DIRECT ORGANOGENESIS AND CALLUS INDUCTION OF COCONUT FROM SEED EMBRYO FOR MASS PROPAGATION

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MASTER OF SCIENCE

(Biotechnology)

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

2021

Direct organogenesis and callus induction of coconut from seed embryo for mass propagation

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology of the Jomo Kenyatta University of Agriculture and Technology

2021

DECLARATION

This thesis is my original work and has not been presented for any award in any university.

Signature…… Date…….……

Caren Chepkemoi Bett

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

Signature……..... Date……

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JKUAT, Kenya

DEDICATION

I dedicate this work to my loving husband Gilbert, my daughter Agatha and my son Marcus and my parents, Elon and Caroline Too. Thank you for your immeasurable support.

ACKNOWLEDGEMENT

I wish to acknowledge the Almighty God for the unmerited favour and good health he granted me during the entire research period.

I indebtedly acknowledge my supervisors: Dr. Cecilia Mweu and Prof. Aggrey B. Nyende for their unwavering support, encouragement and dedicating their time to refine my research work, preparing the scientific manuscript, and compiling the final thesis.

My gratitude goes to the National Research Fund (NRF) and the Research Production Extension (RPE) Division of JKUAT for the financial support accorded to me during the research.

I wish to thank Dr. Neondo, Mr. Rotich, Ms. Kidaha, Ms. Njambi, Ms. Catherine and Mr. Kamau for their excellent commitment and support by assisting and availing the relevant information and priceless technical assistance towards the fulfillment of this research.

I also acknowledge my friends June, Liz and other colleague students Eunice, Ng'etich, Agnes, and Maggie who assisted me in one way or another.

Finally, I am grateful to Mr. Steven (greenhouse attendant at IBR-JKUAT) for assisting me in setting up greenhouse experiments and teaching me some practical skills.

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LIST OF ABBREVIATIONS

BAP	6-Benzylaminopurine
CIM	Callus Induction Media
2,4-D	2,4 dichlorophenoxyacetic acid
GA3	Gibberellic acid
IBA	Indole-3-butyric acid
KIN	Kinetin
MS	Murashige and Skoog
NAA	α -Naphthaleneacetic acid
PGRs	Plant Growth Regulators
SE	Somatic embryogenesis
TDZ	Thidiazuron
Y3	Euwens 1976 media
µM	Micromolar

ABSTRACT

Coconut (*Cocos nucifera* L) is an important crop in many tropical countries both for food and as a cash crop. However, coconut production in Kenya is facing challenges including lack of enough planting material, diseases and low productivity due to the senile coconut plantations. Therefore, higher efficiency of plantlet production via *In vitro* techniques is required for mass propagation. The purpose of this study to evaluate the regeneration potential of coconut plants via direct organogenesis and indirect somatic embryogenesis for mass production of clean planting material. For regeneration through direct organogenesis and indirect somatic embryogenesis in Euwens Y3 media, embryo explants derived from 9-12 months old coconuts were used. Direct organogenesis resulted in embryo germination and development on Euwens Y3 media supplemented with 0.28µM 2 4D, 0.44µM BAP and 0.03µM GA3 after 16 weeks of culturing in darkness. Regeneration through indirect somatic embryogenesis was established in Euwens Y3 medium supplemented with 100 to 250µM 2, 4 -dichlorophenoxyacetic acid (2, 4-D) alone and in combination with 0.35µM and 0.5µM Gibberellic acid (GA_3), 5µM 6-Benzylaminopurine (BAP) and 9µM Thiadiazuron (TDZ). The highest callus induction was observed in medium containing 150µM 2, 4-D + 5µM BAP while the least was in 2, 4-D alone, and in combination with 0.35µM GA_3. Highest embryogenic calli were observed in media containing 75µM 2, 4-D + 5µM BAP and the least was in medium containing 2, 4-D alone and in combination with 0.35µM GA_3. Multiple shoot maturation was observed in medium containing a combination of 10µM kinetin, 10µM BAP, 0.5µM GA3, and 200µM NAA. Maximum shoot elongation (3.63cm) was recorded in medium containing 10µM BAP and the least (3.06 cm) was in medium with 5µM BAP. Rooting was done in Euwens Y3 medium containing Indole-3-butyric acid (IBA). The highest response was observed in Euwens Y3 medium supplemented with 5µM IBA+ 0.5µM GA_3 both with respect to the number of roots 8.33 and root length 5.10 cm while the least was with 15µM IBA+ 0.5µM GA_3 with regard to the number of roots (3.33) and root length 2.83cm. Tissue culture plantlet acclimatization was achieved in soil: sand: manure ratio (3:1:1) gave a 60% survival rate and vermiculate medium had 50% survival rate. Hence, *In vitro* regeneration of coconut through somatic embryogenesis

and direct organogenesis is possible and can be used as an alternative to provide tissue culture plantlets for mass production.

CHAPTER ONE

INTRODUCTION

1.1 Description of the coconut

Cocos nucifera L. belongs to family Arecaceae and the genus Cocos. It is the only species in the genus cocos and it grows to a height of up to 30m tall with pinnate leaves growing to up to 90cm, having a smooth and columnar trunk (Pannetier & Buffard-Morel, 2012). The life spans of *C. nucifera L.* varies depending on the type. The tall variety can last up to 90 years while the dwarf variety lasts for 50 years. Coconut has three main varieties; tall, dwarf and hybrid (Gachanja et al., 2007; Predeepkumor et al., 2008). The tall variety is mostly cultivated along the coast because of its tolerance to diseases and drought. It is self-pollinating in nature and the nut production starts at 5 – 7 years. On the other side, the dwarf variety also mainly self-pollinating with reduced growth habitat. They grow to a height of 5-10m and nut production begins at 3-5 years. It requires substantial amount of rainfall and well-drained soil (Dasanayaka et al., 2009). Many efforts are ongoing in coconut-growing countries to conserve the rich natural diversity existing in coconut germplasm collections for further utilization in crop improvement programs so that it becomes a more profitable crop for small-farm holders who constitute the vast majority of coconut growers (Batugal & Oliver, 2005). The hybrid variety (*minazi chotara*) has the characteristics of both tall and dwarf varieties. It can live to around 60 years and it starts bearing fruit at the age of 4-5 years. Like the dwarf variety, it requires lots of rainfall and well-drained soil.

1.1.1 Coconut Production and Utility

Coconut plant is a popular plant due to its versatility and utilization of the different parts. It naturally grows in the tropical and sub-tropical climatic conditions found in Africa, India, and Indonesia (Duke, 1983). In Kenya, coconut plant grows along the coast where it is grown for food and as cash crop and for roofing traditional houses. Coconut fruit is rich in vitamins and minerals that are useful. Coconut oil is used for skin care, and hair food in cosmetics and medicinal purposes. In medicine, the fruit is

used to treat a wide range of diseases such as viruses of influenza, measles, hepatitis C, herpes, colds, cough, sore throat, bronchitis, tuberculosis, typhoid, and many others (E. Chan & Elevitch, 2006a). The accumulation of galactomannan in the maturing nut has many novel elite applications in the food, medical and pharmaceutical industries (C. B. Nguyen et al., 2010). The coconut milk and flesh restores the energy and enhances the physical state of the body, rejuvenate it and improve eyesight. Activated carbon extracted from coconut fruit shell can be used as fuel. Coconut oil has health benefits such as strengthening hair follicles and removal dandruff. Both oil and milk have a favorable effect on the human skin, moistening, softening and smoothing out the wrinkles. The coconut flesh, due to the lauric acid content, normalizes the cholesterol level in blood; it is also a good antimicrobial and antiviral agent (Ogbolu et al., 2007). It protects against the ultraviolet radiation and does not have any harmful side effects. In Kenya, coconut wine (popularly referred to by Swahili name as '*mnazi*') is a common beverage for a majority of the coastal people. Other products include nuts, leaves for roofing (Swahili dialect term is '*Makuti'*), brooms, coco wood, and copra which are processed into oil mainly for the soap industry, cosmetics, and candle wax (Muhammed., 2013) Coconut trunks are used for house construction. Coconut water contains sugar, proteins, antioxidants, vitamins and minerals and provides an isotonic electrolyte balance while the coir obtained from the fiber husk of the coconut is useful for making ropes, mats, brushes, sacks, and stuffing fiber for mattresses (Gachanja et al., 2007).

1.2 Economic importance

Coconut has several commercial and traditional uses. Coir (the fiber from the husk of the coconut) is used in ropes, mats, brushes, sacks, as caulking for boats, and as stuffing fiber for mattresses. It also used in horticulture for making compost. Coconut leaves are used for making brooms, baskets, mats and kindling arrows, as well. Copra is the dried meat of the seed and after processing produces coconut oil and coconut meat. Coconut oil, aside from being used in cooking, it is also used in detergent industries. Coconut oil contains lauric acid (which has high efficacy in detergent and surfactant manufacture) can be processed to extract sodium lauryl sulfate to produces shower gels and shampoos. Coconut husks and shells can be used for fuel (Muhammed

et al., 2013a). Activated carbon manufactured from coconut shell is considered extremely effective for the removal of impurities. Coconut trunks are used for building small bridges and huts; they are preferred for their straightness, strength, and salt resistance. The roots are used as a dye, mouthwash and medicinal extracts for diarrhea and dysentery. The coconut shell may also be ground and used as an additive for dead skin exfoliation products. Previous research has provided the newer application of coconut oil in the precautionary treatment of not only heart diseases (Lindeberg & Lundh, 1993) but also health complication such as diabetes, cancer and other infectious diseases. It is now gaining popularity in being used as medicine, foods and cosmetics (Peat, 2004). Coconut oil contains four medium-chain fatty acids MCFAs, namely lauric acid (C-12, 48-53%), capric acid (C-10, 7%), caprylic acid (C-8, 8%), and caproic acid (C-6, 0.5%) which are transformed into corresponding monoglycerides which can kill pathogenic microorganisms including bacteria, fungi, yeasts, viruses and protozoa. Lauric acid has also been shown to boost immunity for infants during the first six months of life when their immunity is still low (Enig, 1996).

1.3 Production constraints in Kenya

Coconut farming is facing major challenges in the country. These constraints include high cost of inputs, limited availability of clean planting materials, pests and diseases, poor agronomic practices, poor knowledge base, poor market access, low farm-gate prices and low yields. The processors constrains include lack of modernized processing equipment and inadequate skills. Marketing constraints include high transport costs, inadequate storage facilities, lack of skills in preservation and packaging (Mwachiro, 2011). Currently, major constraints faced by farmers include lack of access to enough clean planting materials where they generally rely on their current crop to reproduce seedlings. The linkage between the farmer and the processor has not been fully exploited resulting in low yield (productivity) and quality of coconut fruits (Mwachiro, 2011). Besides, lack of a reliable marketing system for coconut products exposes farmers to low price margins when they sell their products on an individual basis. This has created an opportunity for exploitation by the intermediary traders, known as middlemen, who have continued to reap the maximum benefit from the products. Absence of structured marketing cooperatives denies farmers the

negotiating ability and opportunity to sell their products beyond the local centers or at the farms. The same case is witnessed by coconut farmers across the East African region where value addition and marketing framework lack or weak support through government policies (Mwachiro, 2011).

In addition, there are no well-established technologies for mass propagation and dissemination of coconut in Kenya (Gachanja et al., 2007). The challenge of pest and diseases has also constraint the coconut sub-sector. Several pests diseases reduce the productivity of the plant, therefore, it cannot reach the optimal level of production (Mwachiro, 2011). These include bole rot disease caused by *Marasmiellus cocophilus* Pegler, sp. nov. which is the main cause of many dead standing trees in the coastal part of Kenya. It affects the young seedlings up to 8 years old (Warui & Gethi, 1980). Insect pests including rhinoceros beetle Orctes monoceros (Huger, 2005) locally known as "chongwa" and the coreid bugs which damages terminal buds interfering with plant growth (Gitau et al., 2009; Gopal et al., 2001; Kandan et al., 2010). Lethal yellowing disease was first identified in Jamaica, it is caused by mycoplasma (Maust et al., 2003; Nipah et al., 2007). Similar diseases affect palms in West Africa, Tanzania (East Africa), part of southern India but luckily the disease hasn't been reported in the coastal part of Kenya but instead, there is a lethal disease which has the same effect and symptoms (Harrison et al., 2014).

1.4 Problem Statement.

In Kenya, the lack of access to quality planting materials is the main challenge facing coconut farming (Mwachiro, 2011). Farmers use seeds exclusively from the coconut parent plant (current crop) to reproduce coconut seedlings. Although coconut has a high local socioeconomic reputation, its production is experiencing many challenges and consequently, the area planted with this crop is declining. The conventional breeding approach using seed to replant land is very expensive due to the low production multiplication of seed coconut for planting, and even when elite germplasm is available it takes decades to multiply up enough planting material for new areas (Nguyen et al., 2015). In addition, the linkage between the farmer and the processor has not been fully exploited due to low-quality coconut seeds (raw materials) production (Muhammed et al., 2013). Aged/senile orchards that are poorly managed,

high pest infestations and disease in existing orchards excessive harvesting of old coconut orchards for timber and young nuts (Oduor & Githiomi, 2006) finally, inadequate technologies for mass production of clean seedlings which can be distributed to the farmers for planting.

