IN VITRO CONSERVATION OF GROUNDNUT (*ARACHIS HYPOGAEA* L.).

By

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DECLARATION

I declare that the work in this thesis entitled *IN VITRO* CONSERVATION OF GROUNDNUT (*ARACHIS HYPOGAEA* L.) has been performed by me in the Department of Plant Science. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other Institution.

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CERTIFICATION

This thesis entitled "*IN VITRO* CONSERVATION OF GROUNDNUT (A*RACHIS HYPOGAEA* L.)" by Maimuna Mohammed ABDULMALIK, meets the regulations governing the award of the degree of Doctor of Philosophy of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literacy presentation.

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DEDICATION

To

My Late Father

Alhaji Abdulmalik Mohammed

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ABSTRACT

Experiments were carried out between 2009 and 2013 in the Biotechnology laboratory, Department of Plant Science, Ahmadu Bello University Samaru, Zaria with the aim of developing tissue culture regeneration, cryopreservation and slow growth protocols for conservation of groundnut. Embryonic axes obtained from seeds of Four (4) groundnut varieties; (SAMNUT 10, SAMNUT 21, SAMNUT 22 and SAMNUT 23) were used in the study. In the regeneration experiment, results showed that maximum shoot multiplication was obtained from Murashige and Skoog (MS) medium supplemented with 15 mg/L BAP. Among the groundnut varieties evaluated SAMNUT 23 and SAMNUT 22 recorded the maximum shoot height (4.7 and 4.8cm, respectively). For *in vitro* rooting, MS media supplemented with 1 mg/L NAA produced the highest root-induction frequency (82%). While the hormone free medium recorded the longest roots (7.4cm). A Significant genotypic effect was also observed, with SAMNUT 22 having the highest root-induction frequency (60%).

In the cryopreservation study, the desiccation technique gave the highest survival of 97% - 100% and shoot formation of between 92% and 97% of cryopreserved embryonic axes at an average moisture content of 17% after 4 - 5 hr desiccation. Among the varieties evaluated, SAMNUT 23 and SAMNUT 22 recorded the highest survival of 65% and shoot formation of 57-60% respectively. The results of the vitrification technique showed that the highest survival (70%) and shoot formation (65%) were obtained from embryonic axes treated for 2 hr in PVS2. SAMNUT 22 and 23 gave the highest survival of 74% and 76% and shoot formation of 72% and 73% after cryopreservation. A significant interaction was observed

between the varieties and dehydration time in PVS2 on survival and shoot formation. Highest shoot formation was observed in SAMNUT 10, SAMNUT 21 and SAMNUT 23 after 2 hr dehydration in PVS2. In the case of SAMNUT 22 this was achieved after 3hr dehydration in PVS2. In the encapsulation-dehydration, the highest survival of 73% and shoot formation of 63% of encapsulated cryopreserved embryonic axes of groundnut was obtained after 6hr air dehydration to moisture content of 20%. Among the varieties evaluated SAMNUT 23 recorded the highest survival of 67% and shoot formation of 61% respectively. In the encapsulation-vitrification technique, maximum survival rate of cryopreserved encapsulated embryonic axes was 48% obtained after 1hr dehydration in PVS2. Among the varieties evaluated SAMNUT 22 and SAMNUT 23 produced the highest survival rates of 44% and 42% respectively. A Slow growth experiment was conducted with four conservation media (MS medium+30g/L sucrose, MS medium+20 g/L sucrose +10 g/L mannitol, MS medium+15 g/L sucrose +15 g/L mannitol and MS medium+10 g/L sucrose +20 g/L mannitol) and for four conservation periods (3, 6, 9 and 12 months). Results revealed that the highest survival rate (34%) and lowest shoot height (3.03cm) were obtained on MS medium supplemented with 10g/L sucrose and 20 g/L mannitol. Among the varieties evaluated SAMNUT 23 recorded the highest survival (33%). With regard to the effect of different conservation periods, the highest survival (74%) was obtained from cultures conserved for 3 months.

This study indicates that MS medium supplemented with 15mg/L BAP and MS medium supplemented with 1mg/L NAA is suitable for shoot multiplication and *in vitro* rooting of the groundnut varieties respectively. The techniques of desiccation, vitrification and encapsulation could be used for the cryopreservation of embryonic axes of groundnut. Also, MS medium supplemented with 10g/L sucrose and 20g/L mannitol could be used for the

medium term conservation of groundnut with subculturing intervals of three (3) to six (6) months.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Groundnut Production Statistics and constraints

Groundnut is an important monoecious annual legume crop currently grown under a wide range of environmental conditions between 40ºS and 40ºN latitude in over 100 countries in Asia, Africa, Australia and Americas (Waliyar *et al*, 2007). Total world production of the crop was estimated at 45.7 million metric tonnes produced from 25.5 million hectares in 2013, of which China (37.3%), India (20.8%), Nigeria (6.6%) and the United States (4.2%) are the largest producers.

An overview of the production trend in groundnut showed that there was no increase in production and yield per hectare between 2004 and 2013 in Nigeria (Figure1.1). Production of groundnut was 3.3 million metric tonnes with an average yield of 1.5 metric tons/ha produced from 2.1 million ha in 2004. From 2005 to 2006 there was a significant increase in the production (3.5-3.8 million metric tonnes) and yield (1.6-1.7 metric tons/ha) even though the area of production increased by 0.1 million hectare. However, from 2007 to 2009 groundnut production and yield steadily declined to 2.8-2.9 million metric tonnes and 1.3-1.1metric tons/ha respectively, even though the area of production increased from 2.2 to 2.6 million ha. In 2010 groundnut production increased to 3.8 million metric tonnes produced over an area of 2.8 million ha. The yield however, remained low at an average of 1.3 metric tons/ha. From 2011 to 2012 groundnut was grown on 2.3-2.6 million ha with a total production of 2.9- 3.3 million metric tonnes and an average yield of 1.3-1.2 metric tons/ha. While in 2013 the annual production of groundnut was estimated at 3.0 million metric tons with an average yield of 1.3 tons/ha produced from 2.3 million ha (FAOSTAT, 2015).

Despite its relative importance and wide utilization, yields from Africa and Nigeria in particular still remain low. FAOSTAT (2015) revealed the average yield of 1.27tons/ha from Nigeria was lower than the world average yield of 1.79tons/ha in 2013. This low yield has been attributed to several factors, including non-availability of the seed of improved varieties, poor soil fertility, drought, inappropriate crop management practices, pests, and disease. The most serious and economically important diseases are groundnut rosette, leaf spots and aflatoxin, while important pest includes the aphids, millipedes, termites, thrips, leafhoppers, white grubs and nematodes (Echekwu and Ike, 2005). Seed quality has also been identified as an important constraint regarding low production of oil seeds, as the seeds which are often the source of planting material deteriorate rapidly during storage, thereby reducing the quality of seeds (Irfan *et al*., 2004).

Most of the abiotic and biotic constraints can be overcome through crop breeding. Generally, crop improvement programs rely on the availability of an array of plant genetic resources (PGR) which in recent years these are fast eroding due to human activities and climate change with its accompanying stresses. Unfortunately, adequate sources of resistance to various stresses are lacking in the cultivated species of groundnut. The lack of resistances in the cultivated germplasm is attributed to its narrow genetic base (Kochert *et al*., 1991). It, therefore, becomes imperative to secure existing germplasm for future breeding programs. Advances in biotechnology now provide new options for improved conservation of PGR using *in vitro* culture techniques such as slow growth for short- and medium-term conservation and cryopreservation for long-term conservation.

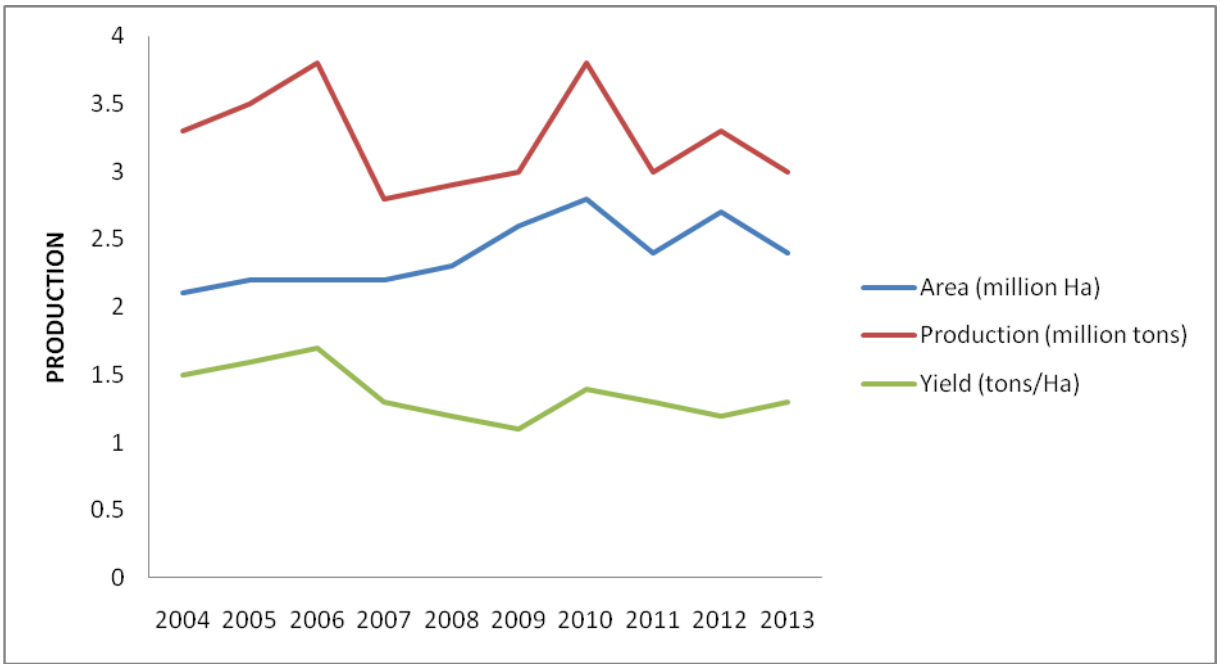

Figure1.1 Groundnut production trend in Nigeria (FAOSTAT 2015)

1.2 Plant Genetic Resources (PGR) Conservation

PGR are the most important components of agro-biodiversity. PGR comprise a diversity of genetic material contained in traditional varieties, modern cultivars, crop wild relatives, and other wild species. Genetic diversity provides plant breeders with options to develop, through selection and breeding, new and more productive crops, that are resistant to virulent pests and diseases and adapted to changing environments (Rao, 2004). With the unabated growth in human population, the world's PGR needs to be secured and more thoroughly exploited to meet the inevitable expansion in future food need. Genetic resources are disappearing at unprecedented rates. The reasons for this loss are many and include deforestation, developmental activities such as hydroelectric projects, road laying, urbanization and finally modern agriculture and the introduction of new and uniform varieties (Rao, 2004).

There are two approaches for the conservation of PGR, namely *in situ* and *ex situ*. *In situ* conservation involves maintaining genetic resources in the natural habitats where they occur, whether as wild and uncultivated plant communities or crop cultivars in farmers' fields as components of the traditional agricultural systems (Rao, 2004). *Ex situ* conservation, on the other hand, involves conservation outside the native habitat and is generally used to safeguard populations in danger of destruction, replacement or deterioration. Approaches to *ex situ* conservation include methods like seed storage, field gene banks, and botanical gardens. Among the various *ex situ* conservation methods, seed storage is the most convenient for long-term conservation of PGR (Rao, 2004). This involves desiccation of seeds to low moisture contents and storage at low temperatures. However, there are a large number of important tropical and sub-tropical tree species which produce recalcitrant seeds that quickly lose viability and do not survive desiccation, hence conventional seed storage strategies are not possible (Roberts, 1973). There are also a number of other important crop species that are sterile or do not easily produce seeds, or seed is highly heterozygous and clonal propagation is preferred to conserve elite genotypes. Examples of crop species in this category are the banana, sweet potato, sugarcane, cassava, yam, potato, and taro. These species are usually conserved in field gene banks. Although field gene banks provide easy access to conserved material for use, they run the risk of destruction by natural calamities, pests, and diseases. These field gene banks do not represent the entire range of genetic variability within the respective crop gene pool. For this reason, safety duplicates of the living collections are established using alternate strategies of conservation and it is in this area that biotechnology contributed significantly by providing complementary *in vitro* conservation options through tissue culture techniques. *In vitro* conservation also offers other distinct advantages. For example, the material can be maintained in a pathogen-tested state, thereby facilitating safer distribution. Further, the cultures are not subjected to environmental disturbances (Withers

and Engelmann, 1997). Several *in vitro* techniques have been developed for storage of challenging crop species. In general, they fall under two categories: (i) slow growth procedures, where germplasm accessions are kept as sterile plant tissues or plantlets on nutrient gels; and (ii) cryopreservation where plant material is stored in liquid nitrogen.

1.3 Justification and Objectives

Groundnut is an important crop worldwide for the production of oil and protein from the seed. Groundnut production is hampered by the multitude of biotic and abiotic stresses, of which there are few improved varieties that are resistant to these stresses and this is mainly due to the narrow genetic base of the cultivated groundnut. Securing existing and novel groundnut germplasm is essential for future improvement programs. Conventionally, groundnut germplasm is conserved in gene banks. However, even under gene bank condition groundnut seeds cannot be stored for long like other true orthodox seeds owing to its high lipid content and thin seed coat. *In vitro* techniques such as slow growth and cryopreservation should be considered as important complementary strategies for efficient conservation of groundnut germplasm. Slow growth procedures provide short- and medium-term storage options, and offer several advantages such as: free from genetic erosion, the possibility for the establishment of core collection with long-term gene banks, free from fungi and bacteria, not expensive when *in vitro* facilities are already present, easy and convenient for international distribution. While cryopreservation enables long-term storage of the plant material and these require less space, labour and it's cheaper in the long run.

Successful *in vitro* conservation of cultivated and wild species of groundnut using cryopreservation has been reported (Gagliardi *et al*., 2000; 2002; 2003; Ozudogru *et al*.,

2009). However, *in vitro* regeneration and conservation techniques are strongly genotypic dependent. This study aims at developing efficient *in vitro* conservation techniques for Nigerian groundnut cultivars that when applied alongside with the conventional seed bank approach will improve the conservation of the crop.

The specific objectives of the study are as follows;

I. To test the amenability of groundnut varieties to tissue culture.
II. To develop an *in vitro* regeneration protocol that will form the basis for establishing an *in vitro* conservation protocol for groundnut.
III. To identify an efficient cryopreservation protocol for groundnut.
IV. To establish a slow growth *in vitro* conservation protocol for groundnut.

CHAPTER TWO

2.0 LITERITURE REVIEW

2.1 Origin and Distribution of Groundnut Crop

The cultivated groundnut or peanut (*Arachis hypogaea* L.) originated in South America. The term Arachis is derived from the Greek word "Archos", meaning a weed, and *hypogaea*, meaning underground chamber, i.e. in botanical terms, a weed with fruits below the soil surface. There are two most common used names for this crop, i.e. groundnut or peanut. The term groundnut is used in most countries of Asia, Africa, Europe and Australia while in North and South America it is commonly referred to as peanut. The term groundnut refers to the pods with seeds that mature underground; the connotation of peanut is because this crop belongs to the leguminous family which includes also other crops such as peas and beans. It is a legume crop and the terminology nut is due to its unusual growing habit where flowers are formed above the ground and after fertilization the gynoecium penetrates the soil and form pods which contain kernels (Prasad *et al*., 2010).

