: Georgiev *et al*. (2014).