1.5. Justification of the study

Coconut is currently propagated mainly through coconut seeds (Perera et al 2009). Tissue culture of coconut is of prime importance for rapid multiplication and distribution of the best genotypes obtained through conventional breeding such as selected parental exhibiting resistance to biotic and/ or abiotic stresses and for increasing the yield. It also increases the number of quality planting materials to enhance plant yield, the areal extent of farming fields and supports large scale planting. Tissue culture technology is a reliable technique that has enabled the availability of quality and quantity planting materials. In addition, appropriate field management of tissue cultured plantlets reduces yield losses caused by pests and diseases. Tissue culture technology has enabled farmers to access large quantities of superior (quality) seedlings that are early maturing compared to the conventional ones and better yield. Hence this research aimed to evaluate the regeneration of coconut via direct organogenesis and indirect somatic embryogenesis for mass propagation of clean planting material which allows the production of multiple plantlets from a single embryo which will then increase the quantity of seedlings for future expansion of coconut farming and contribute to food security and income generation in the coastal region and Kenya at large. Micropropagation enables mass production of plantlets in a short period with characteristics such as fast growth with a short and uniform production cycle. Thus replanting of this region with new, high yielding, disease-resistant cultivars will be an important part of re-establishing the traditional coconut-based farming system both to the individual farmers who will benefit directly from it and to the coastal region at large who rely directly or indirectly on coconut to boost their economic value.

1.6 Objectives

1.6.1 General objectives

In vitro regeneration of the Kenyan coconut germplasm for mass propagation

1.6.2 Specific objectives

1. Evaluating the *In vitro* regeneration of coconut via direct organogenesis.
2. Investigating the *In vitro* regeneration of coconut through indirect somatic embryogenesis using different hormonal media combination and concentrations.
3. Assessment of the coconut tissue culture seedlings acclimatization on different soil media in the greenhouse.

1.7 Null hypothesis

1. No difference exists in regeneration of coconut when using different organogenesis regimes and different hormonal concentrations.
2. No difference exists in regeneration of coconut during indirect somatic embryogenesis using different hormone combination and concentrations.
3. There is no difference between the performances of the tissue-cultured plantlets using different acclimatization media in the greenhouse.

CHAPTER TWO

LITERATURE REVIEW

2.1 Origin and Distribution

Coconut (*cocos nucifera L*) has been referred to as a 'tree of life' because it is an important cash crop which provides almost all the necessities of life such as food, beverages, oil, medicine, construction materials, fuel and even utensils for domestic use (Bandupriya et al., 2016). It is an important palm in tropics and it provides a source of livelihood to more than 50 million smallholder farmers worldwide. It is grown in more than 12 million ha in 90 countries worldwide mainly in the Asian pacific regions. Indonesia is ranked as the largest coconut growing region followed by Philippines and thirdly India (Exposomics, 2017). In Kenya, coconut has a variety of uses locally where the coconut water is commonly known as 'mnazi' is used as refreshment and it's highly rich in energy. The coconut also contains proteins, antioxidants, vitamins and minerals other uses include the copra meat and copra oil which are processed into oil for cosmetic, food and even soap industries. The coconut leaves are used for roofing and for making brooms (Muhammed et al., 2013)

In distribution, coconut is almost exclusively found in the coastal part of Kenya. However, recent studies have shown that there is potential for coconut farming in other parts of the country. Piloting trials have indicated encouraging results in other places like in Makandume village near Mututa swamp in central Imenti, there are 500 coconut palms of East African Tall variety that sits in a 10-acre land. The area has a warm and humid climate with temperatures rising to 28 degrees Celcius hence creating the perfect ecological conditions for coconut farming. Nyeri County will soon be planting coconut trees in Kanyai forest in Kieni West constituency. The county has launched a Sh15 million program funded by both Nuts and Oil Crops Directorate and the National Drought Management Authority (NDMA) where more than 2,000 farmers will benefit from it (Daily Nation, 11 September 2017). Other areas where coconut thrives well in Kenya include Nyanza, Western and parts of the Rift Valley.

2.2 Ecology

A year-round warm and humid climate favours the growth of coconut. Mean annual temperature of 27°C and evenly distributed rainfall of 1500–2500 mm per annum, and relative humidity above 60% provide the ideal climatic conditions for the vigorous growth and yield of the palm. A permanent water table within easy reach of the coconut roots can offset inadequate rainfall, while amounts above 2500 mm could result in diseases of the fruit and leaves. Such conditions are normally found in 20 C° north and south of the equator (Chan, 2006). Periods with mean daily temperatures below 21°C adversely affect the growth and yield of the coconuts. Frost is fatal to seedlings and young coconuts when the growing point is still close to the ground. At the equator, coconut can grow and yield well up to an altitude of 600 m (1970 ft) but will only do so at sea level at latitude 23 C°. At the extremes of the latitudinal range, coconut only grows well on the coast of large landmasses (e.g., east coast of Australia, Africa, South America, etc.) and on islands where the sea exerts a moderating influence on temperature and humidity (Chan, 2006).This palm has a remarkable ability to adapt to a wide range of soil types. Although coarse sand is its natural habitat, best growth is obtained on deep soils with good physical and chemical properties. It is thus widely grown on loams as well as well-drained clays. It requires free drainage; it tolerates alkaline soils up to pH 8 (on coralline atolls) and acid soils with pH 4.5 or higher. The ideal pH range is 5.5–7. Coconut grows on a wide range of light, medium, and heavy soils. It tolerates saline and infertile soils (Chan, 2006) but tolerates drought poorly. Symptoms include desiccation of older fronds, spears (emerging fronds) failing to open normally, and shedding of young nuts. It thrives in full sun. Coconut grows under high levels of shade, but yields are severely affected. It does best when not shaded. Young palms will succumb to fire, but mature palms will often survive if the canopy is far enough above the fire to escape the flames. Coconut does not grow well in a waterlogged environment above 1m (3.3ft) from the earth surface. It prematurely dies within two weeks. Coconut is, however, able to withstand salt spray and cyclonic (hurricane) winds, once it has anchored its roots are well into the ground. Flexibility in the stem and fronds reduces the cross-sectional area presented by each tree and thus reduces the drag forces they must endure. In heavy storms, fronds facing or perpendicular to the direction of the wind tend to snap off close to the base. This

reduces drag forces tremendously and helps coconuts survive storms. Most coconuts survive severe storms, although some are uprooted where there is not sufficient rooting depth. In some varieties a percentage of trees will snap off at the base of the root (Chan, 2006).

2.3 Taxonomy and structure of coconut

The cocos nucifera L belongs to kingdom Plantae, Phylum spermatophyte, Classmonocotyledonae, Order arecales, Family Arecaceae, Genus Cocos and species: nucifera (L). There are two types of coconut in Kenya which include *cocos nucifera* L. var .typica (tall variety) and *cocos nucifera* L.var nana (dwarf).

The coconut fruit consists of three layers namely, the exocarp which is smooth and green, mesocarp mainly containing husk and endocarp which surrounds the coconut flesh/meat. Generally, most consumers recognize the coconut by its endocarp, as sold in many grocery stores (Figure 1).

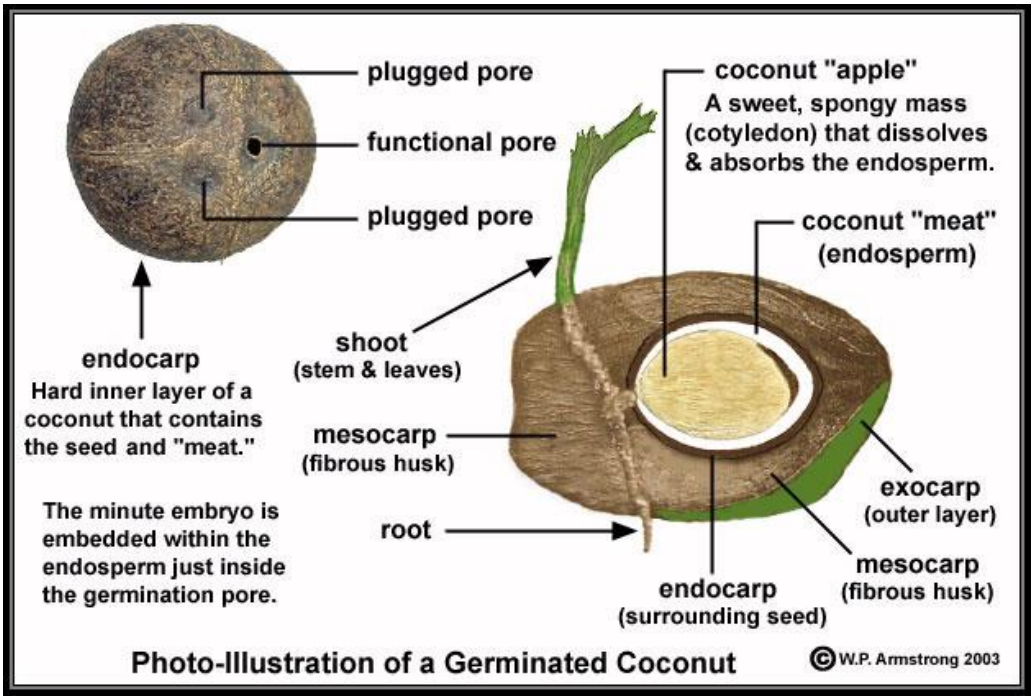

Figure 1: Structure of a coconut fruit (W.P. Armstrong 2003)

2.4 Propagation

Coconut is an important perennial crop grown widely in Asia and the Pacific and it's majorly propagated by seeds and through tissue culture.

2.4.1 Propagation through seeds

Coconuts are mainly propagated conventionally through seeds. The seed nut has less dormancy and requires no special treatment to germinate. However, germination speed of seed nuts varies within and among ecotypes and varieties. Some Tall varieties (e.g., Malayan Talls) germinate while still on the palm, while others like the West African Tall and most Pacific populations take up to 6 weeks. The seeds are mature when the husk begins to lose moisture and the epicarp starts to turn brown. This begins to occur 11 months after pollination where fruits fall to the ground when fully ripe. If large quantities of seed nuts are involved, a two-stage nursery is used to facilitate seedling selection. The first stage is the germination bed, which allows selection based on the speed of germination. The second stage is the nursery where the seedlings are grown to an acceptable size for out planting and removal of plants with vegetative abnormalities. The germination bed should be partially shaded (up to 50%) to prevent the nut water from evaporating before germination occurs, particularly with slow germinating types. The nursery should be in full sun where seedlings may be raised in planting bags or in-ground. Sites used for germination beds and nurseries should be well-drained. Seedlings spacing as shown in(Figure 2) in the nursery are t 60 by 60 cm (Finyange et al., 2019).

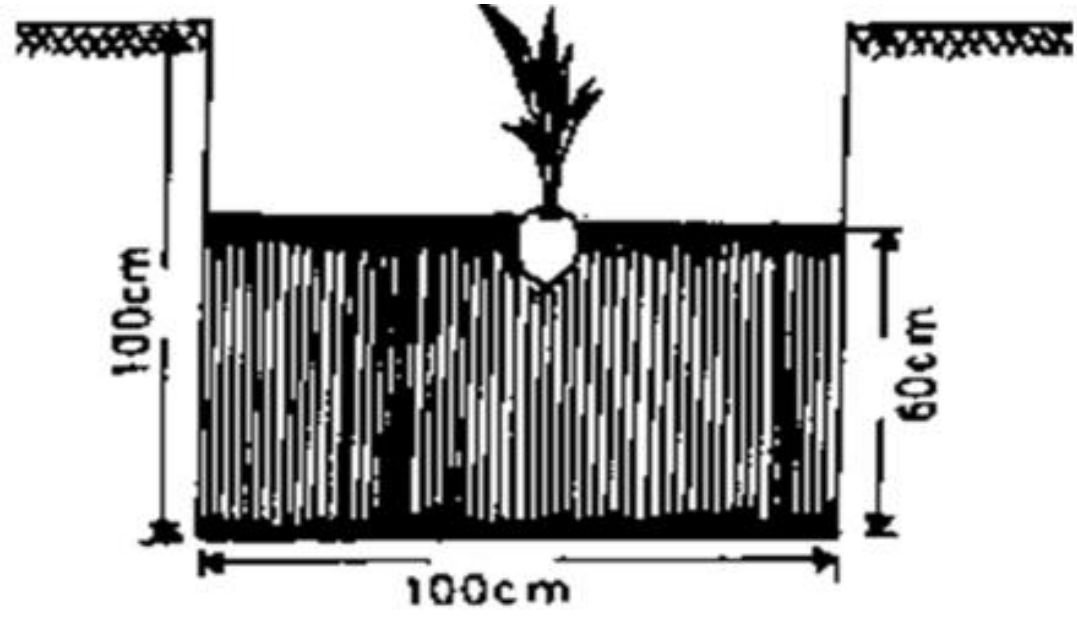

Figure 2: Spacing of coconut seedlings **(Finyange *et al*., 2019)**

Seed nuts are laid flat in rows with 2/3 of the nut buried in coarse sand or soil to reduce the loss of nut water through evaporation. The seed nuts are sown right next to each other. A path every four rows facilitates removal of germinating seed nuts at weekly or fortnightly intervals. Depending on the type, germination can occur 4–6 weeks after sowing and continue over 8 weeks, by which time 75–80% of the seed nuts should have germinated. Regular watering every other day is important to prevent loss of water from the nut cavity through evaporation. Germinating nuts are removed at regular intervals (weekly) when ready for transplanting in the field. The rate of growth depends on whether seedlings are raised in planting bags or in-ground (field nursery). If raised in-ground, seedlings should be out planted not later than 6 months old; if raised in planting bags, at 8–10months. Roots that have developed outside the planting bags are trimmed. In the case of seedlings that are raised in-ground, seedlings are carefully lifted and the exposed roots trimmed. However, Seed propagation of coconut is time consuming (Chan, 2006).