The earliest archaeological records of groundnuts in cultivation are from Peru, dated 3750-3900 years before present (BP). Groundnuts were widely dispersed through South and Central America by the time Europeans reached the continent, probably by the Arawak Indians. There is archaeological evidence of their existence from Mexico, dated 1300-2200 BP. After European contact, groundnuts were dispersed worldwide. The Peruvian runner type was taken to the Western Pacific, China, Southeast Asia and Madagascar. The Spanish probably introduced the Virginia type to Mexico, via the Philippines, in the sixteenth century. The Portuguese then took it to Africa via Brazil. Virginia types apparently reached the Southeast United States with the slave trade (Prasad *et al*., 2010).

2.2 Taxonomy and Classification

The genus *Arachis* belongs to family *Fabaceae*, subfamily *Papilionaceae*, tribe *Aeschynomeneae*, subtribe *Stylosanthinae*. This genus is morphologically well defined and distinguished from other genera by having a peg and geocarpic reproductive growth. The genus Arachis has more than 70 wild species, of which only *Arachis hypogaea* L. is domesticated and cultivated (Prasad *et al*., 2010).

The taxonomy of the genus *Arachis* has been well documented and includes 37 named species and a number of undescribed species. The genus has been divided into nine sections i.e., *Arachis, Caulorrhzae, Erectoides, Extranervosae, Heteranthae, Procumbentes, Rhizomatosae, Trierectoides* and *Triseminalae*. The section *Arachis* comprises an annual and perennial diploid (2n=20) and two annual tetraploids (2n=4x=40) (Prasad *et al*., 2010). There are two major subspecies of *Arachis hypogaea* that mainly differ in their branching pattern (Gibbons *et al*., 1972): *Arachis hypogaea* subsp. *hypogaea* with alternate branching comprising the Virginia group of cultivated groundnut and subspecies *Arachis hypogaea* subsp. *fastigiata* with sequential branching comprising the Spanish-Valencia group of cultivated groundnut. When these lateral branches are upright, the plant is designated bunch or erect type. However, when horizontal and trails the ground, the plant is referred to as a runner or trailing type (Prasad *et al*., 2010).

2.3 Botanical Description

Groundnut is an annual herbaceous legume. It has a central, upright stem and many lateral branches. In runner types, the lateral branches are prostrate, and in bunch types they are more or less erect in the young plants but tend to become prostrate at a later stage. Mature stems

are cylindrical and hollow, sometimes slightly hairy or pubescent (Okonmah and Eruotor, 2012). The Valencia and Spanish groundnut are bunch types, while the Virginia groundnut consists of both the bunch and runner types (Chapman and Carter, 2000). The plant has pinnately compound leaves made up of four leaflets in opposite pairs, with pubescent petioles, oval edges extremely rounded, with slightly pointed tips. Stipules are prominent, linear and adnate to some length to the petiole and become free at the pulvinus. The leaf exhibits nyctiotrophic (sleep) movements, where the adaxial of the leaflets comes together and the petiole drops during each night. The leaves of the Virginia types tend to be darker green, whereas Spanish and Valencia groundnut tend to have lighter leaves (Okonmah and Eruotor, 2012).

The inflorescence is a spike of three to five flowers, borne either singly or in groups in the axils of foliage leaves. The flowers are sometimes white, but more often yellow to orange (Chapman and Carter, 2000; Okonmah and Eruotor, 2012). Groundnut plants are generally self-pollinated (Chapman and Carter, 2000). However, natural cross-pollination occurs at the rate of less than 1% to greater than 6% due to a typical flower and action of bees (Coffelt, 1989). After pollination and fertilization, the flower drops, the corolla close, the calyx tube bends and the flower withers. The region behind the ovary which is a meristematic region begins to grow and becomes the stalk-like structure (the gynophore) that bends downwards (positive geotropic) and forces the ovary into the soil. The ovary at the tip of the peg grows into a pod containing the seeds. The gynophore is commonly referred to as peg and the stage of the plant development at which the gynophore is activated and elongates is referred to as pegging (Chapman and Carter, 2000; Okonmah and Eruotor, 2012). The pod is an elongated sphere with different reticulation on the surface and /or constriction between the seeds, and contains one to six seeds (Okonmah and Eruotor, 2012). The primary root system is tap root,

but with abundantly branched lateral roots, the roots are concentrated in the 5 to 35cm zone below the soil surface. Groundnut roots do not have typical root hairs, but rather tufts of hair, which are produced in the root axils (Okonmah and Eruotor, 2012; Prasad *et al*., 2010).

2.4 Utilization

Groundnut is the world's 13th most important food crop, 6th most important source of edible oil and 3rd most important source of vegetable protein (Waliyar *et al*., 2007). It is an important food and cash crop in Nigeria. It is cultivated as a sole crop, intercrop or mixed crop. Almost every part of the groundnut plant is economically important. The seeds contain high quality edible oil (47-53%), easily digestible protein (25-36%) and carbohydrate (10-15%), besides vitamins (B and E) and mineral (Phosphorus) (Prasad *et al*., 2010). Haulms (leaves and stalk) are utilized as fodder while, the cakes formed after the oil extraction is a high protein animal feed. The shells are used as fuel, as filler in feed industry and in making cardboards. Being a legume with root nodules, it enriches the soil by fixing atmospheric nitrogen, thereby contributing to soil fertility (Waliyar *et al*., 2007).

Groundnut is processed into numerous products for human consumption, like vegetable oil, peanut butter, flour used in confectionery etc. The Kernels are consumed directly as food or snacks in the form of boiled nuts or roasted nuts also in the preparation of local dishes e.g. groundnut soup, Kunun gyada (groundnut porridge), candy, kuli-kuli (groundnut cake) etc.

2.5 Tissue Culture Technique

Plant tissue culture is a technique of culturing plant cells, tissues and organs on synthetic nutrient media under aseptic environment and controlled conditions of light, temperature and

humidity (Dagla, 2012). Plant tissue culture is based on the concept of totipotency i.e. all plant cells given the correct stimuli have the genetic potential to regenerate into whole organisms. Plant regeneration may occur through two pathways, i.e. organogenesis which is the production of shoots, followed by root formation, or through somatic embryogenesis whereby fully formed embryos are generated and subsequently induced to germinate (Razdan, 2002; Slater *et al.*, 2008).

2.5.1 Somatic embryogenesis

Somatic embryogenesis is the process of a single cell or group of cells initiating the developmental pathway that leads to the reproducible regeneration of non-zygotic embryos (somatic embryos) from somatic tissues which are capable of germinating to form complete plants (Razdan, 2002; Slater *et al.*, 2008). These somatic embryos can be produced either directly or indirectly. In direct somatic embryogenesis, the embryo is formed directly from a cell or small group of cells without the production of an intervening callus. Though common form for some tissues (usually reproductive tissues such as the nucellus, styles, or pollen). Direct somatic embryogenesis is generally rare in comparison with indirect somatic embryogenesis. In indirect somatic embryogenesis, callus is first produced from the explant. Embryos can then be produced from the callus tissue or from a cell suspension produced from that callus. Somatic embryogenesis usually proceeds in two distinct stages. In the initial stage (embryo initiation), a high concentration of 2, 4-dichloro-phenoxyacetic acid (2, 4-D) is used. In the second stage (embryo production), embryos are produced in a medium with no or very low levels of 2, 4-D. In many systems, it has been found that somatic embryogenesis is improved by supplying a source of reduced nitrogen, such as specific amino acids or casein hydrolysate (Slater *et al*. 2008).

2.5.2 Organogenesis

Organogenesis relies on the production of organs, either directly from an explant or from a callus culture. There are two types of shoot organogenesis, direct and indirect. In direct organogenesis organs (usually shoots) are generated directly from the meristematic primordia of the explant. Indirect organogenesis involes the formation of shoots is indirectly from an intermediary callus that first develops on the explants (Davies *et al.,* 1997; Kane *et al.*, 1994).Organogenesis relies on the inherent plasticity of plant tissues, and is regulated by altering the components of the medium

The ratio of auxin to cytokinin in the medium determines which developmental pathway the regenerating tissue will take. It is usual to induce shoot formation by reducing the auxin to cytokin ratio of the culture medium. A high auxin to cytokinin ratio promotes rooting or rhizogenesis (Slater *et al.,* 2008). Shoot organogenesis offers several advantages compared to somatic embryogenesis viz; shoot organogenesis is more easily achieved in most species than somatic embryogenesis; shoot production is more synchronous than somatic embryo formation; indirect shoot organogenesis is associated with higher genetic variability and has more chance for selection of somaclonal variants; survival rate is higher among plantlets produced via shoot organogenesis than those via somatic embryogenesis after transferring to greenhouse (Shen, 2007).

2.6 Groundnut tissue culture

In groundnut, regeneration can be effected by either direct organogenesis (Banerjee *et al.*, 2007; Matand *et al.,* 2013; Palanive *et al.*, 2014) or by indirect organogenesis (Karthikeyan

and Darveena, 2015) through the development of shoots from the callus tissue. Regeneration can also occur through somatic embryogenesis (Iqbal *et al.,* 2011).

Tissue culture response in the groundnut is strongly influenced by the plant genotype (Banerjee *et al.,* 2007; Nazir *et al.*, 2011; Okello *et al.,* 2015), the age of the explant source (Mroginsky *et al.,* 1981) and the plant growth regulator levels of the culture medium (Mroginsky *et al.*, 1981; Mckently *et al.,* 1990). Further, the effect of a particular plant growth regulator depends not only on the concentrations applied, but also on the presence of other plant growth regulators as well as its interaction with endogenous growth regulators (Roy and Banerjee, 2003).

2.6.1 Factors influencing organogenesis in groundnut.

Genotype: Regeneration capacity of plants shows a wide range among families, species and even within genotypes from the same species. Generally dicotyledons regenerate more easily than monocots. Plants from some dicotyledonous families such as *Solanaceae, Cruciferae, Gesneriaceae, Begoniaceae,* and *Crassulaceae* have a high regeneration capacity (Yildiz, 2012). Genotypic difference in response to tissue culture in groundnut has been reported by several authors (Banerjee *et al.,* 2007; Marka *et al.,* 2013) and this culture could also be due to different levels of endogenous plant growth regulators within the explants (Banerjee *et al.*, 2007). Verma *et al.* (2009) reported maximum multiplication of in three varieties of groundnut on MS medium supplemented with 15mg/L 6- benzyl aminopurine (BAP) while in the remaining two at 10mg/L BAP. Marka *et al.* (2013) also reported maximum number of multiple shoot formation in one cultivar of groundnut at 15mg/L BAP while in the other at 20 mg/l BAP in comparison to all other concentrations and combinations of plant growth

regulators used. On the contrary, Banerjee *et al.* (2007) reported a maximum multiplication potential in four varieties at 25mg/L BAP while in the fifth variety at 10mg/L BAP. *In vitro* rooting in groundnut is also strongly influenced by the plant genotype (Verma *et al.*, 2009).

Explant: Regeneration capacity depends on the source, type and age of the explant, generally regeneration capacity of older plants is often low. As the organ used for explant source gets older, regeneration capacity decreases. (Yildiz, 2012; Yildiz *et al.*, 2002). Four explant types, including, the whole cotyledon, mono-side-cut cotyledon, diced cotyledon and two-side-cut cotyledon, and root segments from germinating embryos of groundnut were evaluated and mono-side-cut cotyledon was overall the most responsive explant for direct shoot formation (Matand *et al.*, 2013). Venkatachalam *et al.* (1998) while evaluating two explant types of groundnut on plant regeneration reported that although both explant types (epicotyl and hypocotyl) produced multiple shoots at all the five levels of BAP tested, however epicotyl produced the highest number of shoot bud primordia (at 5.0 mg/L). Swathi *et al.* (2006) reported that both the percent regeneration, as well as the average number of shoot buds per explant of groundnut, was found to be higher (82% regeneration, 28 ± 1.5), when the adaxial side of the cotyledon was in direct contact with the medium compared to the abaxial side (58% regeneration, 8.75 ± 2.4).

Plant growth regulators: Plant growth regulators are the most important inducing signal for shoot organogenesis. Yadav *et al.* (2013) reported maximum multiple shoot induction from nodal segments of groundnut in MS medium supplemented with 1.0 mg/L BAP, 3.0 mg/L kinetin (KIN), 1.0 mg/L α-napthaleneacetic acid (NAA) and 0.5 mg/L NAA+2.0 mg/L BAP but best response was observed in medium containing 0.5 mg/L NAA + 2.0 mg/L BAP (Average number of shoots 3.52±0.39, shoot length 7.34±0.30cm). While maximum rooting

was on MS medium with 1.0 mg/L indole-3-butyric acid (IBA). McKently *et al.* (1990) reported maximum root induction frequency on MS medium supplemented with 1mg/L NAA. On the contrary, Gagliardi *et al.* (2000) reported a high development of roots on MS hormone-free medium. Two different cytokinins BAP, KIN and auxins NAA, IBA were tested; among these higher multiple rates were recorded at 5.0 mg/L BAP and maximum rooting at 0.5 mg/L NAA (Venkatachalam and Kavipriya, 2012). Regeneration with cotyledonary explant was standardized by testing various combinations of growth regulators like BAP, KIN, 2,4-D and NAA, with MS medium fortified with BAP at 4 mg/L in combination with 0.1 mg/L NAA the most effective out of the various BAP and NAA combinations tested in multiple shoot bud formation (Swathi *et al*., 2006). Several studies have reported that plant growth regulator BAP is the most suitable cytokinin for shoot induction and multiplication in groundnut (Banerjee, 2014; Banerjee *et al*., 2007; Verma *et al*., 2009). However, the optimum concentration of BAP in shoot multiplication varies with the genotype of groundnut (Banerjee *et al*., 2007; Verma *et al*., 2009). The superior effect of BAP over other cytokinins for shoot bud induction has been attributed to the abilities of plant tissues to metabolize the natural hormone more readily than other synthetic growth regulators or to the ability of BAP to induce endogenous production of zeatin (Barna and Wakhlu, 1994).

2.7 Cryopreservation

Cryopreservation or storage in liquid nitrogen (LN) at temperature of -196^0C, at which all the cells are in a state of suspended animation, is the most promising method of *in vitro* germplasm storage (Engelmann, 2004). The advantages of cryopreservation include small space requirements, low maintenance costs, and only a modest number of replicates is

required to conserve a plant effectively (Shibli and Al-Juboory, 2000). It also has an advantage of long-term storage without subjecting to frequent subculturing which is known to induce somaclonal variation (Jain, 2011). Besides germplasm conservation, cryopreservation may also be used to rejuvenate cell and callus cultures, cryoselect plant material with special properties and for cryotherapy (elimination of pathogens from plants). Galerne *et al*. (1992) mentioned that regrowth of cryopreserved Norway spruce callus was higher compared to that of non-cryopreserved controls. Aguilar *et al*. (1993) showed that production of somatic embryos from cryopreserved Citrus embryogenic callus was higher compared to that of non-cryopreserved cultures. This is due to the selection performed by liquid nitrogen exposure between different cell types. In cryopreserved samples, liquid nitrogen exposure kills more differentiated cells, which do not participate in culture growth, whereas more meristematic cells, which are responsible for growth, remain alive and reinitiate growth very rapidly and intensely after rewarming. By contrast, non-cryopreserved cell and callus cultures grow more slowly, because of the large proportion of differentiated cells, which do not proliferate actively (Engelmann, 2004). Kendall *et al*. (1990) could select freezing tolerant callus of *Triticum aestivum* by repeating their exposure to liquid nitrogen, and the cryoselected callus regenerated plants with enhanced cold hardiness. The seed progeny of some of the lines tested exhibited significantly enhanced tolerance up to −12°C. This could be an interesting strategy to induce cold tolerance in sensitive species (Cruz-Cruz *et al*., 2013). Recently, cryopreservation has been applied in the elimination of pathogens (Ding *et al*., 2008; Helliot *et al*., 2002). Classical cryopreservation techniques have been developed in the 70-80s. They comprise a cryoprotective treatment followed by slow freezing, which is carried out using a programmable freezing apparatus (Kartha, 1985). They are based on chemical cryoprotection and freeze-induced dehydration of samples during cooling. The most commonly employed cryoprotective substances are dimethylsulfoxide (DMSO), sucrose, mannitol, polyethylene

glycol (PEG) and sorbitol. For most materials, optimal freezing conditions consist of a slow cooling rate (0.5 to 2^0C/Min) down to around -40^0C, followed by rapid immersion of samples in liquid nitrogen. Classical cryopreservation techniques are mainly used for freezing undifferentiated cultures such as cell suspensions and calluses (Kartha and Engelmann, 1994).