2.4.2 Tissue culture

Tissue culture regeneration occurs through organogenesis or somatic embryogenesis.

2.4.2.1 Organogenesis

Organogenesis is the process whereby the three-germ tissue in the embryonic layer that is mesoderm, endoderm and ectoderm form the internal organs of an organism. In plant tissue culture, organogenesis refers to the ability of tissues to develop organs *In vitro*. Direct organogenesis therefore refers to the formation of adventitious organs directly from explant without an intervening callus phase e.g. roots or shoot, while indirect organogenesis refers to the formation of organs through a callus intervening stage e.g. the use of hormones to induce callus culture (mass of undifferentiated cells) such as anther culture, and then organs. Direct and indirect organogenesis using plumule has often be reported in coconut as compared to somatic embryogenesis(Das et al., 2014). Direct organogenesis has been reported to be more effective in many plant species. This technique is preferred due to minimal soma clonal variation and cost effective. However, regeneration of coconut through tissue culture technique is still a major challenge because it is a recalcitrant species (Solís-Ramos et al., 2012).

Isolation and culturing of zygotic embryos from coconut fruit dates back to coconut fruit date back to the 1950s (Cutter Jr & Wilson, 1954) where it took another decade before *In vitro* plantlets could be regenerated and converted into viable plants. Most studies revealed that zygotic embryos which were harvested 10–14 months post-pollination were used for the establishment of cultures, with the greatest *ex vitro* success coming from embryos taken at 12 months (Engelmann et al., 2011). These studies further revealed that the nutritional requirements used for embryo germination and plantlet growth varied in the different studies which were undertaken (Balzon et al., 2013). Many types of culture media have been used to support the germination of zygotic embryos but the most commonly used is the Y3 medium developed by (Eeuwens, 1976). In comparison to MS (Murashige & Skoog, 1962) medium, the ammonium and nitrate nitrogen contents in Y3 medium are half, while micro-elements such as iodine, copper and cobalt are tenfold greater in concentration which better reflects the conditions of a coastal soil which favours the coconut germination. Supplementing the culture media with sucrose is very essential for the embryo germination. Activated charcoal in the media has also been showed to help prevent tissue necrosis (Antonova, 2009). Agar (1.6–0.8 % w/v) is often used to create a solid medium for the early stages of germination, however, recent studies (Muhammed *et al*., 2013) report the use of two stages which include culturing of embryo explants in a liquid medium to obtain germination then transfer to an agar medium or nutrient-saturated vermiculite for seedling growth. Other gelling agents such as gelrite (y Aké et al., 2007) and supplementation of growth regulators such as gibberellic acid (0.5 µM) have been reported to promote the rate and number of embryos germinating while certain auxin analogues such as NAA (naphthalene acetic acid) or IBA (indole-3-butyric acid) have been revealed to promote rooting in the later stages of germination and early seedling growth (Ashburner *et al.* 1993; Rillo 1998). Also, some significant endosperm fatty acid, lauric acid (75 µM), have been shown to enhance the growth and development of plantlets (López-Villalobos et al., 2011). In order to optimize embryo germination and plantlet growth, warm temperatures (25–31°C) have been reported to be the most suitable environmental conditions, first in the dark (for 5–8 weeks), and then in the light (45–90 lmol m-2 s-1) once the primary signs of germination are seen. The technique and advancement of embryo culture approach is

essential for the sample collection of coconut germplasm from the field sites and the nature of transport back to the laboratory. Transport of the fruit samples, a traditional approach, create challenges due to the limited number of samples and contaminations transfer through pests and diseases within the fruit. However, (Adkins & Samosir, 2002) proposed an appropriate mode of coconut germplasm collection which involves the isolation of the mature embryo in the field and placement in vials of sterile water or coconut water for transport to the laboratory. Nevertheless, this technique was often ineffective due to the high rate of contamination of embryos during transport. A more efficient protocol was then adopted in the following sequence where embryos were stored in a sterile state, inside a plug of solid endosperm recovered using a 2.5cm diameter cork borer. This method was further modified with on-site surface sterilizing of the endosperm plugs, then placing them in an ascorbic acid solution and holding the plugs at a cool temperature during transport back to the space. This technique still needs to be optimized to achieve a greater number of coconut plantlets flourishing in the soil and hence can serve as a reliable tool for germplasm multiplication. Relevant technology transfer of research outputs to the coconut farmer is a fundamental step towards elevating coconut production.

2.4.2.2 Somatic embryogenesis

Somatic embryogenesis is process by which plant or embryo is derived from a single somatic cell. Somatic embryos are artificially formed from cells which are not normally involved in embryo formation. Direct Somatic embryogenesis in plants is a process by which embryos are formed from somatic cells without a callus intervening stage. Indirect organogenesis on the other hand is a process by which a plant or embryos are derived from a somatic cell through a callus intervening stage. The term somatic embryogenesis (SE) was first introduced by first two independent research groups in Germany and the United States where plantlets were regenerated from cultured carrot (Daucus carota L.) and a mother cell (Quiroz-Figueroa et al., 2006). After the discovery of somatic embryogenesis, the ability to produce somatic embryogenic structures and plantlets from undifferentiated cells has then come to be the point of attention for many researchers in varied species. Though much potential has been realized through SE, there are still other species which show recalcitrance

including coconut. For coconut SE, the first trial took place over 30 years ago at Wye College, UK (Zaid & Al Kaabi, 2006) followed by ORSTOM, France *(Nguyen et al.*, 2015). All the studies done then used many explants to including very young leaves, stem cuts from young seedlings, and young inflorescences to induce embryogenic structures (Gupta et al., 1984). Lately, researchers have focused on the use of immature explants e.g., inflorescences, and zygotic tissue to achieve Somatic Embryogenesis in coconut. Immature embryos responded quickly but their slow growth to mature embryos was enhanced using longitudinal slicing (Adkins & Samosir, 2002) and then by isolation and culture of the plumular tissue (Pérez-Núñez et al., 2006a), (López-Villalobos et al., 2011). Despite the somatic tissues being able to produce true-to-type clones, the focus has yet been to harder-to-use somatic tissue explants such as young inflorescence tissues (Antonova, 2009). In SE just as in embryo culture, Y3 (Eeuwens 1976) and BM72 (Karunaratne & Periyapperuma, 1989) media have been the most frequently used for callus culture. While MS (Murashige & Skoog, 1962) and (Gamborg et al., 1974) have been found to be less effective (Bhalla-Sarin et al., 1986). Activated charcoal (0.1–0.3 %) or ethylene (Sáenz et al., 2010) has been widely used to prevent explanted tissues and callus from browning (a stress-related response caused by the release of secondary plant products such as phenols) However, the presence of activated charcoal in the culture medium interferes with the activity of the exogenously applied plant growth regulators and other media supplements (Nguyen *et al*., 2015), leading to uncertainty in the exact functional concentrations of these additives within the medium (von Aderkas et al., 2002). Differences in particle size and the potency of the various activated charcoal types influence the frequency of somatic embryogenic callus formation (Saenz et al., 2006);Sáenz et al., 2010. Polyvinylpyrrolidone (PVP) (universal toxin absorbing agent), was also tested to have no significant effect on coconut leaf-derived cell suspension cultures). Nevertheless, when PVP was used in zygotic embryo, it gave a positive effect in promoting the rate of SE (Nguyen *et al.,* 2015). Addition of sucrose (3–4 %) is also essential for coconut SE to take place. Although frequent sub-culturing of the cultured explants causes stress during the transfer process, it is a suitable approach to reduce accumulation of toxic phenols (Fernando and Gamage, 2000a; Perera *et al.*, 2006).

Clonal propagation of coconut is divided into three stages. First, the production and proliferation of callus second, the formation, maturation and germination of somatic embryos, and third the acclimatization of the plantlets to ex vitro conditions. Callus formation is generally achieved with a high concentration of auxin (Nguyen *et al.,* 2015), usually 2,4-dichlorophenoxyacetic acid (2,4- D). Working concentration of 2, 4-D varies between different cultivars and explant types. In the case of zygotic embryos of Sri Lanka Tall low 2,4-D (24 µM) treatment gave optimal results in initiating callus (Fernando and Gamage 2000), a much higher dose (125 µM) was needed for Malayan Yellow Dwarf and Buta Layar Tall (Adkins *et al*., 1998; Samosir 1999). For callus production on immature inflorescence tissues and embryo-derived plumules, an even higher concentration of 2,4-D (450 or 600 µM) was required (Verdeil *et al.,* 1994). However, chromosomal aberrations in the cultured tissues have been evident when this is used in high concentrations (Blake and Hornung 1995). Coconut tissues were proven to metabolize 2,4-D into fatty acid analogues, which are subsequently incorporated into triacylglycerol derivatives (López-Villalobos et al., 2011). These molecules represent a stable and stored form of 2,4-D that can continue to arrest somatic embryo formation even when 2,4-D has been removed from the medium. Apart from 2,4- D, other auxins such as NAA (27 µM) in combination with 2,4-D (452 µM) (Nguyen *et al.,* 2015) have been used to promote callus formation on rachillae explants (Gupta *et al.,* 1984). Besides, the study of the ultra-structural changes that took place during the acquisition of SE potential suggested that the gametophyte like conditions produced by 2, 4-D, were required for the successful transition from the vegetative into the embryogenic state (Verdeil *et al.,* 2001). Cytokinin such as 6-benzylaminopurine (BAP), thidiazuron (TDZ), kinetin (Kin), 2-isopentyladenine (2iP), at 5–10 µM are usually supplemented for callus proliferation and maturation. Callus formation is often achieved after one month of initiation in dark 28 ± 2^{o}C (Adkins *et al.,* 1998). Pe´rez-Nu´n˜ez *et al.,* 2006 revealed that dark incubation has been extended to 3 months to achieve greater callus production. To better promote the somatic embryogenic callus production, some studies have also revealed that supplementation of the media with some multi-functional polyamines, particularly putrescine (7.5 mM) or spermine (0.1µM), has been done to protect the explant tissue from ethylene damage (Adkins et al. 1998). To provide a beneficial

environment for callus multiplication and the formation of somatic embryos, ethylene production inhibitors such as aminoethoxyvinylglycine (AVG) and ethylene action inhibitors such as silver thiosulphate (STS) have also been shown to protect the explant tissues Adkins *et al.*,1998). Conversion of undifferentiated callus to somatic embryogenic callus has been achieved by the reduction or removal of 2, 4-D from the culture medium in some studies. Chan *et al.,* (1998) showed that incubating callus under a 12-h photoperiod (45–60 1mol $^{m-2\ s-1}$ photosynthetic photon flux density) also significantly improved the rate of SE, as compared to that produced under darkness. Increasing the amount of BAP (to between 50 and 300 µM) in the medium could also promote SE, leading to a greater number of viable plantlets at the end of the culture phase (Pérez-Núñez *et al., 2006*;Chan et al., 1998). Application of Abscisic acid (ABA) in moderate concentration (5 µM) promotes the formation and the maturation of somatic embryos (Fernando et al., 2004;(Fernando et al., 2003): Samosir *et al.,* 1999; Fernando and Gamage 2000). In addition, the use of osmotically active agents such as polyethylene glycol (PEG) 3 %, osmotically active agent, together with ABA (45 µM) has also been shown to be beneficial in two ways; including the production of somatic embryos and subsequent maturation and germination (Samosir et al., 2006). In a more recent study, (Antonova, 2009) confirmed the importance of using a specific growth retardant ancymidol (30 µM) to promote the rate of somatic embryo germination to 56 % while using the immature inflorescence explants. In raising the rate of SE for some members of the Arecaceae, cell suspension culture system has been successful (Teixeira *et al.*, 1995). Moreover, temporary immersion systems have been used with date palm (Tisserat and Vandercook 1985) and peach palm (D. A. Steinmacher et al., 2011) to raise the rate of plantlet regeneration. If these two techniques could be employed in coconut plantlet regeneration and rapid multiplication of healthy plantlets, it could then create a platform for mass clonal propagation.

2.5 Greenhouse Acclimatization

Acclimatization also known as climatic adaptation is a process by which an individual organism adjusts to a change of environment in order to retain the performance across

a range of environmental conditions. In plant tissue culture, acclimatization is a process by which the in vitro plantlets are transferred and maintained *ex vitro.*

However, the ex vitro acclimatization of somatic embryo-derived plantlets has yet to be refined, with present rates of success of around 50 % so far, (Fuentes et al., 2005). Several potting media containing a mixture of peat moss and soil (1:1, w/w) and nursery conditions have been used in the acclimatization of the *In vitro* plantlets of a wide range of coconut and also in raising the tissue cultured plantlets. According to Talavera e*t al.,* 2005, the *ex vitro* establishment and survival rate of seedling were improved by transferring plantlets through a series of different conditions, firstly in a mist chamber, then a shaded nursery and finally a nursery under full sunlight. Elevation of seedling photosynthesis has also been showed to be a contributing factor to acclimatization success. Early establishment of a photosynthetic based metabolism was significant during in vitro plantlet development (Triques et al., 1997). Use of fatty acids, especially lauric acid, in the plantlet maturation medium (López-Villalobos et al., 2011) further improved the ex vitro establishment.

The success of getting uniform *In vitro* shoots has not been the case for most woody tree species and especially those with long intermittent phases such as coconut palm as compared to other herbaceous species (Brent H. McCown, 2000; Neondo et al., 2011; Solís-Ramos et al., 2012; Tulecke, 1987).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample collection

The coconut embryos were obtained from 10 to 11 months old nuts harvested from the clean coconut mother-plants of the East African Tall Variety from the coastal region of Kenya which had been identified to be highly productive by the Kenya Coconut Development Authority, a Kenya Government Authority in charge of the coconut sector. By using a purposive sampling method, the nuts were collected from South Coast including Msambweni Majikuko Village (4°28'11.34"S, 39°28'46.68"E),) and the North Coast including Birini, (3°45'04.14"S, 39°35'27.27"E) Mmleka (3°58'24.36"S, 39°44'00.95"E) and Kinarani (3°43'35.35"S, 39°34'21.66"E) region of Mombasa. The nuts with husks were packaged and labeled accordingly. The purpose for collection, location, farmers name and code. A total of 320 nuts were transported to Jomo Kenyatta University of Agriculture and Technology (JKUAT) Institute of Biotechnology Research (IBR) laboratory for tissue culture and subsequent analysis.

3.2 Media preparation

The Euwens Y3 media, (Eeuwens, 1976), (Appendix 1) was supplemented with 4 % (w/v) sucrose, 1g/l activated charcoal and 0.28% (w/v) gel rite. The pH value for all the media was adjusted to 5.8 using 0.1M HCl or 0.1M NaOH and 10 ml of media was dispensed in 70 ml culture jars. The media together with the forceps and cork borers which were covered with aluminum foil were sterilized in an autoclave at 121˚C for 15 minutes before use. The sterilized media was then kept in the culture room for three days before use.

3.3 Culture conditions

The embryo explants were initiated on both callus induction and direct embryo development media. The lighting conditions were 16 hours period of light and 8 hours period of darkness provided by cool white fluorescent tubes, 36 lmol s^{-1} $m^{-2,}$ and kept in dark at 25±2˚C in the culture room.

3.4 Experimental design

Experiments on embryo germination, callus induction, embryo formation, shoot formation, shoot multiplication and elongation, root formation and acclimatization were all set up in a completely randomized design in 3 replicates. A total number of 125 explants were used for evaluation of in *vitro* regeneration of coconut via direct organogenesis while total number of186 explants were used for indirect somatic embryogenesis. A total of 9 explants were lost during embryo extraction process.

3.5 Embryo preparation.

The nuts were first dehusked and cleaned thoroughly by gently brushing under running tap water. The cork borers were inserted through the "coconut eye" as shown in (figure 3).

Figure 3: Dehusked coconut and embryo extraction.

From each mature nut, an endosperm was extracted using a 1.5mm, 2mm and 2.5mm diameter sterile cork borer and then washed with distilled water. The endosperms were soaked in 10% savlon with 100µl of Tween® 20 for 30 minutes and rinsed thrice with double distilled water. They were then placed in 95% ethanol with gentle swirling for 1 minute and rinsed thoroughly with double distilled water then soaked in 10% sodium

hypochlorite containing 100µl of Tween® 20 for 20 minutes and then rinsed thrice with double distilled water as shown in the (Figure 4).

Under aseptic conditions in the clean bench, the embryos were excised from the endosperm cylinders using a sterile scalpel. The embryos were finally sterilized using 1% sodium hypochlorite for 1 minute and rinsed thrice with double distilled water.

Figure 4: Surface sterilization of embryo explants

They were initiated in Y3 media and kept in dark (placed inside the boxes to mimic the dark) at 25±2˚C in the culture room as shown below (Figure 5). The embryos were used for both subsequent experiments for direct organogenesis and indirect somatic embryogenesis.

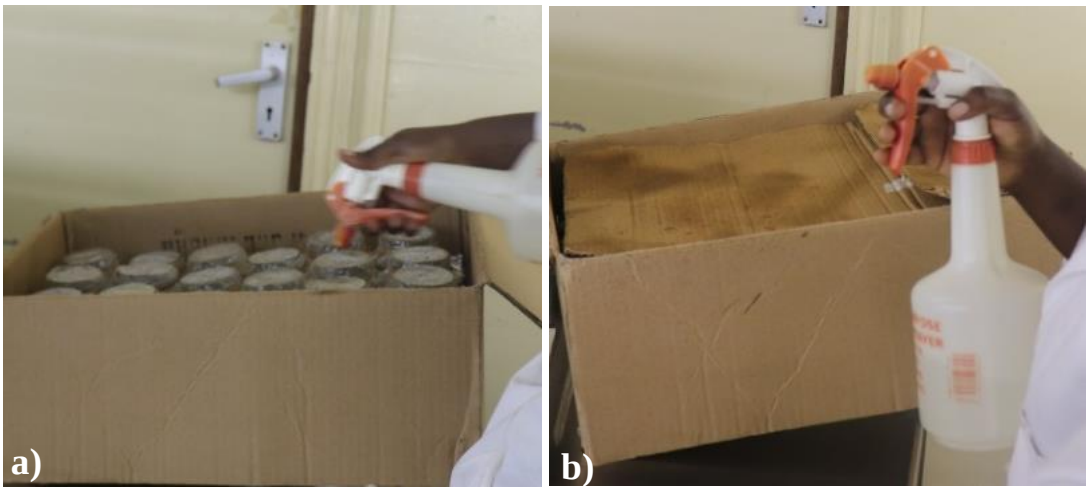

Figure 5: a) spraying the cultures b) spraying the covers

3.6. Evaluation of in *vitro* regeneration of coconut via direct organogenesis.

3.6.1. Embryo germination.

The embryos were extracted and sterilized as discussed in 3.5. Embryo explants were initiated on Y3 media supplemented with 4 % (w/v) sucrose, 1g/l activated charcoal, 0.28 % (w/v) gelrite, 0.28µM 2, 4-D, 0.44µM BAP and 0.03µM GA3 and control without hormones. Growth of embryos was recorded at an interval of 4 weeks while contamination was recorded weekly. The cultures were kept in darkness at 25±2˚C for a period of 3 months with one subculture after 8 weeks.

3.6.2. Embryo development

The embryos with the signs of growth exhibited by swelling, cracking at the tips and colour change were subcultured to the freshly prepared media and kept for 4 weeks in darkness at 25±2˚C in the culture room without sub culturing. The developed embryos were then subcultured into a hormone-free media and transferred to light which was also kept for 4 weeks at 25±2˚C. Observations on shoot formation and data recording were made weekly until shoots were formed which were then taken for multiplication and elongation.

3.6.3 Shoot multiplication and elongation

For shoot multiplication, the developed embryos with a bigger diameter or width were chosen. These shoots were then cut into two equal parts. All the developed embryos

were subcultured into Y3 media supplemented with three different concentrations of BAP; 5µM BAP + 0.5µM GA_3, 10µM BAP + 0.5µM GA_3, and 15µM BAP + 0.5µM GA_3 each for shoot elongation. The data on shoot elongation was measured at an interval of two weeks. The cultures were then kept in light for 8 weeks at 25±2˚C.

3.6.4 Rooting

The elongated shoots, 5-6cm long were transferred to rooting media containing Y3 medium supplemented with 5µM, 10µM and 15µM IBA alone and in combination with 0.5µM GA_3. All the media were supplemented with 4 % (w/v) sucrose, 1g/l activated charcoal and 0.28% (w/v) gel rite. The cultures were incubated in the culture room under a photoperiod of 16 hours of light and 8 hours of darkness. Subsequently, the plantlets were taken to the greenhouse for 3 months.

3.7 Indirect somatic embryogenesis.

3.7.1 Effect of plant growth regulators on Callus induction.

After sterilization, five experiments and a control were set up with six treatments each and a control consisting of hormone free Y3 media. For every treatment, 3 explants were used and each was replicated three times. Initiation was done on the Y3 medium (Euwens, 1976) containing Morel and Whites vitamins (White, 1951) for callus induction. Five treatments including a range of 100µM,-250µM 2, 4-D were tested alone and in combination with 0.35µM GA_3, 0.5µM GA3, 5µM BAP, and 9µM TDZ. All the media were supplemented with 4% (w/v) sucrose, 1g/l activated charcoal and 0.28% (w/v) gel rite and kept in darkness under conditions as described in section 3.3 above.

3.7.2 Effect of plant growth regulators on somatic embryo formation

The formed callus were transferred to embryo formation media containing half 2, 4-D (50-125µM) concentrations alone and in combination with other each of the following Plant growth regulators, 0.35µM GA_3, 0.5 µM GA_3, 5µM BAP, and 9µM TDZ. The cultures were kept in dark for 8 weeks as described in section 3.3 above.

3.7.3 Effect of plant growth regulators on embryo germination

The embryos formed were transferred to embryo germination media containing BAP+ GA_3 for 4 weeks and then subcultured into Y3 medium supplemented with 10µM kinetin + 10µM BAP + 0.5µM GA_3 and 200µM NAA for 8 weeks. All the cultures were kept under 16 hours of light and 8 hours of darkness.

3.8 Assessment of the tissue cultured plantlets

3.8.1 Hardening and Greenhouse acclimatization of the plantlets

The fully developed tissue cultured plantlets which were at least 5cm with three leaves and 3cm roots were de-flasked, rinsed with double distilled water and drenched in a mixture of Y3 nutrients and a mixture of BAP and NAA hormones
for 1 minute before transfer to potting bags containing sterilized potting media. Two different substrates were tested including sterilized soil: sand: manure in the ration of 3:1:1(A) and a vermiculate (B). Both media were sterilized in the autoclave at 121ºC for 15 minutes. The plantlets were acclimatized at 28±2ºC and relative humidity ranging from 70 - 80%.

During this period, liquid fertilizer (foliar) was sprayed on to plantlets after every 21days for 3 months. Watering of plantlets (0.5litre per plantlet in a potting bag), was made after every 2 days for those in soil: sand: manure media and every day for those in vermiculate media because it drains water easily. Observations were made for a period of 12 weeks and data on plant growth rate including color, height, and the number of leaves formed was recorded at 2 weeks interval.

3.9 Data collection and analysis

Data collected on embryo development and germination were the number of swollen embryos and the percentage of swollen embryos was calculated as the total number of swollen embryos divided by the total number of initiated embryos multiplied by 100. The number of contaminated embryos also calculated as the total number of the contaminated embryos divided by the total number of initiated embryos multiplied by 100.The the number of days taken for colour change, and the number of the dormant embryos was calculated as the total number of the dormant embryos divided by the total number of initiated embryos multiplied by 100.

For callus and somatic embryo formation, the mean percentage of embryos forming callus, the mean percentage of calli forming embryos, and the colour change were recorded. For the shoot induction and elongation, shoot formation and elongation period and the colour transition period from white to green, the number of leaves formed and the height of the plantlets (cm) were recorded. The rooting parameters were root length taken using a ruler (cm) and the numbers of roots formed was taken by counting. The data recorded were entered in Microsoft excel spread sheet and analyzed using Minitab 17 Statistical software. Analysis of variance (ANOVA) was used to test the significant differences between the various means (%) and Fisher's test at $P<0.05$ was used to separate means. The variability in data was expressed as the percentage mean± standard error (SE).

CHAPTER FOUR

RESULTS

4.1 *In vitro* regeneration of coconut through direct organogenesis

4.1.1 Effect of Euwens Y3 medium on embryo development

After the 4 weeks of initiation of embryo explants in Y3 medium supplemented with 0.28µM 2, 4-D, 0.44µM BAP and 0.03µM GA3 at 26±2°C in darkness, 19.3% of the embryo explants were removed due to contamination. These contaminations were observed after a week of initiation by observing changes in media turbidity. To prevent the spread of contaminations, the contaminated cultures were immediately removed and the other cultures and the culture room were sterilized with 70% ethanol. Exposure of the cultures to non-sterile surfaces was minimized. The signs of growth were first observed on the 8th week. As shown in (Table 1), there was a gradual increase in the response of the explants which was exhibited by swelling of embryos (Figure 6). The rate of swelling was 8.96% on the 8th week followed by 40.3% on the 12th week and finally 50.7% on the 16th week

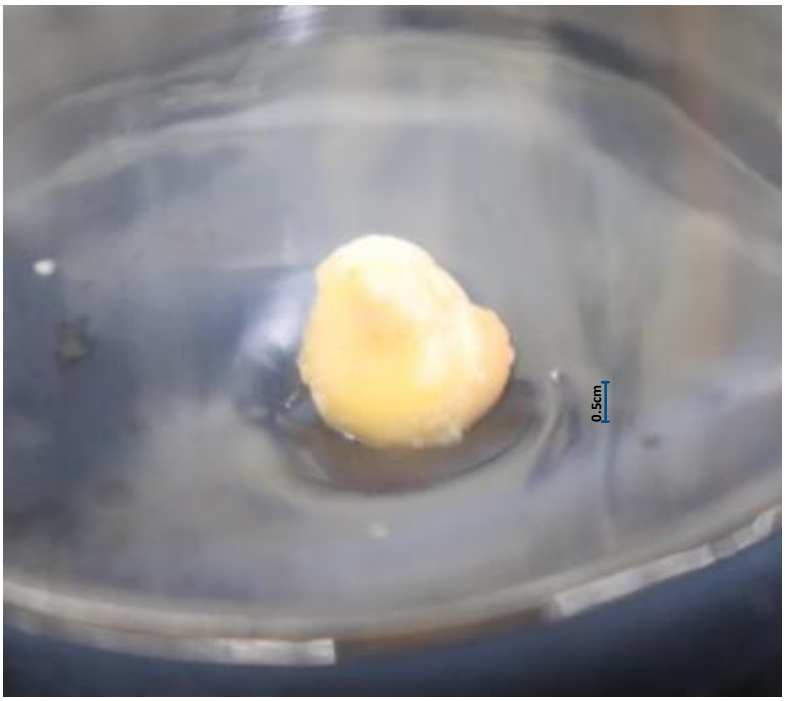

Figure 6: Swollen embryo explant 12 weeks after initiation

However, on the 12th and 16th week, some embryos had not developed any signs of growth nor change in color hence remaining dormant (Figure 6).