In recent years, new techniques have been developed for freezing of differentiated tissues and organs such as apices, zygotic and somatic embryos (Dereuddre, 1992; Engelmann, 1997). They are based on the removal of most or all freezable water by physical osmotic dehydration of explants followed by ultra-rapid freezing, which results in vitrification of intracellular solutes. Their main advantages in comparison to classical procedures are their simplicity since they do not require the use of programmable freezer, and their applicability to a wide range of genotypes. They include encapsulation-dehydration, vitrification, encapsulation-vitrification and desiccation (Engelmann, 2004).

2.7.1 Desiccation technique

Desiccation is the simplest and practicable techniques for cryopreservation, whereby explants are dehydrated by the sterile air current of a laminar air flow cabinet, over silica gel or by flow of sterile compressed air prior to cryo-exposure (Normah and Makeen, 2008). This technique is mainly used for freezing zygotic embryos or embryonic axes extracted from seeds. Desiccation technique has been applied to a wide range of plant taxes, which include embryonic axes of groundnut (Gagliardi *et al*., 2002; Ozudogru *et al.*, 2009) and wild *Arachis* species (Gagliardi *et al*., 2002), citrus (Makeen *et al.*, 2005), zygotic embryos and seeds of jatropha (Prada *et al.*, 2015)

2.7.2 Vitrification technique

Vitrification refers to the physical process by which a highly concentrated cryoprotective solution super cools to very low temperatures and finally solidifies into a metastable glass, without undergoing crystallization at a practical cooling rate (Fahy *et al*., 1984). Thus, vitrification is an effective freeze-avoidance mechanism. As a glass is exceedingly viscous and stops all chemical reactions that require molecular diffusion, its formation leads to metabolic inactivity and stability over time (Burke, 1986).The vitrification procedure involves the addition of cryoprotective solution, vitrification in liquid nitrogen, rapid thawing and unloading. During exposure to highly concentrated cryoprotective solutions, cells are dehydrated sufficiently without causing osmotic injuries, and later vitrified in liquid nitrogen. Certain penetrating solutes, such as dimethysulphoxide (DMSO) and ethylene glycol (EG), will permeate into the cells and provide some antifreeze benefits, thereby widening the window for survival between excessive cell dehydration and intracellular freezing (Meryman and Williams, 1985). The most commonly used vitrification solution in the cryopreservation of plant germplasm is the plant vitrification solution (PVS2) developed by Sakai *et al*. (1990) which contains (v/v) 30% glycerol + (v/v) 15% EG + (v/v) 15% DMSO + 0.4 M sucrose. Many species have been effectively cryopreserved using this solution (Matsumoto *et al*., 1995; Niino *et al*., 1992). Other vitrification solutions have also been developed, such as PVS which contains (v/v) 22% glycerol + (v/v) 15% EG + (v/v) 15% propylene glycol + (w/v) 7% DMSO + 0.5 M sorbitol (Uragami *et al*., 1989) and PVS3 which contains 40% (v/v) glycerol and 40% (w/v) sucrose (Nishizawa *et al*., 1993) and PVS4 which contains 35% (v/v) glycerol, 20% (v/v) ethylene glycol and 0.6 M sucrose (Takagi, 2000). The first step of the vitrification technique is to preculture explants in appropriate media, followed by a step where the explants are osmoprotected with a loading solution, usually containing 2 M glycerol and 0.4 M sucrose. The loading solution contains a lower

concentration of cryoprotectants than the plant vitrification solution (PVS) and is used to minimize the chemical toxicity of the PVS applied in the next step (Sakai *et al*., 2008). After dehydration with a PVS, explants are placed in cryovials with fresh PVS and then plunged into liquid nitrogen. This is followed by rapid warming, removal of the vitrification solution by unloading in a solution, e.g. 1.2 M sucrose (Sakai *et al*., 1991) and then recovery (Sakai *et al*., 2008). According to Volk and Walters (2006), the three mechanisms by which PVS2 aids cryoprotection of shoot tips are; replaces cellular water, changes the freezing behaviour of water remaining in the cells, and impedes water loss during air drying. Because solution components permeate into cells, PVS2 treatment can broaden allowable water contents in cryopreserved shoot tips by lessening the damage from excessive cell shrinkage and limiting the risk of ice formation and growth.

Over the past decade, the vitrification method has become the preferred method for the cryopreservation with more than 160 species and cultivars being successfully cryopreserved (Sakai, 2000; Sakai and Engelmann, 2007). Cultivated and wild *Arachis s*pecies have been cryopreserved using the vitrification technique (Gagliardi *et al*., 2002; Ozudogru *et al*., 2009). Other important crops cryopreserved by vitrification include both temperate and tropical crop species, e.g. embryonic axes of maize (Usman and Abdulmalik, 2010), shoot tips of; grape (Matsumoto and Sakai 2003), blackberry and raspberry (Gupta and Reed, 2006), protocorm-like bodies of orchid (Poobathy *et al*., 2013; Rajasegar *et al*., 2015) and zygotic embryos and seeds of jatropha (Prada *et al.,* 2015)

2.7.3 Encapsulation-dehydration technique

Encapsulation-dehydration (ED) procedure is based on the technology developed for the production of artificial seeds (Dereuddre *et al*., 1990). Explants are encapsulated in alginate

beads, pre-grown in liquid medium enriched with sucrose for several days, partially desiccated down to water content around 20% (fresh weight basis) then frozen rapidly. The survival rate is high and growth recovery of cryopreserved samples is generally rapid and direct, without callus formation. ED offers many advantages in terms of protocol simplification and manipulation of explants (Engelmann *et al*., 2008). ED also avoids the use of harmful cryoprotectants as compared to other methods (Moges *et al*., 2004). The optimizations of the pretreatment procedure and of sample residual moisture content are key factors for successful application of ED (Gonzalez-Benito *et al*., 1998). Removal of most or all freezable water from cells causes vitrification of internal solutes to take place during rapid exposure to liquid nitrogen, thereby avoiding lethal intracellular ice crystallization (Engelmann, 1997). High moisture content has been shown to cause the formation of extra and intracellular ice crystals (Moges *et al*., 2004). ED technique has been successfully applied to numerous temperate and tropical species (Gonzalez-Arnao and Engelmann, 2006). This technique has been developed for various species such as somatic embryos of olive (Shibli and Al-Juboory, 2000) ovules and somatic embryos of citrus (Gonzalez-Arnao *et al*., 2003), protocorm-like bodies of orchid (Subramaniam *et al*., 2011) and *in vitro* fragmented explant of Rose (Mubbarakh *et al*., 2014).

2.7.4 Encapsulation-vitrification technique

Encapsulation-vitrification (EV) developed by Tannoury *et al*. (1991), combines the advantages of both vitrification and ED methods. Explants are encapsulated into sodium alginate beads and sufficiently dehydrated through exposure to a high osmolarity vitrification solution, which helps induce germplasm tolerance towards ice crystal formation during exposure to liquid nitrogen (LN) (Dumet *et al*., 1993). The critical factor determining the success of cryopreservation by EV is the exposure time in the PVS2, longer exposure of plant

material in the PVS2 could cause a drastic decline in both survival and subsequent regeneration. Therefore, it is important to optimize the exposure time in PVS2 for successful cryopreservation of plant material. Exposing cells to high concentrations of cryoprotective additives is potentially injurious due to the phytotoxic effects of individual components or their combined osmotic effects on cell viability (Towill and Jarret, 1992; Sakai, 2000). Preculturing of explants in media enriched with high sucrose concentrations has been reported to increase the total soluble protein and sugar contents in the treated tissues. An increase in the protein level can be considered as one of the earliest physiological responses of osmotically stressed cells, and could be related to the improvement of the freezing tolerance of tissues (Wang *et al*., 2004). It has been applied to apices of pineapple (Gamez-Pastrana *et al*., 2004) and shoot tips of cassava (Charoensub *et al*., 2004)

2.7.5 Genetic stability of cryopreserved materials

Cryopreservation imposes a series of stresses to plant material, which is susceptible to induced modifications in cryopreserved cultures and regenerated plants. It is thus important to verify that the genetic stability of cryopreserved material is not altered before routinely using this technique for long-term conservation of plant germplasm (Engelmann, 2004). The usefulness of cryo-storage only applies if it does not lead to genetic changes in the plant species of interest (Zarghami *et al*., 2008). It is thus recommended to avoid the use of tissue in a non-organised dedifferentiated state, such as callus, and to use organised tissue like shoot tips instead to reduce the likelihood of non desirable genetic mutations (Harding, 2004). No modification has been observed at the phenotype level, biochemical, chromosomal, or molecular level, which could be attributed to cryopreservation in regenerated plants (Engelmann, 1997). The few studies comparing the vegetative and floral development in the field of plants originating from control and cryopreserved material performed with potato

(Mix-Wagner *et al*., 2003) and banana (Cote *et al*., 2000) did not reveal any differences in the characters studied. Most studies have confirmed the presence of genetic stability (Castillo *et al*., 2010; Helliot *et al*., 2002) and where changes in the genome have been found, such as in sugarcane and potato with Restriction Fragment Lengths Polymorphism (RFLP) markers, the changes could not be related to the process of cryopreservation itself (Castillo *et al*., 2010; Harding, 2004). Cejas *et al*. (2013) reported genetic stability of cryopreserved *Phaseolus vulgaris* plants at both phenotypic and molecular levels over time. Random Amplified Polymorphic DNA (RAPD) analysis reported no polymorphism in amplified fragments from *Arachis* species cryopreserved (Gagliardi *et al*., 2003).

2.8 Slow Growth

The first report of successful *in vitro* storage was of shoot tips of *Vitis rupestris* wherein darkness is used to limit growth at 9^0C and cultures were stored up to 290 days (Galzy, 1969). Slow growth offers several advantages such as: free from genetic erosion, the possibility for the establishment of core collection with long term gene banks, free from fungi and bacteria, not expensive when *in vitro* facilities are already present, easy and convenient for international distribution (Malaurie *et al*., 1998). Slow growth procedures allow plant material to be held for 1-15 years under tissue culture conditions with periodic sub-culturing, depending on species. There are several methods by which slow growth can be maintained. In most cases, a low temperature, often in combination with low light intensity (Capuana and Di Lonardo, 2013; Withers and Engelmann, 1997) or even darkness is used to limit growth (Withers and Engelmann, 1997). Slow growth can also be maintained by the addition of osmotic agents such as sucrose, mannitol or sorbitol in the culture medium or in combination with low temperature (El-Dawayati *et al*., 2012; Joshi and Jadhav, 2013; Lopez-Puc, 2013).

The use of low temperature 4ºC in combination with sorbitol as osmoticum gave 100% survival in garlic after 18months of *in vitro* preservation (Hassan *et al*., 2007). It is also possible to limit growth by modifying the culture medium, mainly by reducing the sugar content and or mineral elements concentration and reduction of oxygen level available to cultures by covering explants with a layer of liquid medium or mineral oil. Growth retardants are equally used to limit growth (Withers and Engelmann, 1997). Abscisic acid (ABA) and high concentration of agar in conservation medium were most effective for growth suppression and retention of the ability of regrowth of *Garcinia mangostana* L. and *Lansium domesticum* after 12 months of conservation (Keatmetha *et al.*, 2006). The main objective of slow growth germplasm preservation is to limit the number of subcultures and maintain the genetic diversity of a species in a sterile condition without endangering the plant stability. In vanilla species Minoo *et al*. (2006), reported that shoot cultures were maintained for more than 7 years with yearly subculture on MS medium supplemented with 15g/L each of sucrose and mannitol at 22±2ºC.

2.8.1 Genetic stability of slow growth cultures

The main objective of slow growth *in vitro* conservation is to limit the number of subcultures and maintain the genetic diversity of a species in a sterile condition without endangering plant stability (Moges *et al*., 2003). Using RAPD genetic stability was maintained after slow growth preservation in garlic (Hassan *et al*., 2007). Similarly, Sarkar *et al*. (2001) reported no detection of somaclonal variation using RAPD in potato microplants conserved for 16 months under the combined treatment of ancymidol and low temperature. Determination of stability of slow growth cultures of grape using Inter Simple Sequence Repeat amplification (ISSR) show no variation among the conserved and non-conserved grape (Hassan *et al*., 2014). It is proposed that genetic stability is maintained when the cultures are derived from meristematic

cells. Whereas, when variability is required to be induced, the cultures are grown from explants sources other than meristem and callogenesis is first initiated (Sarwar and Siddiqui, 2004). Generally, organized cultures such as shoots are used for slow growth storage since undifferentiated tissues such as callus are more vulnerable to somaclonal variation.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental Site

The study was conducted at the Biotechnology laboratory, Department of Plant Science Institute for Agricultural Research (11°11´N, 7°38´E; 686m above sea level) Ahmadu Bello University Zaria.

3.2 Experimental Design

The experiments in the study consisted of factorial combinations of treatments which were laid out in a Completely Randomized Design. Each treatment comprised of three (3) explants and this was repeated 10 times.

3.3 Plant Materials

Four (4) IAR improved varieties of groundnut; SAMNUT 10, SAMNUT 21, SAMNUT 22 and SAMNUT 23 were used in the study. Seeds of the varieties were obtained from the groundnut breeding unit of IAR. The origin and description of the varieties are given in Table 3.1.

3.4 Explant

Groundnut seeds were surface sterilized using double sterilization sequence under the laminar flow hood. The first sterilization sequence involves treatment in 70% alcohol for 5min, followed by 10% Chlorox (commercial bleach containing 3.5% NaOCl)) plus 2-3 drops of Tween 20 for 20min with occasional stirring. The seeds were then rinsed thrice with sterile distilled water. This was followed by the second sterilization sequence when the seeds were immersed in 5% Chlorox plus 2-3 drops of Tween 20 for 10min with occasional stirring and then rinsing thrice with sterile distilled water. Embryonic axes were dissected from sterile seeds and used as explants.

3.5 Culture Media

The basal media for all the experiments in this study consisted of Murashige and Skoog (MS) salts and vitamins, containing 3% sucrose and solidified with 0.8% agar (Murashige and Skoog, 1962; Table 3.2). All media were adjusted to pH 5.8 before autoclaving at 121^0C and 15psi air pressure for 15 min.

The stock solution for macro nutrients was prepared in 10x strength by dissolving appropriate quantities of the components in 1L of sterile distilled water. Stock solutions of the macro nutrients and vitamins were prepared in 100x strength by dissolving appropriate quantities of the components in 100ml of sterile distilled water. To prepare 1L of the MS basal medium, 100ml of the macro nutrient stock solution and 10ml of the micronutrients and vitamins stock solutions were used.

3.6 Regeneration Experiment

3.6.1 Shoot induction and multiplication experiment

Embryonic axes were cultured on MS medium containing 30g/L sucrose, supplemented with 6-benzylaminopurine (BAP) at varying concentrations (0.0, 5, 10, 15, 20 & 25mg/L) and solidified with 8g/L agar. The 0.0mg/L BAP served as control. The experiment was arranged as 4 x 6 factorial experiments in a Completely Randomized Design of three replicates.

3.6.2 *In vitro* rooting experiment

Microshoots of 3cm length were subcultured on MS medium plus 30% sucrose and supplemented with different concentrations of NAA (0.0, 1.0 and 1.5mg/L) The 0.0mg/L NAA served as control. The experiment was arranged as Completely Randomized Design

with 4 x 3 factorial combination of treatments in three replicates. Well rooted plantlets were washed under running tap water to remove the medium from the roots.

Table 3.1: Origin and Description of the Groundnut Varieties

Varieties	Characteristic
SAMNUT-10	Developed at IAR, Potential pod yield 2000kg/ha, Late maturing (135-150 days), Rosette resistant; Seed colour-variegated (tan and white); Oil content- 55-60% (Dry matter basis); Adaptation-Guinea savannah and forest.
SAMNUT-21	Developed at IAR & ILRI-ICRISAT; Potential pod yield 2000kg/ha Medium maturing (110-120 days), Seed mass- 45-50g; Seed colour-variegated (tan and white); Oil content-51%; Adaptation- Sudan and guinea savannah.
SAMNUT-22	Developed at IAR & ILRI-ICRISAT; Potential pod yield 2400kg/ha; Potential haulm yield 4000kg/ha; Medium maturing (110-120 days); Seed mass- 45-50g; Seed colour- tan; Oil content: 45%; Adaptation- Sudan and guinea savannah.
SAMNUT-23	Developed at ICRISAT& IAR; Potential pod yield 2000kg/ha; Early maturing (90-100 days); Rosette resistant; Seed mass- 35-38%; Seed colour-red; Oil content- 53%; Adaptation-Sudan and Sahel savannah.

Source: NACGRAB (2014)

Table 3.2: Nutritional components of MS media

Composition	Molecular formulae	Concentration in medium (mg/L)
Macro Nutrients		
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Calcium chloride	$CaCl_2 \cdot 2H_2O$	440
Magnesium sulphate	$MgSO_4 \cdot 7H_2O$	370
Potassium dihydrogen orthophosphate	KH_2PO_4	170
Micro Nutrients		
Potassium iodide	KI	0.83
Boric acid	H_3BO_3	6.2
Manganese sulphate	$MnSO_4 \cdot 4H_2O$	22.3
Zinc sulphate	$ZnSO_4 \cdot 7H_2O$	8.6
Sodium molybdate	$Na_2MoO_4 \cdot 2H_2O$	0.25
Cobber sulphate	$CuSO_4 \cdot 5H_2O$	0.025
Cobalt sulphate	$CoCl_2 \cdot 6H_2O$	0.025
Iron source		
Ferrous sulphate	$FeSO_4 \cdot 7H_2O$	27.8
Sodium EDTA	$Na_2EDTA \cdot 2H_2O$	37.3
Vitamin and Other Supplements		
Myo-inositol	$C_6H_{12}O_6$	100
Nicotinic acid	$C_6H_5NO_2$	0.5
Pyridoxine-HCl	$C_6H_{12}NO_3.HCl$	0.5
Thiamine-HCl	$C_{12}H_{17}CIN_4OS.HCl$	0.5
Glycine	$C_2H_5NO_2$	2
Carbon source		
Sucrose	$C_{12}H_{22}O_{11}$	30000
pH		5.8

Source: Slater, *et al*. (2008).

3.7 Slow Growth Conservation Experiment

To induce slow growth, *in vitro* regenerated shoots 3cm from the four varieties were cultured in sterile test tubes containing 10ml of slow growth media (one shoot per tube). The slow growth media consisted of MS media supplemented with different concentrations of sucrose (30, 20,15,10g/L) in combination with different concentration of mannitol (10,15, 20g/L) and maintained for different conservation periods (3, 6, 9 and 12 months). The different combinations of the media are given in Table 3.3. The cultures were maintained at 26±2ºC. The experiment was arranged as 4 x 4 x 4 factorial experiment in a completely randomized design of three replicates. Data was recorded at the end of each conservation period (four conservation periods).The survived shoots were cultured on recovery medium (MS medium supplemented with 15mg/L BAP) and cultures were incubated under normal growth conditions.

3.8 Cryopreservation Experiment

3.8.1 Desiccation protocol

Embryonic axes were excised under aseptic condition and subjected to desiccation under the air current of a laminar flow cabinet for 0, 1, 2, 3, 4 and 5 hr exposure period. Moisture content (MC) was determined on fresh weight basis after drying in a 100°C oven for 24 hr with 3 replicates of 10 embryonic axes per du-ration. Desiccated and non desiccated (0) embryonic axes were placed in 2 ml sterile cryovial and directly immersed into liquid nitrogen (−196°C) and held for 1 hr. After storing, cryovials were rapidly thawed in a water bath at 40°C for 2 min. Embryonic axes were cultured individually in test tubes containing 10 ml of MS medium supplemented with 15 mg/L BAP and solidified with 8 g/L agar. The

experiment was arranged as 4 x 6 factorial experiment in a Completely Randomized Design of three replicates.

Table 3.3: Media components for the induction of slow growth.

Osmotic agents	
Sucrose (g/L)	Mannitol (g/L)
30 (Standard control)	0
20	10
15	15
10	20

3.8.2 Vitrification protocol

Embryonic axes were excised from the seeds and precultured on solidified MS medium supplemented with 0.3 M sucrose for 24 hr. Precultured embryonic axes were then treated with a loading solution (2 M glycerol + 0.4 M sucrose dissolved in MS medium) for 15 min at 25°C. Treated embryonic axes were transferred to 2 ml cryovials and 1 ml PVS2 [30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethylsulfoxide (DMSO) in MS medium with 0.4 M sucrose] was added with a micropipette and dehydrated for different duration (0, 1, 2, 3, 4 and 5 hr) at 25°C before directly plunged into LN and held for 1 hr. After storing, cryovials were rapidly thawed in a water bath at 40°C for 2 min. PVS2 solution was drained from cryovials, replaced twice with 1 ml of unloading solution (1.2 M sucrose dissolved in MS medium), and held for 10 min. Cryopreserved embryonic axes were then cultured on MS medium supplemented with 15 mg/L BAP for recovery. The experiment was arranged as 4 x 6 factorial experiment in a Completely Randomized Design of three replicates.

3.8.3 Encapsulation-dehydration protocol

Embryonic axes were suspended in 2% (w/v) Na-alginate supplemented with 0.75M sucrose. The mixture including the embryonic axes was encapsulated in 0.1M $CaCl_2$ solution containing 0.75M sucrose. The encapsulated embryonic axes were then precultured in liquid MS medium supplemented with 0.75M sucrose for 72hr. They were then subjected to air drying under sterile air current of the laminar flow cabinet for the following duration; 0, 2hr, 4hr, 6hr, and compared with a control (non encapsulated and none cryopreserved embryonic axes). Dehydrated encapsulated embryonic axes were then placed in 2ml cryovial, directly immersed in liquid nitrogen (-196^{0}C) and held for 1hr. After storing, cryovials were rapidly thawed in a water bath at 40^{0}C for 2min. Encapsulated embryonic axes were then cultured on MS medium supplemented with 15mg/L BAP for recovery. The experiment was arranged as 4 x 5 factorial experiment in a Completely Randomized Design of three replicates.

3.8.4 Encapsulation-vitrification protocol

Embryonic axes were precultured on semisolid hormone free MS medium supplemented with 0.3M sucrose for 24hr in the dark. Precultured embryonic axes were then suspended in calcium-free MS inorganic medium supplemented with 2% (w/v) Na-alginate and a mixture of 2M glycerol plus 0.4M sucrose as cryoprotectants. The mixture including the embryonic axes were then dispensed with a sterile pipette into 0.1M $CaCl_2$ solution supplemented with 2M glycerol plus 0.4M sucrose and held for 1 hr at 25^{0}C. The encapsulated embryonic axes were then dehydrated with PVS2 solution in a 100ml flask at 25^{0}C for the following duration; 0, 1hr, 2hr, 3hr and compared with a control. The mixture was gently shaken 2 or 3 times per hour during dehydration. After which 5 beads were placed in a 2ml cryovial and 1ml PVS2

solution was added to cool rapidly. The cryovials were directly immersed into LN (-196^{0}C) and held for 1hr. After storing, cryovials were rapidly thawed in a water bath at 40^{0}C for 2min. PVS2 solution was drained from cryovials and replaced twice with 1ml of 1.2M sucrose solution (unloading solution) and held for 10min. Thereafter encapsulated embryonic axes were then cultured on MS medium supplemented with 15mg/L BAP for recovery. The experiment was arranged as 4 x 5 factorial experiment in a Completely Randomized Design of three replicates.

3.9 Culture Condition

Cultures were maintained in a growth chamber at 26±2^{0}C under 16hr light/8 hr dark photoperiod provided by cool white fluorescence tubes for 4 weeks. After 7days cultures with contamination were discarded.

3.10 Acclimatization

Plantlets were transferred to sterilize riverside sand and covered with transparent plastic bags to retain humidity and maintained at room temperature. After 4 days the humidity of the microclimate was reduced gradually by punching holes in the bags by 10 days the plastic bags were completely removed and plantlets transferred to bigger pots in the screen house.

3.11 Data Collection

Data were collected on regeneration frequency (%), expressed as percentage of responsive embryonic axes producing shoot after four weeks of culture initiation and on number of microshoots/explant which was obtained by counting the number of shoot developing from the explant after four weeks and the average was taken for five test tubes per replicate. For

microshoot height (cm), this was measured with a ruler from the primordial leaf node to the base of the microshoot and the average was taken for five test tubes per replicate. Number of axillary branches/shoot was counted per microshoot four weeks after shoot initiation and the average was taken for five test tubes per replicate while root induction frequency (%) was expressed as percentage of microshoots producing roots within two weeks of subculture. Number of roots/plantlet was counted as number of roots produced from the plantlet after four weeks and the average was taken for five test tubes per replicate and root length (cm) was measured with a ruler from the base of the shoot to the tip of the longest root and the average was taken for five test tubes per replicate. Survival rate (%) was determine by the appearance of green colour, increase in size, callusing and development of the root or shoot pole and expressed as a percentage of embryonic axes that survived within two weeks of culturing. Shoot formation (%) was expressed as percentage of embryonic axes regenerating shoots within one month after cryopreservation. Moisture content (MC) was determined after each dehydration period, in the desiccation and ED experiments. Embryonic axes or encapsulated embryonic axes were weighed and dried in a 100°C oven for 24hr and then reweighed. The moisture content was calculated using the following formula;

$$MC\ (\%) = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fresh weight}} \times 100$$

3.12 Statistical Analysis

Data collected were subjected to analysis of variance (ANOVA) using the general linear model (GLM) procedure of the statistical analysis system (SAS Institute Inc., 1990). Means were compared using Duncan's multiple range test (DMRT) at 0.05 probability level.

CHAPTER FOUR

4.0 RESULTS

4.1 Effect of 6-benzylaminopurine (BAP) and variety on microshoot height and multiplication potential of embryonic axes of groundnut (*Arachis hypogaea* L.).

Shoot regeneration by direct organogenesis was observed 4 weeks after culture, and 100% regeneration was recorded in all the treatments including the control (BAP-free medium). As shown in Table 4.1 and Plate I, the presence and concentration of the BAP in the media significantly (($p \leq 0.05$) affected shoot height with the BAP-free medium producing the tallest shoots (9.7cm). The addition of BAP in the media decreased shoot height progressively from 5mg/L BAP to 25mg/L BAP, which recorded the least value (2.3cm). The number of shoots/explant, was also significantly (($p \leq 0.05$) influenced by the BAP concentration (Table 4.1). BAP application from 5mg/L increased the number of shoots/explant progressively up to 15mg/L which produced the highest number of shoots/explant (8.3). Further addition of BAP in the media caused a decrease in the number of shoots with medium supplemented with 25mg/L BAP recording the lowest number of shoots. On the other hand the BAP-free medium failed to produce multiple shoots. Table 4.1, shows the effect of BAP on number of axillary branches. The addition of BAP in the culture media from 5mg/L also progressively increased the number of axillary branches with the maximum number of axillary branches (3.1) obtained in medium supplemented with 15mg/L BAP which was comparable to that obtained at 20mg/L BAP. Further increase in BAP to 25mg/L decreased the number of axillary branches. While on the BAP-free medium no axillary branching was observed in the microshoots regenerated.

Results shown in Table 4.1 indicated a significant difference (($p \leq 0.05$) among the varieties in response to the BAP treatment. SAMNUT 22 (4.8cm) and SAMNUT 23 (4.7cm) produced

the tallest shoots followed by SAMNUT 10 (4.0cm) and SAMNUT 21 (4.0cm) which are statistically similar. In terms of the number of shoots/explant, SAMNUT 22 recorded the highest number of shoots/explant (5.1) than SAMNUT 10 (3.4), which was statistically similar to that observed in SAMNUT 23 (4.6) and SAMNUT 21 (4.2). As regards to number of axillary branches, SAMNUT 23 produced the highest number of axillary branches (2.6), which was similar to the value recorded by SAMNUT 21 (2.3). While SAMNUT 21, SAMNUT 10 (2.2) and SAMNUT 22 (2.0) are statistically at par with each other.

Table 4.1: Effect of BAP concentrations and variety on microshoot height and multiplication potential of embryonic axes of groundnut.

Treatment	Shoot height (cm)	Mean Number of shoots/explant	Mean number of axillary branches/shoot
BAP concentration (mg/L)			
Control	9.7a	1.0d	0.0d
5	4.2b	3.5c	2.4c
10	3.9bc	4.5c	2.5c
15	3.4c	8.3a	3.1a
20	2.8d	6.0b	3.0ab
25	2.3d	3.6c	2.6bc
SE±	0.22	0.56	0.18
Variety (V)			
SAMNUT 10	4.0b	3.6b	2.2b
SAMNUT 21	4.0b	4.3ab	2.3ab
SAMNUT 22	4.8a	5.3a	2.0b
SAMNUT 23	4.7a	4.8ab	2.6a
SE±	0.18	0.45	0.15
BAP x V	NS	NS	NS
CV (%)	12.29	31.58	19.07

Means followed by the same letter(s) within a column are not significantly different at $p \leq 0.05$ level of significance using DMRT. NS, not significant

Plate I: Groundnut microshoots regenerated 4weeks after culture on MS media supplemented with different concentration of BAP. a: hormone free medium b:MS+5mg/L BAP c:MS+10mg/L BAP d:MS+15mg/L BAP e;MS+20mg/L BAP f: MS+25mg/L BAP

4.2 Effect of Naphthalene Acetic Acid (NAA) and variety on *in vitro* rooting of regenerated microshoots of groundnut (*Arachis hypogaea* L.).