4.1.2 Germination

When all the swollen and the dormant embryos were further subcultured into the same freshly prepared media as in section 3.6.1 and kept in darkness for further embryo development and germination for additional 4 weeks, a 37.31% germination rate was recorded (Table 1).

Table 1: Embryo development after 16 weeks in Euwens Y3 media

Treatments	Total No. of weeks	LDC (%)	SE (%)	DE (%)	GE (%)	COLOUR
Y3+2,4D +BAP+GA3	4	17.91	0	0	0	White
	8	0	8.96	0	0	White
	12	0	40.3	11.19	0	White
	16	0	50.75	24.62	37.31	Cream
Control (Y3 only)	4	1.49	0	0	0	White
	8	0	0	0	0	White
	12	0	0	0	0	White
	16	0	1.49	3.73	0	White

% SE-percentage of swollen embryos; % LDC-percentage loss due to contamination; % DE-percentage of dormant embryos and % GE- Percentage of germinated embryos

Germination was observed on the sprouting of the plumule and a signs of radicles emerging (Figure 7A).Upon transferring the germinated explants from dark to light at 16 hours light and 8 hours of darkness, the color of the germinated embryos began to change from white, to pale green, after 1 week and eventually to green after 2 weeks (Figure 7A, Figure 7B, and Figure 7C) respectively.

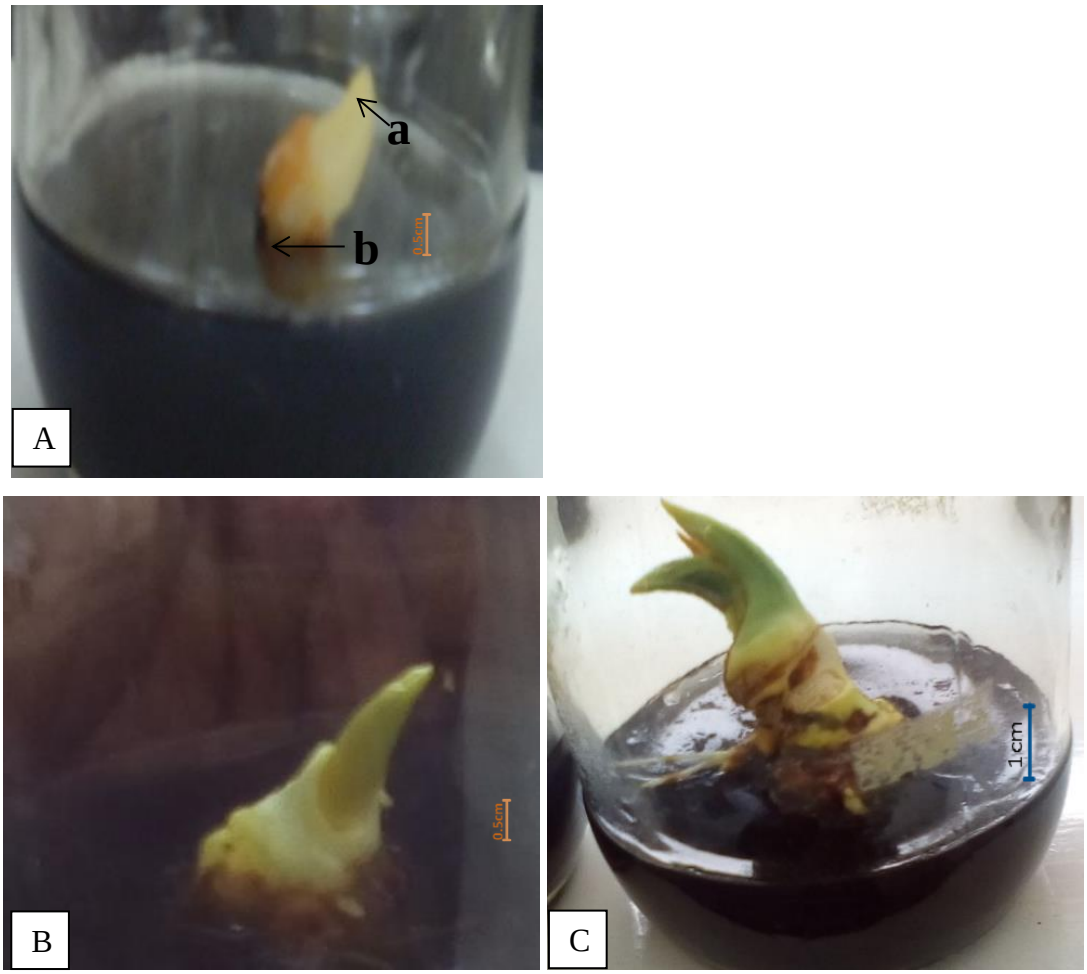

Figure 7: Embryo germination stages

A) a) emerging plumule b**)** emerging radicle, B**)** Pale green germinating embryos
C) Green germinating embryo.

4.1.3 Shoot multiplication

When the 5 months old, green developed/germinated embryos were multiplied by subdividing into two on elongation media (Figure 8), none of the embryos induced shoots. No growth was also evident. Instead they became brown after 3 weeks and eventually died. The other plantlets which were not subdivided induced shoots and were further subcultured in shoot elongation media containing three different levels of BAP.

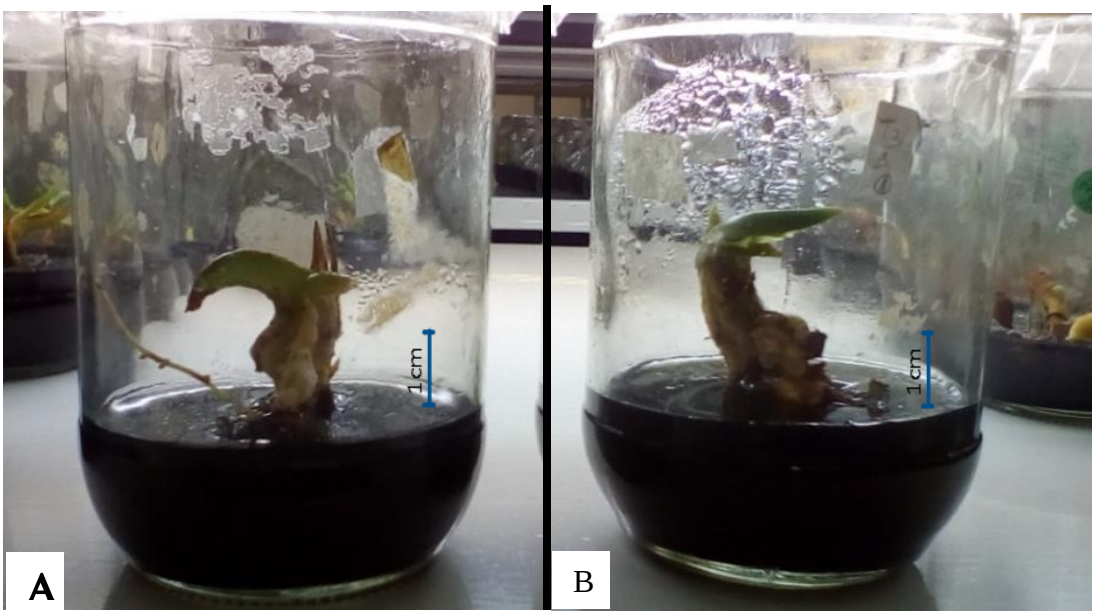

Figure 8: A and B Shoot multiplication through equal subdivision

4.1.4 Effect of Plant growth regulators on shoot elongation.

The 6 months old, green, well-germinated shoots with an average height of 2.5cm described in section 4.1.3 above successfully elongated when subcultured in: Y3 + (5 to 15µM) BAP + 0.5µM GA_3 and the control (Y3 media without any hormones) (Figure 9). Among the three treatments, highest mean length of 3.63cm was exhibited in Y3 + 10µM BAP + 0.5µM GA_3 followed by 3.06cm in Y3 + 15µM BAP + 0.5µM GA_3. However, the control experiment showed the lowest mean length of 0.1cm (Table 2).

Table 2: Shoot elongation in Y3 medium supplemented with BAP and GA_3

Parameters	**Treatments**			
	5µMBAP+0.5µM GA3	**10µMBAP +0.5µM GA3**	**15µMBAP +0.5 µM GA3**	**Control (Y3)**
Shoot height(cm)	3.13 ± 0.45^a	3.63 ± 0.47^a	3.06 ± 0.28^a	0.1 ± 0.1^b
Number of leafs	2.67 ± 0.33^a	2.67 ± 0.33^a	2.67 ± 0.33^a	0^b

Mean values followed by the same letter within the same row are not significantly different by Fisher's test ($P<0.05$) n=36.

Figure 9: Tissue cultured Plantlets in the shoot elongation media

4.1.5 Rooting.

A successful root induction was observed upon subculturing the 7-month-old shoots with at least 5cm in height and 3 developed leaves onto Y3 media supplemented with 5µM 1BA + 0.5µM GA_3, 10µM 1BA + 0.5µM GA_3, and 15µM 1BA + 0.5µM GA_3.

On root length, analysis of data revealed that the concentration with significant longer roots was Y3 + 5µM IBA+ 0.5µM GA_3 (5.10cm) while the lowest (2.83) was observed on t 15µM IBA. The highest number of roots induced was in the treatment containing 5µM IBA + 0.5µM GA_3 while 15µM 1BA + 0.5µM GA_3 treatment had the least (2.83) number of roots induced. However, the control treatment did not induce roots (Table 3).

Table 3: Effect of IBA in combination with 0.5µM GA_3 on root induction

parameters	Treatments			
	5µMIBA+0.5µM GA3	**10µMIBA+0.5µM GA3**	**15µMIBA+0.5µM GA3**	**Control**
Root height(cm)	5.10 ± 1.99^{a}	3.27 ± 0.43^{ab}	2.83 ± 0.72^{ab}	0^{b}
Number of roots	8.33 ± 2.0^{a}	7.33 ± 2.18^{a}	3.33 ± 0.88^{ab}	0^{c}

Mean values followed by the same letter within the same row are not significantly different by fisher's test ($P<0.05$) n=36.

When Y3 media supplemented with three different concentrations of 5µM, 10µM, and 15µM IBA alone were tested, analysis of data revealed that the concentration with the longest roots was 5µM IBA with a mean length of 2.80 cm while the lower among three concentrations were at 10µM IBA with a mean of 0.96cm. However, the control experiment did not induce roots (Table 4).

On the other hand, analysis of data revealed that the hormonal concentration that included the highest number of roots was 15µM IBA. However, the control experiment did not induce any roots.

Table 4: Effect of IBA alone on root induction

Parameters	Plant growth regulators (µM)			
	Y3+5µM IBA	**Y3+10µM IBA**	**Y3+15µM IBA**	**Control**
Root height(cm)	2.80 ± 0.792^{a}	1.833 ± 0.391^{ab}	$0.956 \pm 0.354b^{c}$	0^{c}
Number of roots	3.11 ± 1.44^{ab}	4.89 ± 2.34^{ab}	6.67 ± 1.6^{4a}	0^{b}

Mean values followed by the same letter within the same row are not significantly different by Fisher's test ($P<0.05$) n=36.

Notably, all three levels of IBA induced roots with no difference in their morphological appearance (Figure 10). The plantlets were subsequently taken for acclimatization in a greenhouse

Figure 10: Tissue cultured Plantlets with roots in the root induction media

4.2 In vitro regeneration through indirect somatic embryogenesis

4.2.1 Callus induction

Culturing embryos in Y3 media supplemented with 150µM 2, 4-D + 5µM BAP and 150µM 2, 4-D + 0.5µM GA_3 resulted in 83% callus formation after 12 weeks while the control experiment did not induce any callus (Table 5).

Table 5: Effect of plant regulators on callus induction media after 12 weeks

2,4-D concentration ranging from 100 to 250µm	% Callus induction using different Plant growth regulators in (µM)				
	2,4-D alone	**2,4-D +0.35 µM GA3**	**2,4-D + 0.5 µM GA3**	**2,4-D + 5 µM BAP**	**2,4-D + 9 µM TDZ**
Control	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}
100	33.3 ± 0.21^{abc}	16.7 ± 0.17^{bc}	50.0 ± 0.22^{abc}	33.3 ± 0.21^{abc}	66.7 ± 0.21^{ab}
125	16.7 ± 0.17^{bc}	16.7 ± 0.17^{bc}	66.7 ± 0.21^{ab}	50.0 ± 0.22^{abc}	50.0 ± 0.22^{abc}
150	16.7 ± 0.17^{bc}	16.7 ± 0.17^{bc}	83.3 ± 0.17^{a}	83.3 ± 0.17^{a}	50.0 ± 0.22^{abc}
175	16.7 ± 0.17^{bc}	16.7 ± 0.17^{bc}	66.7 ± 0.21^{ab}	66.7 ± 0.21^{ab}	50.0 ± 0.22^{abc}
200	16.7 ± 0.17^{bc}	33.3 ± 0.21^{abc}	50.0 ± 0.22^{abc}	66.7 ± 0.21^{ab}	33.3 ± 0.21^{abc}
250	16.7 ± 0.17^{bc}	33.3 ± 0.21^{abc}	33.3 ± 0.21^{abc}	66.7 ± 0.21^{ab}	33.3 ± 0.21^{abc}

Mean values followed by the same letter within the column are not significantly different by fisher's test ($P<0.05$) (n=186).