The presence and concentration of NAA in the medium significantly influenced the rooting ability of microshoots. Rooting was observed within two weeks in responsive microshoots. Medium supplemented with 1mg/L NAA recorded the highest root induction frequency (82.4%) compared to 1.5mg/L NAA treatment (63.5%). Similarly, 1.5mg/L NAA treatment produced higher root induction frequency compared to the control (NAA-free medium) which recorded the least (Table 4.2). The effect of NAA on the number of roots/plantlet was significant. Medium supplemented with 1mg/L NAA or 1.5mg/L NAA produced the highest number of roots/ plantlet (9.3 & 9.4) compared to the NAA-free medium (Table 4.2; Plate II). However, the presence and concentration of NAA in the medium decreased the root length with the NAA-free medium, producing the longest root length (7.4cm) among the treatments (Table 4.2).

A significant difference (($p \leq 0.05$) was observed among the groundnut varieties in response to the NAA treatment. SAMNUT 22 and SAMNUT 21 recorded the highest root induction frequency of 60.0 and 55.2% respectively, and were significantly higher than SAMNUT 23 and SAMNUT 10 (Table 4.2). Similarly, the effect of NAA on the number of roots/plantlet was significant, with SAMNUT 22 and SAMNUT 23 producing more number of roots/plantlet than SAMNUT 10 which was statistically similar to SAMNUT 21. However, SAMNUT 10 and SAMNUT 21 produced the longest root lengths compare to the other varieties (Table 4.2).

The regenerated plantlets were transferred to plastic cups and acclimatized and subsequently established in the screen house in larger pots, the plants flowered and set pods. Stages in the regeneration by direct organogenesis are shown in Plate III.

4.2.1 Effect of variety x NAA interaction on root induction frequency of microshoots of groundnut.

Significant ($p \leq 0.05$) interaction was observed between the varieties and NAA treatment on root induction frequency in the groundnut plantlets (Table 4.3). All the varieties were statistically similar on both the NAA-free medium and medium supplemented with 1mg/L NAA. However, on medium supplemented with 1.5mg/L NAA significant difference were observed with SAMNUT 21 and SAMNUT 22 recording higher root induction frequency than SAMNUT 23 and SAMNUT 10.

Keeping the hormonal level constant and varying the variety it was observed that SAMNUT 10, SAMNUT 21 and SAMNUT 23 recorded higher root induction frequency on medium supplemented with 1mg/L NAA than on the other media. On the contrary SAMNUT 22 recorded higher root induction frequency on media supplemented with 1mg/L NAA and 1.5mg/L NAA than on the NAA-free medium (Table 4.3).

4.2.2 Effect of variety x NAA interaction on the number of roots/plantlet of groundnut.

Table 4.4 presents the effect of variety x NAA interaction on the number of roots/plantlet of groundnut. The NAA-free medium and medium supplemented with 1mg/L NAA did not have any significant effect on the number of roots/plantlet in the groundnut varieties. However, when the medium was supplemented with 1.5mg/L NAA significant difference was observed

with SAMNUT 23 producing more roots/plantlet than SAMNUT 21 and SAMNUT 10, while SAMNUT 22 was statistically comparable to SAMNUT 23 and SAMNUT 21.

Keeping the hormonal level constant and varying the variety, it was observed that SAMNUT 10 and SAMNUT 21 significantly produced the highest number of roots/plantlet on medium supplemented with 1mg/L NAA while SAMNUT 22 produced the highest number of roots/plantlet on media supplemented with 1mg/L NAA and 1.5mg/L NAA than on the NAA-free medium. On the contrary, SAMNUT 23 recorded the highest number of roots/plantlet on medium supplemented with 1.5mg/L NAA (Table 4.4).

4.2.3 Effect of variety x NAA interaction on root length of groundnut plantlets.

There was a significant ($p \leq 0.05$) interaction between the varieties and NAA treatment on root length of groundnut plantlets (Table 4.5). On the NAA-free medium SAMNUT 10 recorded more root length than SAMNUT 21 and SAMNUT 23 while SAMNUT 22 is statistically similar to all the other varieties. On the other hand, when microshoots were cultured in medium supplemented with 1mg/L NAA it was observed that SAMNUT 21 recorded longer root length compared to the other varieties. However, when microshoots were cultured in medium supplemented with 1.5mg/L NAA the highest root length was observed in SAMNUT 10 and SAMNUT 21.

Comparing the varieties while maintaining the hormonal level constant, it was observed that SAMNUT 10 recorded longer root length on the NAA-free medium compared to the NAA supplemented media. A similar trend was also observed in SAMNUT 22 and SAMNUT 23. While SAMNUT 21 recorded longer root length on both the NAA-free medium and medium supplemented with 1mg/L NAA (Table 4.5).

Table 4.2: Effect of NAA concentrations and variety on root induction in microshoots of groundnut.

Treatment	Root induction frequency (%)	Mean number of roots/plantlet	Root length (cm)
NAA concentration (mg/L)			
Control	10.4c	1.7b	7.4a
1	82.4a	9.3a	5.3b
1.5	63.6b	9.4a	4.9c
SE±	2.71	0.75	0.15
Variety (V)			
SAMNUT 10	45.0b	5.2b	6.6a
SAMNUT 21	55.2a	6.4ab	6.3a
SAMNUT 22	60.0a	7.6a	5.5b
SAMNUT 23	48.3b	7.9a	5.1c
SE±	3.13	0.86	0.18
NAA x V	*	*	**
CV (%)	12.72	26.94	6.36

Means followed by the same letter(s) within a column are not significantly different at $p \leq 0.05$ level of significance using DMRT **highly significant.

Table 4.3: Effect of variety x NAA interaction on root induction frequency in microshoots of groundnut.

	NAA (mg/L)		
Variety	Control	1	1.5
SAMNUT 10	8.3d	79.4ab	47.2c
SAMNUT 21	8.3d	85.8a	71.4b
SAMNUT 22	16.7d	84.5a	78.9ab
SAMNUT 23	2.3cd	80.0ab	56.7c
		SE± 5.42	

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT.

Table 4.4: Effect of variety x NAA interaction on number of roots/plantlet of groundnut.

	NAA (mg/L)		
Variety	Control	1	1.5
SAMNUT 10	1.0d	9.7ab	5.0c
SAMNUT 21	1.0d	9.3ab	9.0b
SAMNUT 22	2.3cd	9.0b	11.3ab
SAMNUT 23	2.3cd	9.0b	12.3a
		SE± 1.49	

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT.

Table 4.5: Effect of variety x NAA interaction of root length of groundnut plantlets.

	NAA (mg/L)		
Variety	Control	1	1.5
SAMNUT 10	7.9a	6.2c	5.7cd
SAMNUT 21	7.0b	6.6b	5.3de
SAMNUT 22	7.5ab	5.0e	4.0f
SAMNUT 23	7.0b	3.3g	4.8e
		SE± 0.31	

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT.

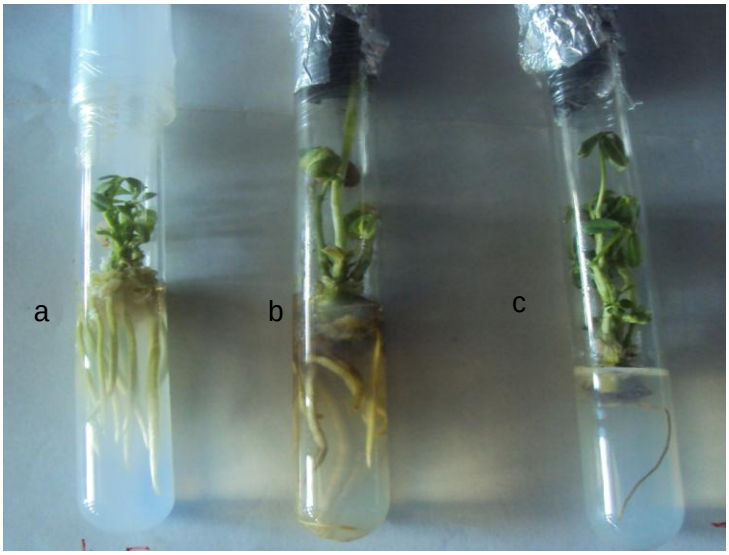

Plate II: Groundnut plantlets regenerated on root induction media. a-plantlet on MS medium+1.5mg NAA. b-plantlet on MS medium+1mg NAA. c-plantlet on control medium.

Plate IIIa: regenerated plantlets of groundnut (*Arachis hypogaea* L.) b:plantlets transferred to cups containing riverside sand for hardening. c: plantlets acclimatized in air bags. d: regenerated plants flowering

4.3 Cryopreservation of Embryonic axes of Groundnut (*Arachis hypogaea* L.) by Desiccation

Time of desiccation under the sterile air of laminar hood had a significant influence on the extent of water loss of embryonic axes of groundnut, their survival and subsequent shoot formation after storage in liquid nitrogen (Table 4.6). The control (non desiccated embryonic axes of groundnut) and embryonic axes desiccated for 1 hr failed to survive the cryogenic treatment. However, desiccation of embryonic axes from 2 hr to 5 hr rapidly caused significant loss of moisture content. This greatly improved the survival and shoot formation of cryopreserved embryonic axes. The highest survival (96.67% - 100%) and subsequent shoot formation (91.67% - 96.67%) was obtained at an average moisture content of 17% after 4 to 5 hr desiccation rates.

Results obtained from this study showed significant differences among the varieties evaluated (Table 4.6). SAMNUT 23 recorded the lowest moisture content compared to SAMNUT 22, SAMNUT 10 and SAMNUT 21 which were statistically the same. Significant survival of cryopreserved embryonic axes was observed among the varieties. SAMNUT 22 and SAMNUT 23 which were at par with each other had the highest survival. This was followed by values observed in SAMNUT 21 while the lowest survival value was observed in SAMNUT 10. Similarly, significant shoot formation of cryopreserved embryonic axes was observed among the varieties with SAMNUT 22 recording higher shoot formation value than SAMNUT 21 and SAMNUT 10.

4.3.1 Effect of variety x time of desiccation on moisture content of cryopreserved embryonic axes of groundnut.

There was a significant interaction between the varieties and time of desiccation for moisture content of cryopreserved embryonic axes of groundnut. In the control treatment SAMNUT 10 embryonic axes had higher moisture content than that of SAMNUT 21 and SAMNUT 23 which are statistically similar to SAMNUT 22. At the 1hr desiccation time, embryonic axes of SAMNUT 10 and SAMNUT 22 recorded higher moisture content than that of SAMNUT 23 which was statistically similar to that of SAMNUT 21.Thereafter, from 2 hr to 5 hr desiccation time all the varieties recorded comparable low moisture content (Table 4.7).

Comparing the varieties while maintaining the desiccation time constant it was observed that SAMNUT 10 and SAMNUT 22 recorded lower moisture content from 2hr desiccation time. On the contrary, SAMNUT 21 and SAMNUT 23 recorded lower moisture content from 1hr desiccation time (Table 4.7).

4.3.2 Effect of variety x time of desiccation on survival of cryopreserved embryonic axes of groundnut.

As shown in Table 4.8 the effect of variety x time of desiccation on survival of cryopreserved embryonic axes was significant. At 2hr time of desiccation SAMNUT 22 and SAMNUT 23 recorded higher survival rate than SAMNUT 21 and SAMNUT 10. A similar trend was also observed in 3hr time of desiccation treatment. While in the other treatments no significant difference was observed among the varieties.

Keeping the time of desiccation constant and varying the varieties, SAMNUT 10 and SAMNUT 21 recorded significantly higher survival rate at the 4hr and 5hr time of desiccation. On the contrary, SAMNUT 22 and SAMNUT 23 recorded higher values from 2hr to 5hr time of desiccation (Table 4.8).

4.3.3 Effect of variety x time of desiccation in shoot formation of cryopreserved embryonic axes of groundnut.

Effect of variety x desiccation interaction in shoot formation of cryopreserved embryonic axes was significant (Table 4.9). At 2hr time of desiccation SAMNUT 21, SAMNUT 22 and SAMNUT 23 comparably produce more shoots than SAMNUT 10. While at a 3hr desiccation time SAMNUT 22 and SAMNUT 23 had higher shoot formation than the other varieties. All the groundnut varieties comparably recorded high shoot formation at the 4hr desiccation time, with the exception of SAMNUT 10 which significantly recorded the least shoot formation. However, at a 5hr desiccation time, all the groundnut varieties recorded comparably high shoot formation after cryopreservation. When embryonic axes were desiccated for 4hr all the varieties with the exception of SAMNUT 10 comparably recorded very high shoot formation. In the control, 1hr and 5hr time of desiccation treatments no significant difference was observed among the varieties.

Varying the variety and keeping the time of desiccation constant, SAMNUT 10 recorded highest shoot formation at the 5hr time of desiccation while SAMNUT 21 recorded significantly higher values at 4hr and 5hr time of desiccation. In contrast SAMNUT 22 and SAMNUT 23 recorded higher values from 3hr to 5hr time of desiccation.

Table 4.6: Effect of desiccation rate and variety on moisture content, survival and shoot formation of cryopreserved embryonic axes of groundnut

Treatment	Moisture content (%)	Survival (%)	Shoot formation (%)
Time of desiccation (hr) (D)			
Control	27.3a	0.0d	0.0e
1	22.5b	0.0d	0.0e
2	18.3c	76.1c	59.4d
3	17.4c	83.8b	78.8c
4	17.3c	96.7a	91.7b
5	16.7c	100.0a	96.7a
SE±	0.74	2.34	1.30
Variety (V)			
SAMNUT 10	20.7a	50.4c	44.6c
SAMNUT 21	19.9a	57.7b	56.3b
SAMNUT 22	21.1a	64.8a	59.5a
SAMNUT 23	18.0b	64.7a	57.3ab
SE±	0.60	1.91	1.06
G x D	**	**	**
CV (%)	9.06	9.64	5.87

Means followed by the same letter(s) within a column are not significantly different at $p \leq 0.05$ level of significance using DMRT. NS non significant, ** highly significant

Table 4.7: Effect of variety x desiccation interaction on the moisture content of cryopreserved embryonic axes of groundnut.

	Time of desiccation (hr)					
Variety	Control	1	2	3	4	5
SAMNUT 10	31.3a	24.7b	18.7c	17.7c	16.0c	15.7c
SAMNUT 21	26.0b	22.0bc	18.3c	17.0c	17.7c	18.3c
SAMNUT 22	27.7ab	26.0b	19.0c	18.7c	18.0c	17.0c
SAMNUT 23	24.3b	17.3c	17.0c	16.3c	17.3c	15.7c
			SE± 1.47			

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT.

Table 4.8: Effect of variety x desiccation time interaction on the survival of cryopreserved embryonic axes of groundnut.

	Time of desiccation					
Variety	Control	1	2	3	4	5
SAMNUT 10	0.0e	0.0e	53.8d	60.0cd	86.7ab	100.0a
SAMNUT 21	0.0e	0.0e	71.4c	75.0bc	100.0a	100.0a
SAMNUT 22	0.0e	0.0e	88.9ab	100.0a	100.0a	100.0a
SAMNUT 23	0.0e	0.0e	88.3ab	100.0a	100.0a	100.0a
			SE± 4.68			

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT.

Table 4.9: Effect of variety x desiccation interaction on the shoot formation of cryopreserved embryonic axes of groundnut.