Signs of callusing were first observed from the 6th week in the callus induction media. This was accompanied by embryo enlargement and color change from white to cream (Figures 11 A and B). Upon sub culturing for additional 4 weeks, on the freshly prepared media of the same constituents, the callus became more profound and could easily be differentiated from the swollen embryos (Figure 11B). However, some of the embryos remained dormant even after 16 weeks in the callus induction media.

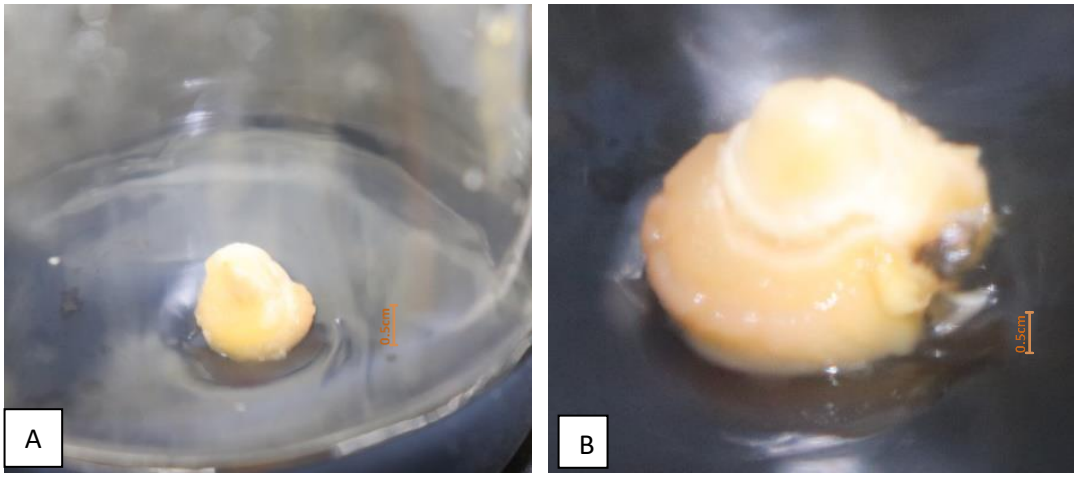

Figure 11: Callus induction

A; Swollen embryos at the 6th week and B; enlarged callus at the 8th week

4.2.2 Effect of PGRs on embryo formation

The formed calli in section 4.2.1 above when subcultured in to Y3 media supplemented with 50 to 125µM 2,4D alone all and in combination with 0.35µM GA3, 0.5µM GA3, 5µM BAP and 9µM TDZ. The best hormone combination and concentration for embryogenic calli formation was 75µM 2, 4-D+5µM BAP which resulted in the highest mean of 83% embryogenic calli while those subcultured in Y3 media alone did not induce embryogenic calli after 12 weeks (Table 6).

Table 6: Effect of plant growth regulators on embryo formation

2,4-D concentration ranging from 100 to 250µm	**Plant growth regulators in (µM)**				
	2,4D alone	**2.4-D + 0.35µM GA3**	**2.4-D + 0.5µM GA3**	**2.4-D + 5µM BAP**	**2.4-D+ 9µM TDZ**
Control	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}
50	0^{c}	16.7 ± 0.17^{bc}	50.0 ± 0.22^{abc}	33.3 ± 0.21^{abc}	66.7 ± 0.21^{ab}
62.5	16.7 ± 0.17^{bc}	16.7 ± 0.17^{bc}	50.0 ± 0.22^{abc}	50.0 ± 0.22^{abc}	50.0 ± 0.22^{abc}
75	16.7 ± 0.17^{bc}	16.7 ± 0.17^{bc}	66.7 ± 0.21^{ab}	83.3 ± 0.17^{a}	50.0 ± 0.22^{abc}
87.5	16.7 ± 0.17^{bc}	16.7 ± 0.17^{bc}	50.0 ± 0.22^{abc}	66.7 ± 0.21^{ab}	50.0 ± 0.22^{abc}
100	16.7 ± 0.17^{bc}	33.3 ± 0.21^{abc}	33.3 ± 0.21^{abc}	66.7 ± 0.21^{ab}	33.3 ± 0.21^{abc}
125	16.7 ± 0.17^{bc}	33.3 ± 0.21^{abc}	33.3 ± 0.21^{abc}	66.7 ± 0.21^{ab}	16.7 ± 0.17^{bc}

Mean values followed by the same letter within the column are not significantly different by Fisher's test ($P<0.05$)

The embryogenic calli became more evident with both friable, (Figure 12A) and compact (Figure 12B) textures, however, some calli developed into roots (Figure 12C). The embryogenic calli formed in media containing 2, 4-D alone and in combination with GA_3 and BAP were compact while those formed in the media containing 2, 4-D in combination with TDZ were friable (figure 12).

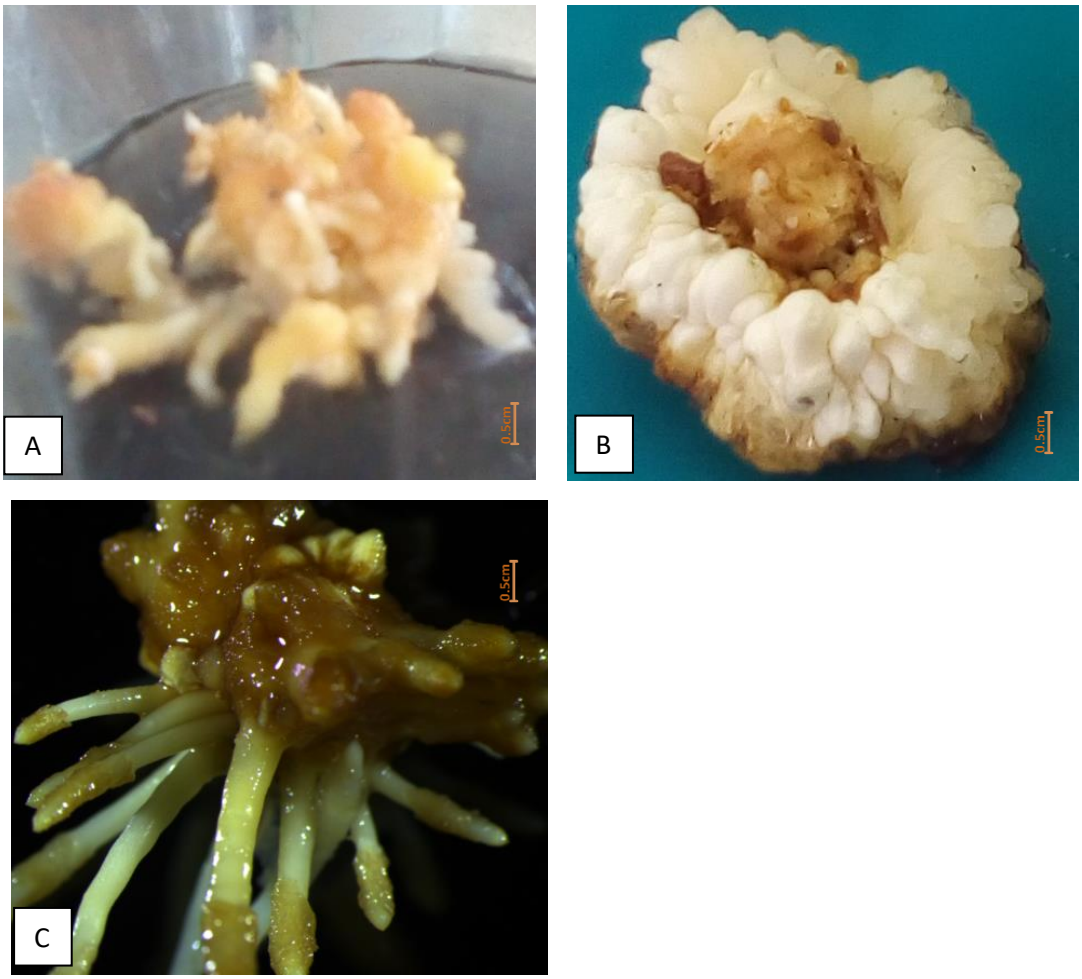

Figure 12: A) friable calli, B) compact calli C) root-like structures

When viewed under the oil-immersion lens (100X) of a compound microscope the embryogenic calli showed different stages of somatic embryo formation which include; the globular stage which had round and shiny protrusions, heart shape and torpedo stages (Figure 13).

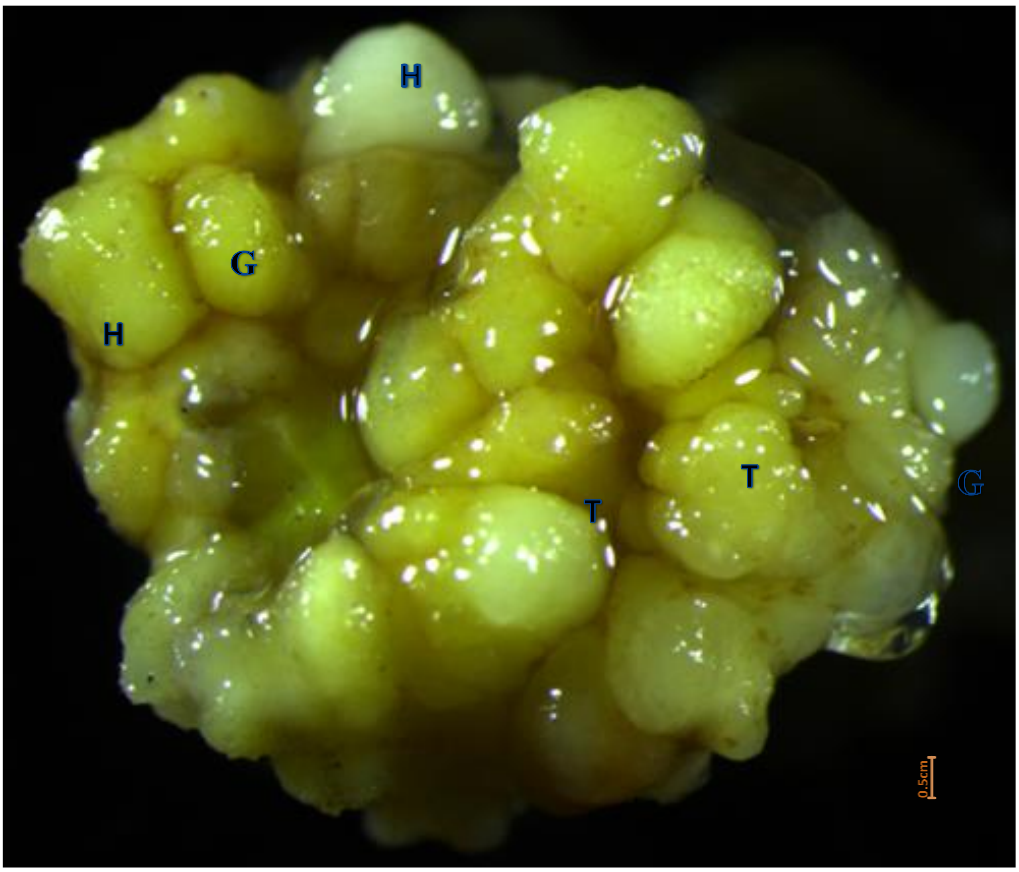

Figure 13: Stages of embryogenic callus development

G-globular, H-heart shape and T-torpedo stages.

4.2.3 Effects of PGRs on embryo germination/ maturation shoot induction

Upon sub culturing of the embryogenic calli on shoot induction media, compact embryogenic calli from media supplemented with 50 to 125µM 2,4 D and 0.5µM GA_3 became more prolific and the color changed from white and eventually to green (Figure 14A-D). After a series of subcultures on shoot induction media, they became dark green with more visible embryos which continued to enlarge but did not induce shoots (Figure14E). On the other hand, embryogenic calli from the media that was supplemented with 50 to 125µM 2,4D and 5µMBAP induced one shoot at the center of the calli which eventually became a plantlet. The rest of the calli continued to enlarge with visible embryos but with no further shoot formation and eventually hardened despite sub culturing onto freshly prepared media. At such a stage, substitution of GA3 with ABA is therefore recommended.