	Time of desiccation					
Variety	Control	1	2	3	4	5
SAMNUT 10	0.0f	0.0f	47.5e	60.0cd	70.0bc	90.0a
SAMNUT 21	0.0f	0.0f	62.5cd	75.0b	100.0a	100.0a
SAMNUT 22	0.0f	0.0f	66.7bcd	90.0a	100.0a	100.0a
SAMNUT 23	0.0f	0.0f	60.7cd	90.0a	96.7a	96.7a
			SE± 2.61			

Means followed by the same letter(s) are not significantly different at $P<0.05$ level of significance using DMRT.

4.4 Cryopreservation of Embryonic axes of Groundnut (*Arachis hypogaea* L.) by Vitrification

To determine the optimum time of exposure to the PVS2, embryonic axes of groundnut were dehydrated with PVS2 for different duration prior to a plunge into LN. Dehydration duration in PVS2 significantly (($p \leq 0.05$) affected the rates of survival and subsequent shoot formation of the cryopreserved embryonic axes (Figure 4.1). Very low survival and shoot formation was observed when embryonic axes were treated in PVS2 for 1hr. The highest survival (70%) and shoot formation (65%) was obtained when embryonic axes were dehydrated in PVS2 for 2hr and which was comparable to that recorded in the control treatment. Increasing the dehydration time from 3hr upwards significantly (($p \leq 0.05$) decreased the rate of survival and shoot formation.

Significant varietal differences were also observed among the varieties in response to the PVS2 treatment (Figure 4.2). SAMNUT 22 and 23 which are statistically the same had the highest survival (74.4 and 75.6%) and shoot formation (72.2 and 72.8%), than SAMNUT 10 and SAMNUT 21.

4.4.1 Effect of variety x dehydration time in PVS2 on survival rate of cryopreserved embryonic axes of groundnut.

Significant (($p \leq 0.05$) interaction was observed between variety and dehydration time in PVS2 on survival rate (Table 4.10). In the control treatment SAMNUT 22 and SAMNUT 23 comparably recorded higher survival rates than the other varieties. A similar trend was also observed when embryonic axes were dehydrated for 1hr, 2hr and 5hr in PVS2. When the embryonic axes were subjected to 3hr dehydration in PVS2, SAMNUT 22 recorded higher

value than the other varieties. During the 4hr dehydration treatment, SAMNUT 23 recorded higher survival rate than the other varieties.

Keeping the dehydration time in PVS2 constant and varying the variety it was observed that SAMNUT 10 recorded higher survival values after 2hr dehydration compared to the other treatments. SAMNUT 21 recorded higher survival rates in the control treatment than in the other treatments. SAMNUT 22 also recorded comparable high survival values in the control, 2hr and 3hr dehydration treatment. In SAMNUT 23 comparably high values were observed in the control and at a 2hr dehydration time in PVS2 compared to the other treatments.

4.4.2 Effect of variety x dehydration time in PVS2 on shoot formation rate of cryopreserved embryonic axes of groundnut.

A significant interaction was observed between the variety and dehydration time in PVS2 on shoot formation (Table 4.11). In the control and 3hr dehydration treatment SAMNUT 22 recorded significantly high shoot formation rate than the other varieties. When the embryonic axes were dehydrated for 1hr in PVS2, it was observed that SAMNUT 22 and SAMNUT 23 which were at par with each other recorded higher shoot formation compared to the other varieties. A similar trend was also observed in 2hr and 5hr dehydration treatments while in the 4hr dehydrating treatment the highest value was recorded by SAMNUT 23.

Comparing the varieties while maintaining the dehydration time in PVS2 constant it was observed that SAMNUT 10 significantly recorded higher shoot formation rate at a 2hr dehydration time in PVS2 than in the control and other dehydration treatments. SAMNUT 21 and SAMNUT 23 recorded higher values in the control treatment than in the other treatments.

SAMNUT 22 also recorded highest shoot formation in the control treatment, but this value was similar to that observed in 3hr dehydration treatment.

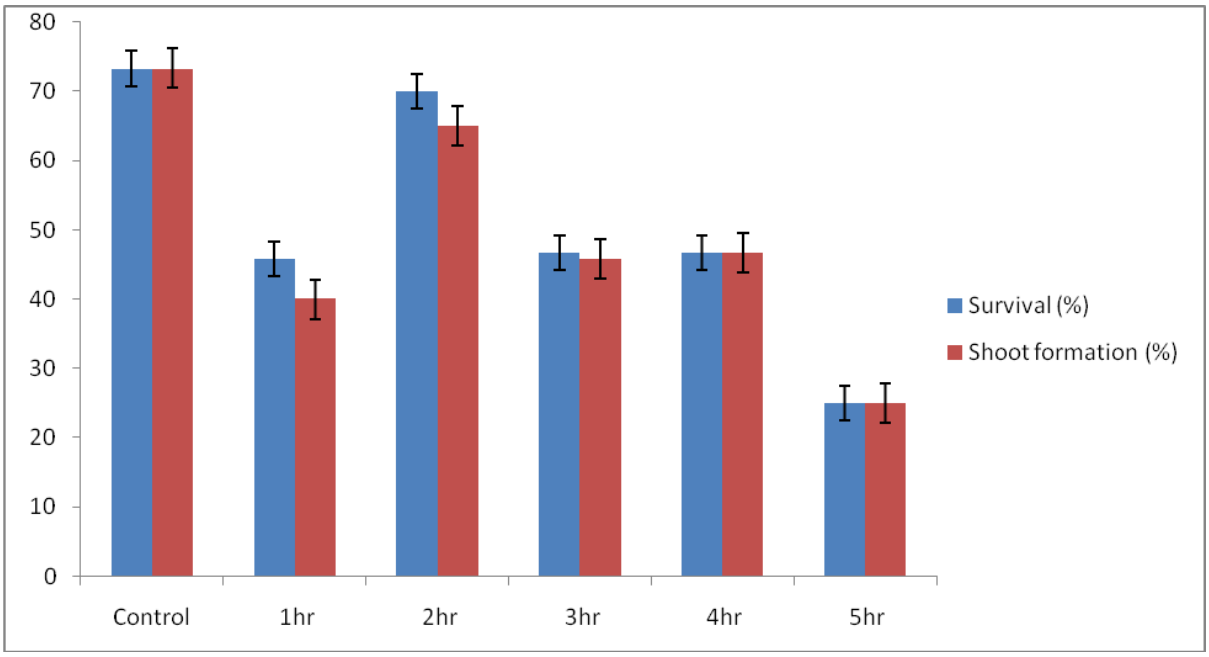

Figure 4.1: Effect of dehydration time in PVS2 on Survival and Shoot formation of cryopreserved embryonic axes of groundnut. Vertical bars represent ±SE of means of 3 replicates.

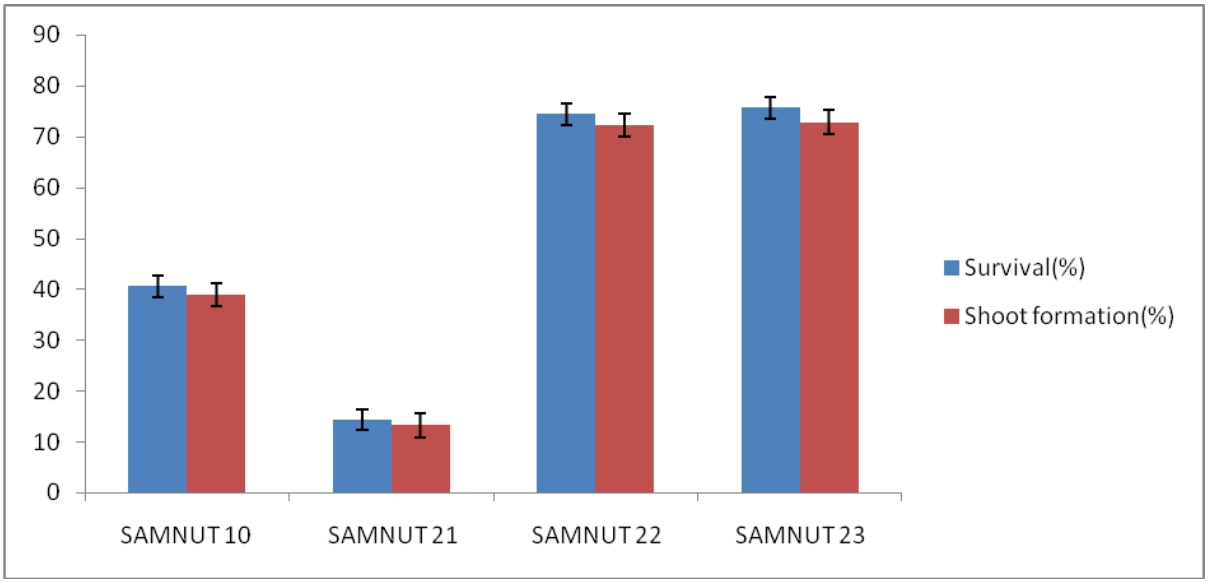

Figure 4.2: Response of groundnut varieties to PVS2 treatment. Vertical bars represent ±SE of means of 3 replicates.

Table 4.10: Effect of variety x dehydration in PVS2 interaction on the survival of cryopreserved embryonic axes of groundnut.

	Dehydration in PVS2 (hr)					
Variety	Control	1	2	3	4	5
SAMNUT 10	66.7c	43.3d	80.0b	6.7f	46.7d	0.0f
SAMNUT 21	46.7d	6.7f	26.7e	6.7f	0.0f	0.0f
SAMNUT 22	93.3a	66.7c	86.7ab	90.0a	60.0c	50.0d
SAMNUT 23	86.7ab	66.7c	86.7ab	83.3b	80.0b	50.0d
			SE± 6.67			

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT.

Table 4.11: Effect of variety x dehydration in PVS2 interaction on the shoot formation of cryopreserved embryonic axes of groundnut.

	Dehydration in PVS2 (hr)					
Variety	Control	1	2	3	4	5
SAMNUT 10	66.7d	33.3g	80.0c	6.7j	46.7f	0.0j
SAMNUT 21	46.7f	6.7i	20.0h	6.7j	0.0j	0.0j
SAMNUT 22	93.3a	60.0e	80.0c	90.0ab	60.0e	50.0f
SAMNUT 23	86.7b	60.0e	80.0c	80.0c	80.0c	50.0f
			SE± 5.71			

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT.

4.5 Cryopreservation of Embryonic Axes of Groundnut (*Arachis hypogaea* L.) by Encapsulation-dehydration

The time of dehydration in laminar hood significantly ($p \leq 0.05$) affected the rates of both survival and shoot formation of encapsulated cryopreserved embryonic axes of groundnut (Table 4.12; Plate IV). Embryonic axes not subjected to the cryogenic treatment recorded the highest survival and shoot formation compared to those subjected to the cryogenic treatment. Among the cryogenic treatments, the highest survival (73%) and shoot formation (63%) of encapsulated cryopreserved embryonic axes of groundnut were obtained after air dehydration for 6hr to 20% moisture content prior to being plunged into liquid nitrogen. This was followed by the survival (62%) and shoot formation (50%) rates obtained after air dehydration of 4hr to 26% moisture content respectively. Dehydration time of 2hr significantly produced the lowest survival (45%) and shoot formation (40%) rates at 43% moisture content. While encapsulated non dehydrated embryonic axes (0) of groundnut with moisture content of 66% failed to survive the cryogenic treatment.

Significant ($p \leq 0.05$) variation was observed among the varieties in survival levels in response to the dehydration time (Table 4.12). SAMNUT 23 produced the highest survival rate, which was statistically at par with SAMNUT 22, followed by SAMNUT 10 while SAMNUT 21 produced the lowest survival rate. Similarly, significant variation was also observed among the varieties evaluated in terms of shoot formation with SAMNUT 23 producing the highest shoot formation followed by SAMNUT 22. While SAMNUT 10 only produced higher shoot formation than SAMNUT 21. Results obtained in this study also show that the moisture content of embryonic axes was significantly influenced by the variety. SAMNUT 10 embryonic axes had the highest moisture content, followed by SAMNUT 21 which was

statistically comparable to SAMNUT 22. The least moisture content was recorded by SAMNUT 23.

4.5.1 Effect of variety x dehydration time in the laminar hood interaction on the survival rate of cryopreserved encapsulated embryonic axes of groundnut.

A significant interaction was observed between variety and time of dehydration in the laminar hood on survival of cryopreserved encapsulated embryonic axes of groundnut (Table 4.13). In the control treatment all the varieties comparably recorded higher survival rates with the exception of SAMNUT 21 which was statistically different while in the encapsulated non dehydrated treatment (0) no significant difference was observed among the varieties. When embryonic axes were dehydrated for 2hr and 6hr SAMNUT 22 and SAMNUT 23 comparably recorded higher survival compared to the other varieties while at 4hr dehydration time SAMNUT 23 recorded the highest value.

Comparing the varieties while maintaining the dehydration time constant it was observed that SAMNUT 10 and SAMNUT 21 recorded higher survival in the control treatment than the other dehydration treatments. SAMNUT 22 recorded higher survival rates in both the control treatment and 6hr dehydration time. While SAMNUT 23 recorded higher values in 4hr and 6hr dehydration time, which was comparable to that observed in the control treatment.

4.5.2 Effect of variety x dehydration time interaction on the shoot formation of cryopreserved encapsulated embryonic axes of groundnut.

There was a significant ($p \leq 0.05$) interaction between the varieties and time of dehydration under the sterile air of the laminar hood on shoot formation of cryopreserved encapsulated embryonic axes of groundnut (Table 4.14). In the control and non dehydrated treatments no significant difference was observed among the varieties for shoot formation, while in 2hr and 6hr dehydration time SAMNUT 22 and SAMNUT 23 comparably recorded higher shoot formation than the other varieties. On the contrary, at the 4hr dehydration time only SAMNUT 23 recorded higher value than the other varieties.

Comparing the varieties while maintaining the dehydration time constant it was observed that all the varieties with the exception of SAMNUT 23 recorded their highest shoot formation in the control treatment while in SAMNUT 23 the highest value was observed in the control treatment but this was statistically similar to that recorded in 4hr and 5hr dehydration time.

Table 4.12: Effect of dehydration time and variety on survival and shoot formation of cryopreserved encapsulated embryonic axes of groundnut.

Treatment	Survival (%)	Shoot formation (%)	Moisture Content (%)
Air Dehydration (hr) (**D**)			
Control	85.0a	85.0a	26.4c
0	0.0e	0.0e	65.8a
2	45.0d	40.0d	43.2b
4	61.7c	50.0c	26.4c
6	73.3b	63.3b	20.0d
SE±	3.16	2.58	0.99
Variety (**V**)			
SAMNUT 10	46.7b	42.7c	40.0a
SAMNUT 21	37.3c	30.7d	36.8b
SAMNUT 22	61.3a	56.0b	34.6bc
SAMNUT 23	66.7a	61.3a	33.9c
SE±	2.83	2.31	0.89
D x V	**	**	NS
CV (%)	14.62	13.27	6.73

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT, NS non significant, ** highly significant. 0 – none dehydrated encapsulated embryonic axes stored in Liquid nitrogen. Control- none cryopreserved regenerated embryonic axes

Table 4.13: Effect of variety x dehydration time interaction on the survival rates of cryopreserved encapsulated embryonic axes of groundnut.