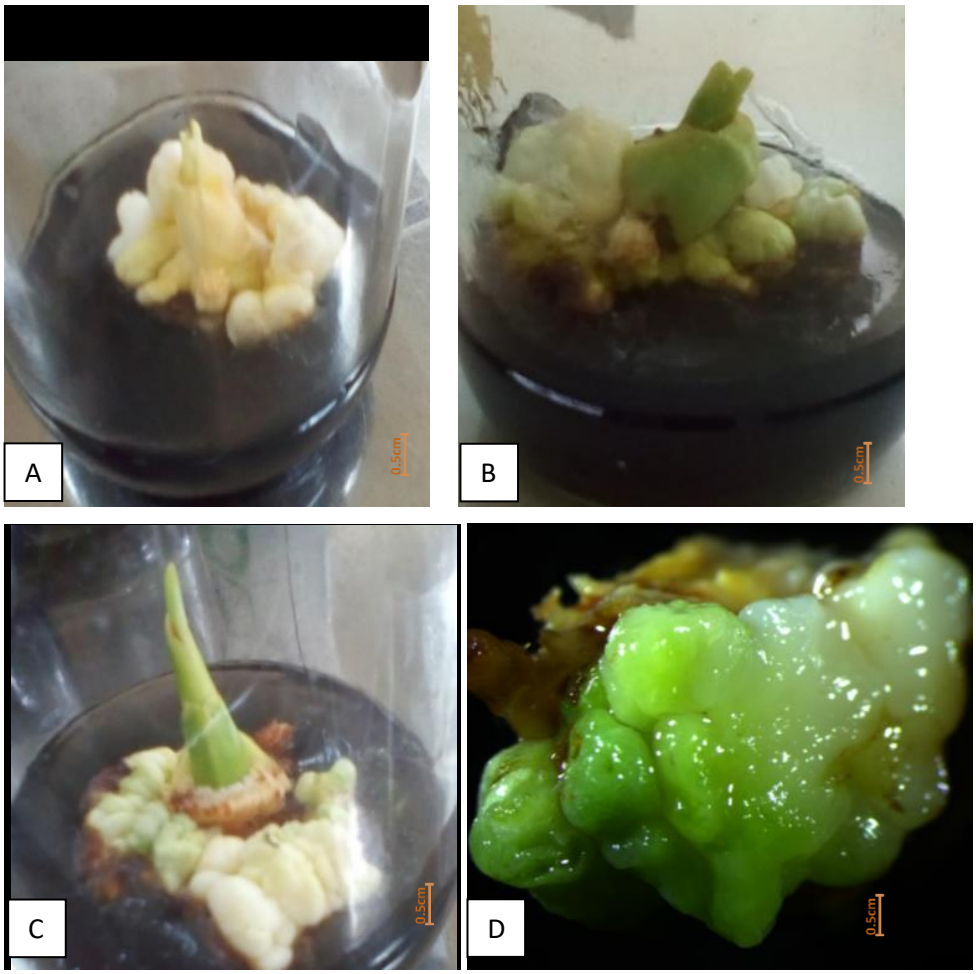

Figure 14: Embryo maturation

A) white, B) cream white; C) pale green; D) green compact embryogenic calli forming one shoot and E) Green enlarged embryogenic calli without shoot.

When both friable and compact calli were subcultured onto shoot induction media, the friable calli from media supplemented with 2,4D and TDZ became enlarged and green. When the calli were further subcultured into a freshly prepared Y3 media containing 10µM kinetin+ 10µM BAP+ 200µM NAA + 0.5µM GA_3 and placed in light, multiple shoots were induced from friable embryogenic calli (Figure 15 A and B) as the color changed from cream white to pale green and finally to green.

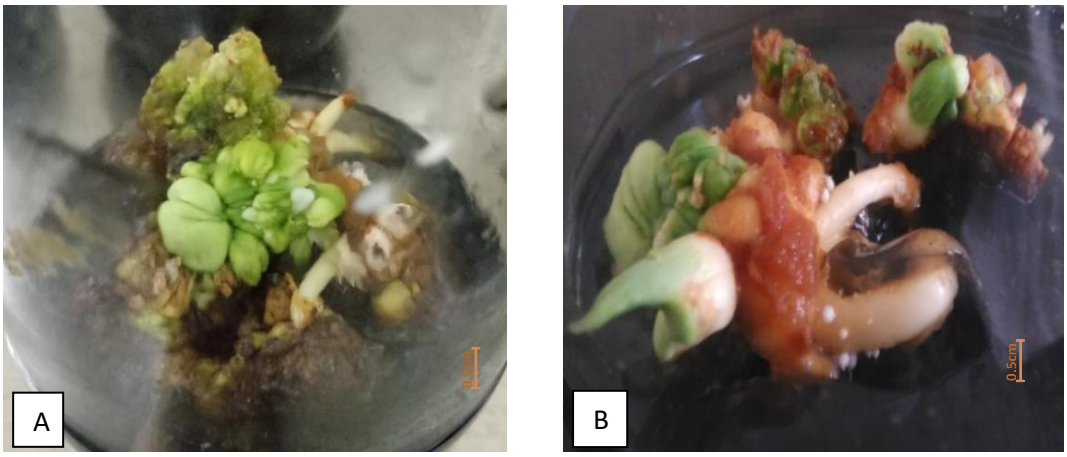

Figure15: Multiple shoot induction from friable embryogenic calli

4.2 Effect of different media on acclimatization of coconut tissue cultured plantlets

Thirty tissue cultured plantlets from both direct organogenesis and indirect somatic embryogenesis which at 5cm to 12 cm in height, with 5 roots and at least 3 leaves were selected for green house acclimatization. At least 10 plantlets were placed in each of the three media selected. After 1 week of acclimatization in the greenhouse, there was 100% survival of all the plantlets in all the media with no change in both plant height and number of leaves. The average height of plantlets transplanted on soil: sand: manure media (3:1:1 ratio) was 7.97cm while on vermiculate was 6.37cm and finally on a control media (soil only) was 6.01cm after 1 week of transplanting in the greenhouse. Notably, by the end of 12 weeks, the average height of plantlets on soil: sand: manure media was 12.28cm while those on vermiculate media were 7.7cm. All the plantlets transplanted on the control died. The highest mortality rate was observed between the 2nd week and the 6th week with 100% mortality rate on the control media whiles the soil: sand: manure media and vermiculate media had 40% and 50% respectively. Notably, there was a gradual loss of plantlets on soil: sand: manure media (10%) compared to those in vermiculate media (50%) after 4 weeks. The highest mortality rate was due to the rotting of the plantlets from the roots and full acclimatization was actually observed from the 8th week. Though plantlets continued to grow increasing height, no new leaves formed. (Figure 16 and Figure 17) Also, there

were no more deaths and hence the survival rate in both soil: sand: manure and vermiculate media was 60% and 40% respectively.

Table 7: Effect of media on greenhouse acclimatization

Weeks	Data recorded after every 2 weeks on different acclimatization media					
	Control (soil only)		soil: sand: manure 3:1:1 ratio		Vermiculate media	
	Mean height	Survival rate	Mean height	Survival rate	Mean height	Survival rate
0	6.01±0.78	100	7.97±1.31	100	6.37±2.37	100
2	6.01±0.87	50	8.63±1.41	90	6.49±0.75	60
4	7.0±0.83	30	8.68±1.42	90	6.13±0.97	50
6	0	0	8.83±2.08	60	6.02±1.21	50
8	0	0	10.6±3.41	60	6.85±1.96	40
10	0	0	11.65±3.84	60	7.7±2.61	40
12	0	0	12.28±4.06	60	7.7±2.61	40

Mean values followed by the same letter within the column are not significantly different by Fisher's test ($P<0.05$) (n=30)

Figure 16: Plantlet acclimatization on soil: sand: manure media

Figure 17: Plantlet acclimatization on vermiculate media

CHAPTER FIVE

DISCUSSION

5.1 *In vitro* regeneration of coconut through direct organogenesis

The first step in tissue culture procedure is to get microbial free explants. The level of contamination here was 19.4% when the plant growth regulators were co-autoclaved with the media within the first 4 weeks of initiation. This was lower as compared to 54% when using co -autoclaved plant growth regulator and 51% when using microfiltered plant growth regulators contamination level in study by (Muhammed et al., 2013) who attributed the same to systemic contamination. For this study, major contaminants were fungal. Various studies have also revealed that fungal contaminants are the main cause of plant mortality especially in woody plants (Ray et al., 2017). On a study done by Omamor et al., 2007 on oil palm the major contaminants were reported to be *Penicillium* sp with 40.8% followed by *Curvularia sp 14.5%.*

For the direct organogenesis using embryo explants, signs of growth which include swollen embryos, cracking and colour change were observed from 8^{th} to 16^{th} weeks in Y3 media supplemented with GA3, BAP and 2,4D. This is in contrast with findings by Muhammed et al., 2013 who used MS media whereby no signs of growth were observed until the embryos were transferred to liquid media giving 84% germination and on semi solid 27%. Embryo germination was observed from the 16 to 24 weeks which was seen by plumule and signs of radicle emergence and this was also reported by Danso et al., 2009. For this study, there was 37.31% germination rate which was also lower as compared to Molla et al., 2004 whose embryo germination rate was 89% in their study on *In vitro* regeneration of coconut in Bangladesh using Y_3 basal nutrient medium and higher as compared to (Muhammed et al., 2013) on Zygotic embryo culture in Kenya with 27% germination rate in solid MS media. Low germination rates could be attributed to several factors including genotype, the effect of genotype, embryo maturity and culture medium on *In vitro* embryo germination of Sri Lankan coconut (Cocos nucifera L.) varieties using the embryo explants revealed the germination rate for each genotype was: san Ramon Tall (SNRT) (77.48 %), Sri

Lanka Red Dwarf (SLRD) (67.28 %), Sri Lanka Green Dwarf (PGD) (71.85 %) and King Coconut (RTB) (52.5 %), embryo maturity; where 97.67% germination was evident when using 12 month old coconuts as compared to 10 month old which resulted in 52.17%; culture medium and the sucrose levels where the solid media recorded 91.66% and 92.22% in 75 g/L and 60 g/L sucrose respectively as compared to liquid media which recorded 56.66 % and 60.46 % in 75 g/L and 60 g/L sucrose, respectively as reported by (Vidhanaarachchi et al., 2016).

This study recorded 33% dormancy whereby some explants did not respond. This could be as a result of the age of the nuts either too old young as reported by Steinmacher et al., 2007 were he recommended use of young explants because they are more responsive for callus formation and other *In vitro* manipulation because their cells are rapidly dividing, unlike the mature explants which cause dormancy when subjected to *in vitro* propagation. In previous studies dormancy was attributed to the use of nuts with mature embryos and endosperm which are likely to contain inhibitory substances leading to dormant cells. For this study, Gibberellic acid ($GA_{3)}$ was added to the culture medium to promote the germination of somatic embryos. This phytohormone was also was used for breaking dormancy at every stage of embryo development hence improving the performance of coconut micropropagation. This is in agreement with (Fernando and Gamage, 2000) who observed that substances which stimulates growth can be applied in form of natural fluids such as coconut water which contains various hormones that break its own dormancy.

The average shoot heights were not significantly different in Y3 media with various concentrations of BAP. The highest average height was recorded in the media supplemented with 10µM BAP and the lowest was in the media supplemented with 15µM BAP (Table 2). Therefore, it was evident that when BAP was added at low or higher concentration, it inhibited further shoot elongation. This was confirmed when compared to other studies on other woody perennials. In a study done by (Anis et al., 2003) on micropropagation of M alba using shoot and nodal explants, found that the use of 2mg/l BAP gave the best results but (Chitra & Padmaja, 2002) found that the addition of 0.5mg/l BAP was suitable for shoot induction and multiplication from axillary explants. However, (Bhau & Wakhlu, 2003) found out that the addition of

BAP beyond 2mg/l inhibited shoot formation and multiplication in M.albafrom nodal and shoot explants. Most studies have also revealed that the use of sucrose has a significant effect on shoot induction, multiplication and elongation. For this study,40g/l Sucrose was added to the media which resulted in the highest average shoot height of 3.63cm. This was lower as compared to (Vidhanaarachchi et al., 2016) who studied the effect of culture medium on in vitro embryo germination of Sri Lankan coconut varieties, and who recorded an average shoot height of 4.10cm in the culture media supplemented with 75 g/L sucrose and an average height of 4.83cm on a culture media supplemented with 60 g/L sucrose.

Some of the shoots produced spontaneous roots without necessarily the rooting media which was also reported by (Fernando and Gamage, 2000). Successful induction of roots is one key factor that determines the survival rate of the tissue culture plantlets upon acclimatization and field transfer. Successful rooting was achieved by supplementing Y3 media with IBA and GA3 hormones (Table 3). The use of 15µM IBA induced rooting in micro propagation of sandal wood which is also one of the recalcitrant plants as well as coconut palm (Solís-Ramos et al., 2012), contrary to this study, 5µM IBA induced higher mean rooting than at 15µM IBA though not significantly different. The minimum rooting effect was observed in the treatment without the hormones meaning that the rooting hormone was responsible for boosting root induction. This study also revealed that a combination of plant growth regulators in tissue culture media has more affirmative results as compared to using only one hormone (Vidhanaarachchi et al., 2013). GA3 has been reported by other researchers in various topics to promote normal growth, root development and axis development and also elongation which were also evident in this study.

5.2 *In vitro* regeneration of coconut through somatic embryogenesis

For indirect somatic embryogenesis, callusing was evident after 3 months in the experiments performed in the Y3 medium (Euwens, 1976) supplemented 100-250µM 2,4D alone, and in combination with GA_3, BAP, and TDZ. This is similar to (Perera et al., 2008) who reported 22% callusing of coconut using anthers explants after 3 to 8 months. The calli in media supplemented with 2,4D alone and in combination with

GA3 and BAP were compact while those in media supplemented with 2,4D in combination with TDZ were friable.

Thiadiazuron (TDZ) as compared to other plant growth regulators when incorporated in the initiation media also revealed to be a potential plant growth hormone for somatic embryogenesis in coconut. The highest percentage callus induction from embryo explants in the media supplemented with 9µM TDZ was 66.7% and the percentage embryogenic calli was 50% and all were friable. Similarly, Perera 2009 reported the use of 9µM TDZ to be effective in callusing frequency using the ovary explants which resulted in a callusing frequency of 76.4% which was higher as compared to this study. The embryo explants used in medium supplemented with 150µM 2,4D and 0.5µM BAP yielded the highest percentage of 83.3% and the least was those in Y3 media supplemented with 2,4D alone with 33.3% compared to previous studies, various authors have also reported different callusing frequencies (Hornung, 1995) of 75%, (Chan et al., 1998) of 60 % and (Fernando et al., 2004) with 54.3% all-in Euwens Y3 media in combination with other hormones.

In this study, the addition of 0.5µM GA_3 proved to be better in the induction of both callus (83.3%) and embryogenic calli (66.7%) as compared to 0.35µM GA_3 which resulted in callus induction frequency of 16.7% and embryogenic calli frequency of 16.7%. For embryogenic callus induction, Y3 media supplemented with 2,4-D in combination with BAP also yielded the highest embryogenic calli induction of 83.3%. (Perera et al., 2009) also reported higher callus induction and embryogenic calli formation of 50% to 75% using coconut anther explants all in Y3 media.
However, he also reported the use of 9µM TDZ to be effective in callusing frequency using the ovary explants which resulted in a callusing frequency of 76.4%. which was higher as compared to this study which where the media supplemented with 2,4-D in combination with TDZ was 66.7% while using the embryo explants. The highest plant regeneration for this study was 83.3%% and this was in the media which was also previously supplemented with 2,4-D and BAP and also 2,4-D and 0.5µM GA3 the least was in control media which was Y3 devoid of PGRs. However, the friable and creamy white translucent mass of globules developed in TDZ containing media later induced multiple shoots when subcultured into shooting media containing Kinetin,

(Lalitha et al., 2013) found TDZ to be very efficient in multiple shoot induction during *in vitro* regeneration of mulberry which is also a deciduous woody plant. Thidiazuron is one among the most active cytokinin-like substances for woody plant tissue culture. Similarly, (Mweu et al., 2016) also revealed that TDZ is vital for high-frequency induction of callus and multiple shoots in somatic embryogenesis of Jatropha which was in agreement for this study. The successful rate of coconut plant regeneration through somatic embryogenesis is still at a low rate but this study was higher compared to Bandupriya 2008 whose plant regeneration rate was less than 5%. The plant regeneration frequency has however been attributed to challenges of recalcitrance of coconut to tissue culture, high heterogeneity in tissue response, intensive browning of explants, low callus induction and low conversion to somatic embryos, and proliferation of roots instead of shoots (Bandupriya et a1., 2008).

However, the explants initiated in hormone-free media in this study did not induce callus or embryogenic calli. Similarly, this was also reported by (Vidhanaarachchi et al., 2016). The hormone-free media without phytohormonal additives serves occasionally in the germination of shoots but not in the whole process of tissue culture. Plant established *ex vitro* uses the endogenous hormones produced in different pathways aided by the natural environment. Hence, the *in vitro* plantlets produced in the lab also require the phytohormones in their pathways for a complete plantlet formation and also the rapid growth for normal tissues. For this study, phytohormones) were added to the culture medium to promote the germination of somatic embryos and also for breaking dormancy at every stage of embryo development hence improving the performance of coconut micropropagation. However, the effect of GA_3 has not been tested on the formation of somatic embryos (Perera et al., 2009).

5.3 Acclimatization of coconut tissue cultured plantlets

Tissue cultured plantlets acclimatized in soil: sand: manure ratio (3:1:1) and vermiculate after 12 weeks with survival rate of 60% and 40% respectively. When tissue cultured plantlets were transplanted to the greenhouse they started growing gradually. Normally when *In vitro* raised plants are taken for acclimatization their leaves are not fully developed and hence a low level of photosynthesis and undeveloped cuticular wax impairs stomatal mechanisms (Rival 2000). Despite the

addition of Y3 nutrients and hormones to aid in successful acclimatization, yellowing of leaves was still observed 3 months after transplanting and the plantlets started withering. Rival, 2000 observed similar trend and attributed the problem to the photosynthetic mechanism and/or poor nutrition in the potting media used in the acclimatization process. Due to the above characteristics, the nature of the tissue-cultured plantlets during the *ex vitro* transfer of coconut causes prolonged acclimatization duration, similarly Fernando et al., 2004 and Gunathilake et al., 2004. Explants rotting from the roots into the stem were evident and the same was reported by (Steinmacher et al., 2007). After the successful hardening of the plantlets, the plantlets steadily overcame the morphological stress and adapted to the *ex vitro* conditions. Generally, acclimatization is a key challenge in most of the tissue cultured plants due to the shock and stress experienced upon transfer from the *in vitro* environment to the *ex vitro* conditions in the greenhouse. In addition to the recalcitrance of coconut palms to tissue culture, a high rate of plantlet loss upon acclimatization hinders mass production (Fernando et al., 2004) hence availability of clean planting materials becomes a challenge.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusions

The study shows the possibility of regenerating *Cocos nucifera* L using embryo explants via indirect somatic embryogenesis where zygotic embryos were initiated in 100-250µM 2,4D in combination with GA_3, BAP, and TDZ. Lowering the concentrations of 2,4D to half while retaining the concentrations of other growth regulators induced embryogenic calli and finally sub culturing them to a shoot induction media containing kinetin, BAP, and NAA for multiple shoot induction. This is a key milestone of coconut regeneration in Kenya using Eeuwens Y3 media with distinct callus becoming embryogenic and eventually developing multiple shoots. This protocol, however, is reproducible and can be used in the mass propagation of coconut.

For direct organogenesis, embryos germination was achieved in Y3 media supplemented with 2 4D, BAP, and GA3 on 14 to 16^{th} weeks. They were then subcultured in light onto a shooting media where it took 4 weeks to change from white, cream white green and eventually green.

The shooting of coconut produced from both indirect and direct processes in the culture room was achieved in Y3 media supplemented with BAP and GA3 after 8 weeks.

Rooting was successfully induced in Y3 media supplemented with IBA which resulted in the highest number of roots and root length. However, when plantlets were subculture in a rooting media containing IBA in combination GA3, it resulted in the highest number of roots and also the highest mean length as compared to using IBA alone.

The study was able to identify the hormonal concentration and combination which lead to the successful formation and maturation of shoots from a single embryo which was taken through shooting to rooting media and hence a protocol for somatic embryogenesis of coconut using Zygotic embryos.

Finally, successful acclimatization in the greenhouse at 30 ± 2°C and 70-80% humidity was achieved both in soil: sand: manure 3:1:1 ratio and in vermiculate media after 3 months with survival rates 60% and 40% respectively.

6.2 Recommendation

Up scaling the multiplication for direct organogenesis from 4 to a higher number in future through multiplication by subdivision

The callus produced from somatic embryogenesis provides a good platform for further research, and therefore the higher rate of maturation should be optimized till plantlets are formed.

To avoid dormancy, precautions should be taken during sampling (should be 11 month old) to avoid collecting mature nuts.

The ex vitro acclimatization of somatic embryo-derived plantlets still needs to be refined. More research should be done on the right nutritional requirements in the acclimatization media for successful weaning in the greenhouse.

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APPENDICES

Appendix I: Components of Y3 medium (Euwens, 1976)

	COMPONENTS	AMOUNT /litre
1.	MACRO ELEMENTS-1A	g/l
2.	NH4.C1	5.35
3.	$KN0_3$	20.2
4.	KCl	14.92
5.	$NaH_2PO_4.H_2O$	3.12
6.	$CaCl_2.2H_2O$	2.94
7.	$MgSO_4.7H_2O$	2.47
8.	MICROELEMENTS -1B	
9.	H_2BO_3	0.31
10.	$CoCl_2$	0.024
11.	$CuSO_4$	0.025
12.	$MnSO_4$	1.12
13.	$NiCI_2$	0.0024
14.	KI	0.83
15.	ZnSO4	0.72
16.	$Na_2MoO_4.2HO_2$	0.024
17.	MICROELEMENTS -1C	
18.	$FeSO_4$	1.39
19.	Na_2EDTA	3.73

Appendix II: Morel and white vitamins

	Composition	Milligrams/Litre
1.	myo – Inositol	100
2.	Thiamine hydrochloride	1
3.	Pyridoxine hydrochloride	1
4.	Nicotinic acid (Free acid)	1
5.	Biotin	0.01
6.	Calcium pantothenate	1
7.	Total g/l	0.1

Appendix III: Effect of plant growth regulators on callus induction

Plant growth regulators in µM			
Treatments	N	Mean	SE
100µM2,4D	6	33.3	0.21
125µM2,4D	6	16.7	0.17
150µM2,4D	6	16.7	0.17
175µM2,4D	6	16.7	0.17
200µM2,4D	6	16.7	0.17
250µM2,4D	6	16.7	0.17
100µM2,4D +0.35µM GA3	6	16.7	0.17
125µM2,4D +0.35µM GA3	6	16.7	0.17
150µM2,4D +0.35µM GA3	6	16.7	0.17
175µM2,4D +0.35µM GA3	6	16.7	0.17
200µM2,4D +0.35µM GA3	6	50	0.22
250µM2,4D +0.35µM GA3	6	0	0
250µM2,4D +0.5µM GA3	6	50	0.22
100µM2,4D +0.5µM GA3	6	66.7	0.21
125µM2,4D +0.5µM GA3	6	83.3	0.17
175µM2,4D +0.5µM GA3	6	33.3	0.21
200µM2,4D +0.5µM GA3	6	33.3	0.21
250µM2,4D +0.5µM GA3	6	33.3	0.21
100µM2,4D +5µM BAP	6	33.3	0.21
125µM2,4D +5µM BAP	6	50	0.22
150µM2,4D +5µM BAP	6	100	0
175µM2,4D +5µM BAP	6	50	0.22
200µM2,4D +5µM BAP	6	33.3	0.21
250µM2,4D +5µM BAP	6	100	0
100µM2,4D +9µM TDZ	6	66.7	0.21
125µM2,4D +9µM TDZ	6	33.3	0.21

150µM2,4D +9µM TDZ	6	66.7	0.21
175µM2,4D +9µM TDZ	6	50	0.22
200µM2,4D +9µM TDZ	6	33.3	0.21
250µM2,4D +9µM	6	33.3	0.21
Control (Euwens Y3 media only)	6	0	0
p value	0.004		
Means (± SE) followed by the same alphabets in each column were not significantly different at P ≤0.05 using Fisher's test (n=186).			

Appendix IV: Effect of plant growth regulators on embryo formation

Plant growth regulators µM			
Treatments	N	Mean	SE
100µM2,4D	6	0	0.21
125µM2,4D	6	16.7	0.17
150µM2,4D	6	16.7	0.17
175µM2,4D	6	16.7	0.17
200µM2,4D	6	16.7	0.17
250µM2,4D	6	16.7	0.17
100µM2,4D +0.35µM GA3	6	16.7	0.17
125µM2,4D +0.35µM GA3	6	16.7	0.17
150µM2,4D +0.35µM GA3	6	16.7	0.17
175µM2,4D +0.35µM GA3	6	16.7	0.17
200µM2,4D +0.35µM GA3	6	33.3	0.21
250µM2,4D +0.35µM GA3	6	0	0
100µM2,4D +0.5µM GA3	6	50	0.22
125µM2,4D +0.5µM GA3	6	50	0.22
150µM2,4D +0.5µM GA3	6	83.3	0.17
155µM2,4D +0.5µM GA3	6	33.3	0.21
200µM2,4D +0.5µM GA3	6	33.3	0.21
250µM2,4D +0.5µM GA3	6	33.3	0.21
100µM2,4D +5µM BAP	6	33.3	0.21
125µM2,4D +5µM BAP	6	50	0.22
150µM2,4D +5µM BAP	6	83.3	0.17
175µM2,4D +5µM BAP	6	50	0.22
200µM2,4D +5µM BAP	6	33.3	0.21
250µM2,4D +5µM BAP	6	100	0
100µM2,4D +9µM TDZ	6	50	0.22
125µM2,4D +9µM TDZ	6	50	0.21

150µM2,4D +9µM TDZ	6	33.3	0.21
175µM2,4D +9µM TDZ	6	33.3	0.21
200µM2,4D +9µM TDZ	6	33.3	0.17
250µM2,4D +9µM TDZ	6	16.7	0.17
Control (Euwens Y3 media only)	0	0	0
p value	0.003		
Means (± SE) followed by same alphabets in each column were not significantly. different at P ≤0.05 using Fisher's test (n=186).			

Appendix V: Effect of BAP concentration on shoots elongation

BAP Concentrations in µM				
Shoot height	treatments	N	Mean	SE
	5	9	1.178	0.19
	10	9	1.667	0.33
	15	9	0.811	0.19
	Control	9	0.1	0.03
	P value < 0.05	0.001		
No of leaves	treatments	N	Mean	SE
	5	9	2.67	0.33
	10	9	2.67	0.33
	15	9	2.67	0.33
	Control (Euwens Y3 media only)	9	0.1	0.03
	P value < 0.05	0.001		
Means (± SE) followed by same alphabets in each column were not significantly different at P ≤0.05 using Fisher's test.				

Appendix VI: Effect of IBA on root induction after 8 weeks (cm) (n=36)

Plant Growth Regulators IBA µM				
No. of roots	treatments	N	Mean	SE
	10	9	3.11	1.44
	15	9	6.67	1.64
	5	9	4.89	2.34
	Control	9	0	0
	P value	0.038		
length of roots	treatments	N	Mean	SE
	10	9	0.956	0.354
	15	9	1.833	0.391
	5	9	2.8	0.792
	Control (Euwens Y3 media only)	9	0	0
	P Value < 0.05	0.002		
Means (± SE) followed by same alphabets in each column were not significantly different at P ≤0.05 using Fisher's test.				

Appendix VII: Effect of IBA on root induction after 8 weeks (cm) (n=36)

Plant growth regulators IBA (µM) +0.5µMGA3				
Variable				
No. of roots	treatments	N	Mean	SE
	10	9	3.33	0.88
	15	9	7.33	2.18
	5	9	8.33	2
	Control	9	0	0
	P Value < 0.05	0.044		
Length. of roots	Treatments	N	Mean	SE
	10	9	2.83	0.72
	15	9	3.27	0.43
	5	9	5.1	1.99
	Control(Euwens Y3 media only)	9	0	0
	P value	0.018		

Means (± SE) followed by same alphabets in each column were not significantly different at P ≤0.05 using Fisher's test.