	Dehydration time (hr)				
Variety	Control	0	2	4	6
SAMNUT 10	86.7ab	0.0g	40.0e	40.0e	66.7cd
SAMNUT 21	80.0bc	0.0g	20.0f	40.0e	46.7e
SAMNUT 22	86.7ab	0.0g	60.0d	73.3c	86.7ab
SAMNUT 23	86.7ab	0.0g	60.0d	93.3a	93.3a
		SE±	6.32		

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT. 0 – none dehydrated encapsulated embryonic axes stored in Liquid nitrogen. Control- none cryopreserved regenerated embryonic axes

Table 4.14: Effect of variety x dehydration time interaction on the shoot formation of cryopreserved encapsulated embryonic axes of groundnut.

	Dehydration time (hr)				
Variety	Control	0	2	4	6
SAMNUT 10	86.7a	0.0g	26.7e	40.0d	60.0c
SAMNUT 21	80.0ab	0.0g	13.3f	20.0ef	40.0d
SAMNUT 22	86.7a	0.0g	60.0c	60.0c	73.3b
SAMNUT 23	86.7a	0.0g	60.0c	80.0ab	80.0ab
		SE±	5.16		

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT. 0 – none dehydrated encapsulated embryonic axes stored in Liquid nitrogen. Control- none cryopreserved regenerated embryonic axes

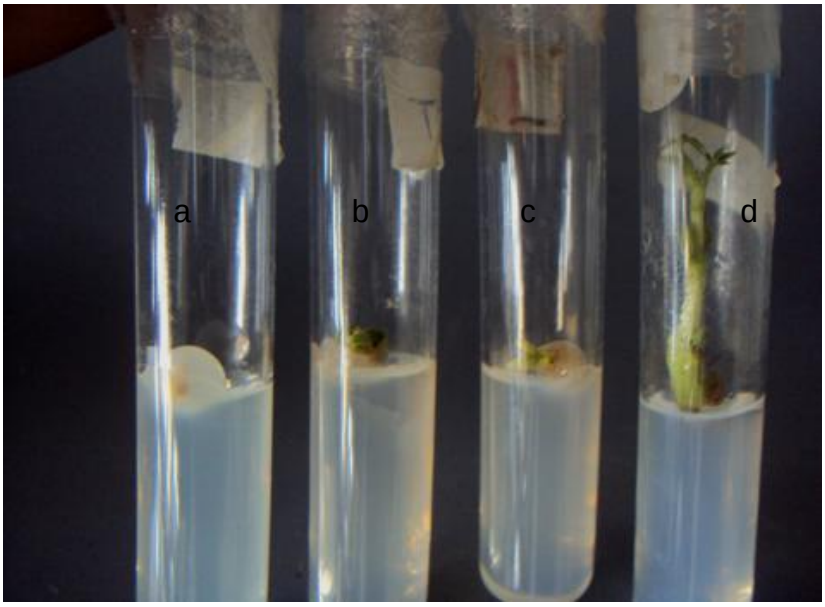

Plate IV: Cryopreserved encapsulated embryonic axes recovering on MS + 15mg/LBAP. a: 24hr after LN storage. b-c: shoot bud formed at 2 weeks. d: true shoot formed after 4 weeks

4.6 Cryopreservation of Embryonic axes of Groundnut (*Arachis hypogaea* L.) by Encapsulation-vitrification

Significant variations in terms of survival for non-cryopreserved (control) and cryopreserved embryonic axes of groundnut were observed with the non cryopreserved embryonic axes significantly ($p \leq 0.05$) producing the highest survival compared with the cryopreservation treatments (Figure 4.3). Dehydration of encapsulated embryonic axes of groundnut in PVS2 prior to a plunge in liquid nitrogen significantly affected their survival, with increasing exposure time there was a drastic reduction in the survival rate. The highest survival (48%) was obtained after 1hr of dehydration in PVS2 compared with survival rates (25%) obtained after 2hr dehydration in PVS2. While dehydration time of 3hr produced the lowest survival rate (15%).On the other hand encapsulated non dehydrated embryonic axes, failed to survive the liquid nitrogen storage. As shown in Plate V none of the survived encapsulated embryonic axes produced shoots.

Significant ($p \leq 0.05$) variation was also observed among the varieties in terms of survival in response to the dehydration time in PVS2. SAMNUT 22 and SAMNUT 23 comparably produced higher survival rates than SAMNUT 10 and SAMNUT 21 (Figure 4.4).

4.6.1 Effect of variety x time of dehydration in PVS2 interaction on the survival of cryopreserved encapsulated embryonic axes of groundnut.

The variety x dehydration time in PVS2 interaction on survival of cryopreserved encapsulated embryonic axes of groundnut was significant (Table 4.15). In the control treatment, SAMNUT 22 and SAMNUT 23 which were statistically similar significantly recorded higher survival than the other varieties. A similar trend was also observed at 1hr

dehydration time in PVS2. From 2hr to 3hr dehydration time SAMNUT 22, SAMNUT 23 and SAMNUT10 comparably recorded higher values than SAMNUT 21.

Varying the variety while maintaining the dehydration time constant it was observed that all the varieties recorded significantly higher survival rates in the control treatment compared to the dehydration treatments.

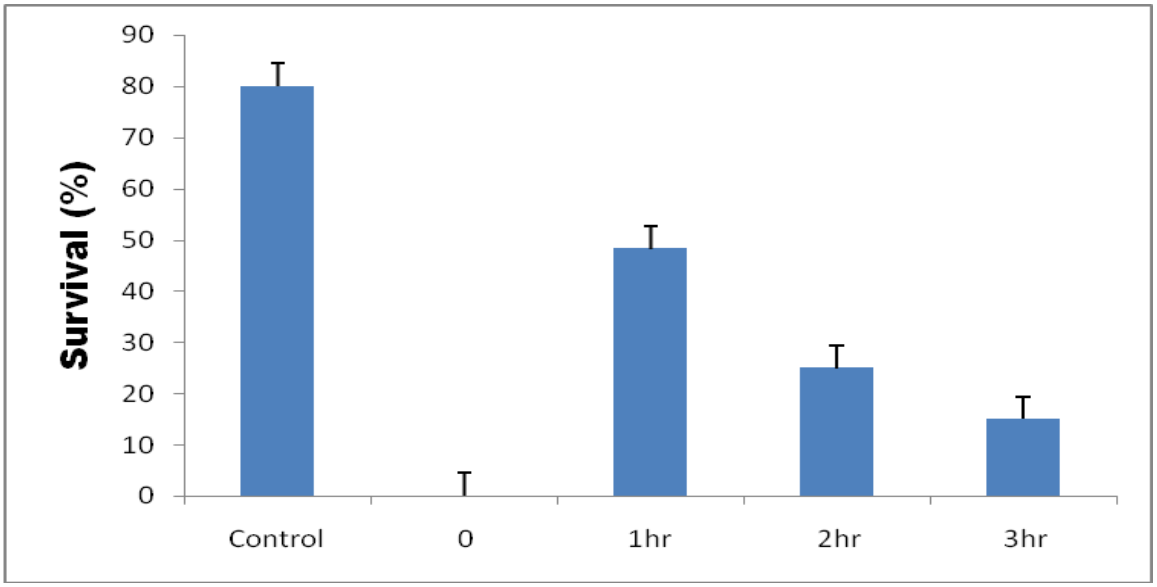

Figure 4.3: Effect of dehydration time in PVS2 on Survival of cryopreserved encapsulated embryonic axes of groundnut. Vertical bars represent ±SE of means of 3 replicates.

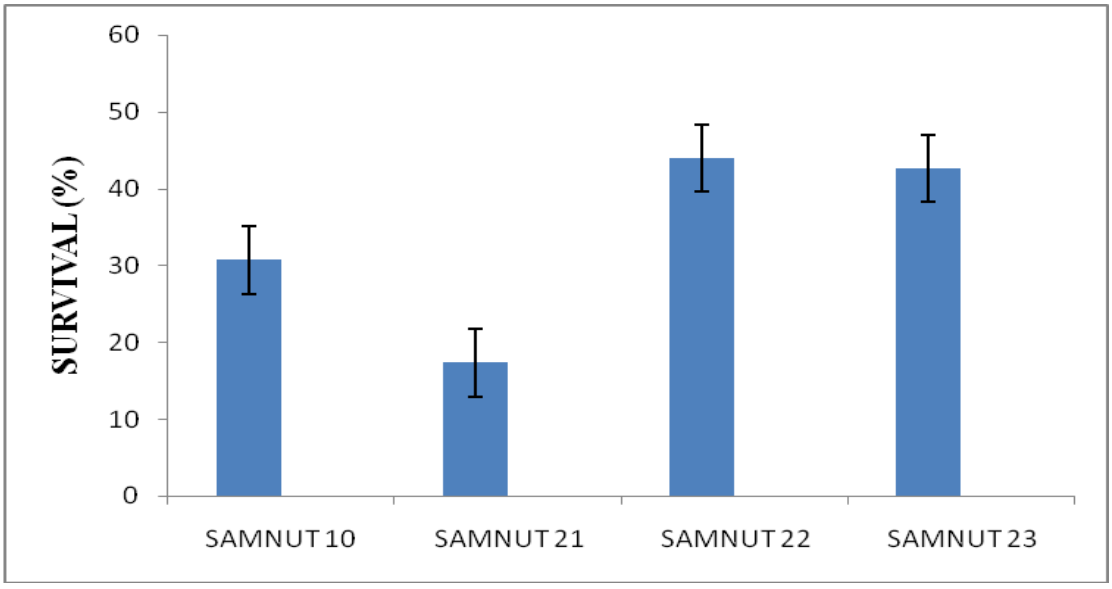

Figure 4.4: Response of groundnut varieties to encapsulation and dehydration in PVS2. Vertical bars represent ±SE of means of 3 replicates.

Table 4.15: Effect of variety x dehydration in PVS2 interaction on the survival of cryopreserved encapsulated embryonic axes of groundnut.

	Dehydration in PVS2 (hr)				
Variety	Control	0	1	2	3
SAMNUT 10	73.3b	0.0e	33.3cd	26.7cd	20.0d
SAMNUT 21	66.7b	0.0e	20.0d	0.0e	0.0e
SAMNUT 22	93.3a	0.0e	73.3b	33.3cd	20.0d
SAMNUT 23	86.7a	0.0e	66.7b	40.0c	20.0d
		SE±	8.94		

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT. 0 – none dehydrated encapsulated embryonic axes stored in Liquid nitrogen. Control- none cryopreserved regenerated embryonic axes

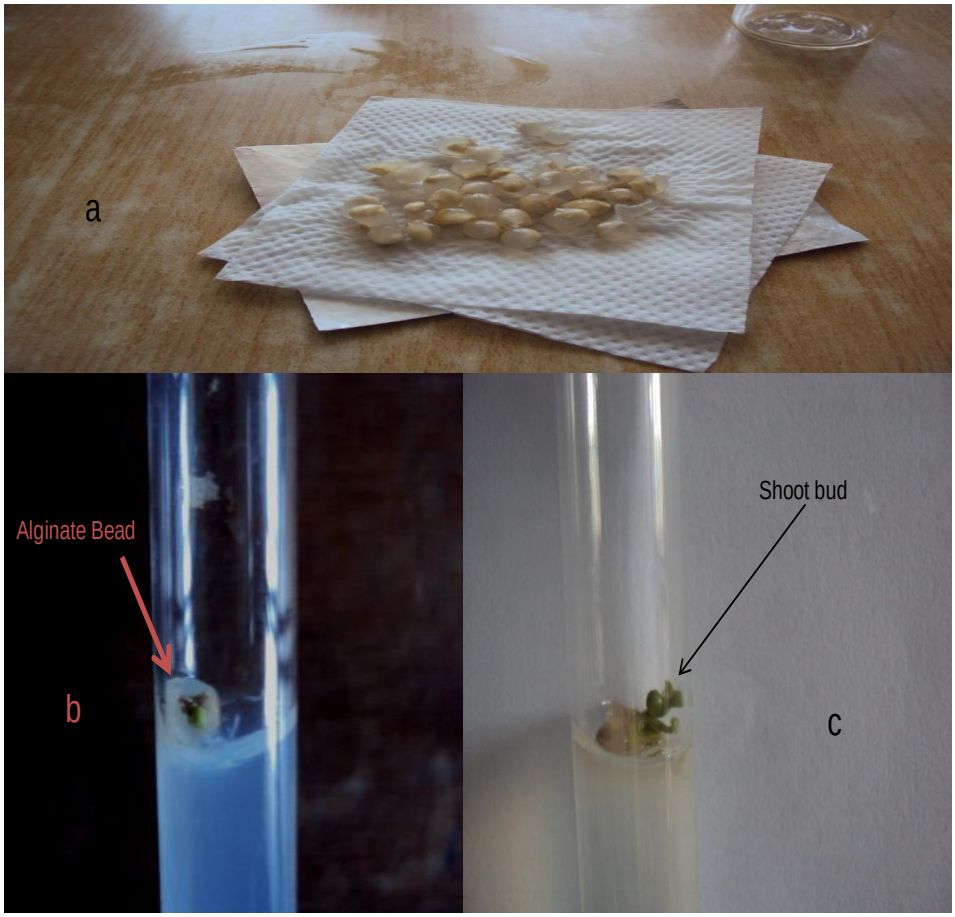

Plate V a: encapsulated embryonic axes. b-c: Shoot bud produced from cryopreserved encapsulated embryonic axe

4.7 Effect of Sucrose and Mannitol Concentrations, Varieties and Conservation period on survival rates of slow growth maintained cultures.

The effect of different sucrose and mannitol concentrations, on survival was observed to be significant (Table 4.16). Results revealed that the highest mean value of survival rate (33.8%) was obtained on MS medium supplemented with 10g/L sucrose and 20g/L mannitol, followed by survival rate (30.6%) recorded from medium supplemented with 20g/L sucrose and 10g/L mannitol and survival rate (29.4%) recorded by medium supplemented with 15g/L sucrose and 15g/L mannitol which were statistically similar. The lowest mean value of survival percentage (25.0%) was observed in the control medium.

As for the effect of variety on survival of cultures, results showed that the highest survival (33.1%) was recorded by SAMNUT 23, followed by SAMNUT 22 (29.4%) and SAMNUT 10 (30.0%) which were statistically the same. The lowest survival rate (26.3%) was recorded by SAMNUT 21.

With regard to the effect of different conservation periods, results in Table 4.16 indicate that the highest survival (73.8%) was obtained from cultures conserved for 3 months, followed by survival rates (45.0%) obtained from 6 months conservation period. Cultures maintained for 9 months and 12 months, failed to survive.

4.7.1 Effect of sucrose and mannitol concentrations x conservation period interaction on survival rates of slow growth maintained cultures.

Significant ($p \leq 0.05$) interaction was observed between the conservation medium and conservation period on survival rates of cultures (Table 4.17). At 6 months conservation period, MS medium supplemented with 10g/L sucrose and 20g/L mannitol significantly

recorded higher survival values than the other treatments. While at 3, 9 and 12 months all the conservation media were not significantly different.

Keeping the conservation period constant and varying the conservation medium it was observed in the control medium that higher survival rates was obtained at 3months conservation period than at the other conservation periods. A similar trend was also observed in the other conservation media.

4.7.2 Effect of variety x conservation period interaction on survival rates of slow growth maintained cultures.

Significant ($p \leq 0.05$) interaction was observed between the varieties and conservation period on survival rates of cultures (Table 4.18). At 6 months conservation period SAMNUT 23 significantly recorded higher survival than SAMNUT 21 which was statistically comparable to SAMNNUT 10 and SAMNUT 22. On the contrary, at 3, 9 and 12 months all the varieties were statistically similar.

Keeping the conservation period constant and varying the variety it was observed that all the varieties recorded higher survival values at 3 months conservation period compared to the other conservation periods. From 9 to 12 months all the cultures failed to survive.

4.7.3 Effect of variety x sucrose and mannitol concentrations x conservation period interaction on survival rates of slow growth maintained cultures.

A significant interaction was observed between variety, conservation medium and conservation period on survival rates of cultures (Table 4.19). At 6 months conservation

period in cultures maintained in the control medium, SAMNUT 23 and SAMNUT 22 comparably recorded higher survival rates than SAMNUT 21 and SAMNUT 10. However, when the medium was supplemented with 20g/L sucrose and 10g/L mannitol the highest significant survival was observed in SAMNUT 23 and SAMNUT 10 compared to the other varieties. In cultures maintained in medium supplemented with 15g/L sucrose and 15g/L mannitol, SAMNUT 23 significantly recorded higher survival rates than the other varieties. While in cultures maintained in medium supplemented with 10g/L sucrose and 20g/L mannitol all the varieties were not significantly different. Also at 3, 9 and 12 months conservation periods, no significant difference was observed among the varieties irrespective of the conservation medium.

Keeping the conservation period constant and varying the varieties it was observed that in SAMNUT 10 that the maximum survival was obtained on medium supplemented with 20g/L sucrose and 10g/L mannitol and medium supplemented with 10g/L sucrose and 20g/L mannitol. While in SAMNUT 21 higher survival values was obtained from medium supplemented with 10g/L sucrose and 20g/L mannitol than on the other conservation media. A similar trend was also observed in SAMNUT 22. In SAMNUT 23 the entire conservation media supplemented with osmotic agents comparably recorded higher survival values than the control.

Table 4.16: Effect of sucrose and mannitol concentrations, variety and conservation period on survival rates of slow growth maintained cultures.

Treatment	Survival (%)
Medium supplements (g/L) (**M**)	
MS + 30 sucrose(Control)	25.0c
MS + 20 sucrose+10 mannitol	30.6b
MS + 15 sucrose+15 mannitol	29.4b
MS + 10 sucrose+20 mannitol	33.8a
SE±	1.14
Variety (**V**)	
SAMNUT 10	30.0b
SAMNUT 21	26.3c
SAMNUT 22	29.4b
SAMNUT 23	33.1a
SE±	1.14
Period (months) (**P**)	
3	73.8a
6	45.0b
9	0.0c
12	0.0c
SE±	1.14
M x V x P	NS
CV (%)	18.83

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT. NS non significant, NS not significant

Table 4.17: Effect of sucrose and mannitol concentrations x conservation period interaction on survival rates of slow growth maintained cultures.

	Period (months)			
Medium supplements (g/L)	3	6	9	12
MS + 30 sucrose(Control)	70.0ab	30.0d	0.0e	0.0e
MS + 20 sucrose+10 mannitol	77.5a	45.0c	0.0e	0.0e
MS + 15 sucrose+15 mannitol	72.5a	45.0c	0.0e	0.0e
MS + 10 sucrose+20 mannitol	75.0a	60.0b	0.0e	0.0e
	SE±	4.56		

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT.

Table 4.18: Effect of variety x conservation period interaction on survival rates of slow growth maintained cultures.

	Period (months)			
Variety	3	6	9	12
SAMNUT 10	75.00a	45.00bc	0.00d	0.00d
SAMNUT 21	70.00a	35.00c	0.00d	0.00d
SAMNUT 22	72.50a	45.00bc	0.00d	0.00d
SAMNUT 23	77.50a	55.00b	0.00d	0.00d
	SE±	4.56		

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT.

Table 4.19: Effect of variety x sucrose and mannitol concentrations x conservation period interaction on survival rates of slow growth maintained cultures.

	Conservation period (months)															
	3				6				9				12			
	Medium supplement (g/L)				Medium supplement (g/L)				Medium (g/L)		supplement		Medium (g/L)		supplement	
Variety	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
SAMNUT10	70.0ab	80.0a	70.0ab	80.0a	20.0d	60.0b	40.0c	60.0b	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e
SAMNUT21	70.0ab	70.0ab	70.0ab	70.0ab	20.0d	20.0d	40.0c	60.0b	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e
SAMNUT22	70.0ab	80.0a	70.0ab	70.0ab	40.0c	40.0c	40.0c	60.0b	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e
SAMNUT23	70.0ab	80.0a	80.0a	80.0a	40.0c	60.0b	60.0b	60.0b	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e
							SE±	4.56								

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT.

1: MS + 30 sucrose (Control)

2: MS + 20 sucrose+10 mannitol

3: MS + 15 sucrose+15 mannitol

4: MS + 10 sucrose+20 mannitol

4.8 Effect of Sucrose and Mannitol Concentrations on Shoot height.

Shoot height was significantly retarded with decreasing concentration of sucrose and increasing concentration of mannitol in the conservation medium (Table 4.20). The highest shoot height (7.83cm) was obtained in the control medium, followed by shoot height (5.25cm) obtained from medium supplemented with 20g/L sucrose and 10g/L mannitol and shoot height (4.16cm) obtained from medium supplemented with 15g/L sucrose and 15g/L mannitol. Whereas medium supplemented with 10g/L sucrose and 20g/L mannitol recorded the lowest shoot height (3.03cm).

4.9 Effect of Sucrose and Mannitol Concentrations, Varieties and Conservation period on Number of Shoot.

The analysis of variance data (Table 4.21) showed that only the variety x conservation period interaction was significant ($p \leq 0.05$) for number of shoots. While there were insignificant difference observed for the effects of sucrose and mannitol concentrations (osmoticums), varieties, conservation period, osmoticums x variety, osmoticums x conservation period and osmoticums x variety x conservation period interactions.

At 3 months conservation period, no significant difference was observed among the varieties. However, at 6 months conservation period SAMNUT 23 recorded a significantly higher number of shoots/explant which is comparable to the number of shoots/explant observed in SAMNUT 22 and SAMNUT 10 (Table 4.22).

Varying the variety and keeping the conservation period constant it was observed that all the varieties recorded a higher number of shoots/explant at 3 months than at 6 months conservation period (Table 4.22).

Table 4.20: Effect of sucrose and mannitol concentrations, on shoot height of slow growth maintained cultures.

Treatment	Shoot height
Medium supplements (g/L)	
MS + 30 sucrose(Control)	7.8a
MS + 20 sucrose+10 mannitol	5.3b
MS + 15 sucrose+15 mannitol	4.2c
MS + 10 sucrose+20 mannitol	3.0d
SE±	0.39
CV (%)	26.53

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT.

Table 4.21. Analysis of variance for shoot height and number of shoots/explant of groundnut plantlets under slow growth

Source of variation	*Degree of freedom*	*Mean Square*	
		Shoot height (cm)	Mean number of shoots/explant
Treatment (T)	3	67.53**	0.40
Variety (V)	3	0.33	0.94
Storage period (P)	1	1.03	0.00
T x V	9	0.55	0.72
T x P	3	0.15	0.17
V x P	3	0.22	2.29**
T x V x P	9	0.05	0.18
Error	32	1.81	1.81

**P≤0.01

Table 4.22: Effect of variety x conservation period interaction of number of shoots/plantlet of slow growth maintained cultures.

Variety	Period (months)	
	3	6
SAMNUT 10	75.0a	45.0bc
SAMNUT 21	70.0a	35.0c
SAMNUT 22	72.5a	45.0bc
SAMNUT 23	77.5a	55.0b
	SE± 1.09	

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Effect of BAP and variety on shoot height and multiplication potential from embryonic axes of Groundnut (*Arachis hypogaea* L.).

The relative concentrations of auxin to cytokinin in regeneration medium strongly influenced shoot organogenesis in plants. Several studies have reported that plant growth regulator BAP is the most suitable cytokinin for shoot induction and multiplication in groundnut. However, the optimum concentration of BAP for shoot multiplication varies with variety of groundnut (Banerjee *et al.,* 2007; Verma *et al.,* 2009). In this study, the effectiveness of different concentrations of BAP was evaluated for shoot multiplication in groundnut. The presence and concentration of the BAP in the nutrient media significantly reduced shoot height with the BAP-free medium producing the tallest shoots. The addition of BAP in the media inhibited shoot elongation progressively from 5mg/L BAP to 25mg/L BAP. The inhibitory effect of higher BAP concentrations on shoot elongation has been reported in groundnut (Verma *et al.,* 2009) and in pineapple (Usman *et al.,* 2013). It is possible that the inhibition of shoot elongation by the high concentration of BAP was due to the production of ethylene. According to Cary *et al.* (1995), ethylene production was stimulated by micro molar concentrations of Benzyladenine and could account, in part, for the inhibition of hypocotyl elongation in *Arabidopsis.* Cytokinin had been implicated in the enhancement of lateral bud formation in plant (Taiz and Zeiger, 2003). The enhancement in the regeneration of multiple shoots in groundnut may possibly be due to a better auxin:cytokinin ratio used, which is a crucial factor in regeneration (Banerjee *et al.,* 2007). In the present study the number of shoots/explant and axillary branching was significantly (($p \leq 0.05$) influenced by the BAP concentrations. It was

observed that supplementing MS media with BAP was necessary in achieving shoot multiplication. As the BAP-free medium did not produce multiple shoots, rather growth of a single shoot was observed. Increased concentration of BAP progressively increased the number of shoots and axillary branching produced, attaining a maximum number of shoots/explant (8.3) and axillary branching (3.1) at 15mg/L BAP. Verma *et al.* (2009) also observed maximum shoot multiplication in groundnut at 10-15mg/L BAP. Marka *et al.* (2013) also reported maximum number of multiple shoots in groundnut at 15-20 mg/L BAP in comparison to all other concentrations and combinations of plant growth regulators evaluated in the study. On the contrary, Banerjee *et al.* (2007) observed maximum multiplication potential in 5 varieties of groundnut in 10 and 25mg/L BAP. Further increase in BAP from 15mg/L caused a decrease in the number of shoots and axillary branch with MS medium supplemented with 25mg/L recording the lowest value. A similar trend in the inhibitory effect of BAP at higher concentrations and failure to produce multiple shoots and axillary branches at lower BAP concentrations or on BAP-free medium have been reported (Banerjee, 2014; Banerjee *et al.*, 2007; Okello *et al.*, 2015; Verma *et al.* 2009). The reduction in the number of shoots could be due to the inhibition of adventitious meristem elongation caused by the higher BAP concentration (Borchetia *et al.*, 2009).

The response of groundnut varieties to regeneration was significantly different with SAMNUT 22 and SAMNUT 23 producing the tallest shoots. In terms of number of shoots/explant and axillary branches, SAMNUT 22 recorded the highest number of shoots/explant while SAMNUT 23 produced higher axillary branches. Genotypic difference in response to tissue culture in groundnut has been reported earlier (Banerjee *et al.*, 2007; Marka *et al*., 2013; Okello *et al.*, 2015). The observed variation among the

varieties for all the parameters evaluated could be attributed to their differential genomic constitution. This phenomenon has earlier been reported in groundnut. (Radhakrishnan *et al.*, 1994). Similarly, differences in the level of endogenous plant growth regulators within the explants have been reported to account for differences in the *in vitro* response in groundnut (Banerjee *et al.*, 2007).

5.2 Effect of NAA and variety on *in vitro* rooting of regenerated microshoots of groundnut (*Arachis hypogaea* L.).

The presence and concentration of NAA significantly influenced *in vitro* rooting in groundnut. Medium supplemented with 1mg/L NAA recorded the highest root induction frequency (82.42%), while the NAA-free medium (10.42%) recorded the least root induction frequency. This result suggests that NAA is essential for *in vitro* rooting of microshoots of groundnut as minimal rooting was observed in its absences. It has been established that auxin stimulates lateral root initiation by activating quiescent pericycle cells to initiate division and then expansion, which facilitate the lateral root emergence (Fukaki and Tasaka, 2009). Therefore, the appropriate synthesis signaling and transport of auxin are all required for root formation (Peret *et al*., 2009). The root induction frequency obtained at 1mg/L NAA in our study was higher than the rooting frequency of 62.5% reported by McKently *et al.* (1990) in groundnut at the same concentration. On the contrary, Gagliardi *et al.* (2000) reported successful *in vitro* rooting of 70% of microshoots of groundnut on MS hormone free medium. Okello *et al.* (2015), reported maximum root production of 70-100% in three groundnut cultivars on MS medium supplemented with 1 mg/L NAA and 2.0 mg/L IBA supported

The effect of NAA on the number of roots/plantlet was significant. Medium supplemented with 1mg/L NAA or 1.5mg/L NAA produced the highest number of roots/plantlet of 9.3 and 9.4 respectively compared to NAA-free medium. The value obtained in our study is higher than number of roots/plantlet of 3.5 reported by McKently *et al.* (1990) in groundnut when rooted in basal medium supplemented with 1mg/L NAA. Auxin is reported to control cell division, cell elongation and certain stages of differentiation (Davies, 2004) which could have accounted for the increased number of roots/plantlet observed in this study. Lewis *et al*. (2011), also reported that auxin increases the number of lateral roots. On the other hand supplementing the media with NAA significantly decreased root length, with the NAA-free medium recording the longest root length (7.35cm). Taiz and Zegler (2003) reported that although plants may require auxin for root induction, but root growth is inhibited at higher concentration. Kollmeier *et al.* (2000) also reported that root elongation phase is very sensitive to auxin concentration and it is inhibited by high concentration of auxin in the rooting medium. This is because auxin can increase the rate of ethylene biosynthesis (Riov and Yang, 1989). Therefore, it could be possible that the high concentrations of NAA inhibited root elongation through the inducement of ethylene biosynthesis, which is inhibitory to root growth.

Significant differences were observed among the groundnut varieties in response to the NAA treatment. SAMNUT 22 and SAMNUT 21 recorded the highest root induction frequency while SAMNUT 22 and SAMNUT 23 produced more number of roots/plantlets than the other varieties. However, SAMNUT 10 and SAMNUT 21 produced the longest root lengths compare to the other varieties. This result confirms

earlier reports that rooting in groundnut is strongly influenced by the plant genotype (Verma *et al*., 2009).

Significant ($p \leq 0.05$) interaction was observed between the varieties and NAA treatment on root induction frequency, number of roots/plantlet and root length of groundnut plantlets. All the varieties recorded highest root induction frequency on medium supplemented with 1mg/L NAA. On the contrary, maximum number of roots/plantlet were observed in SAMNUT 10 and SAMNUT 21 on medium supplemented with 1mg/L NAA while in SAMNUT 22 and SAMNUT 23 on medium supplemented with 1.5mg/L NAA. SAMNUT 10, SAMNUT 22 and SAMNUT 23 recorded longer root lengths on the NAA-free medium. While SAMNUT 21 recorded longer root length on both the NAA-free medium and medium supplemented with 1mg/L NAA. The observed differences could be attributed to genotypic influence or due to the level of endogenous hormone in the different varieties. According to Roy and Banerjee (2003), the effect of a particular plant growth regulator depended not only on the concentrations applied, but also on the presence of the other plant growth regulators as well as its interaction with endogenous growth regulators.

5.3 Cryopreservation of embryonic axes of groundnut (*Arachis hypogaea* L.) by desiccation.

Desiccation rates significantly influenced the reduction of cellular water content in embryonic axes of groundnut, their survival and subsequent shoot formation after storage in liquid nitrogen. Non-desiccated embryonic axes of groundnut failed to survive the liquid nitrogen storage. This result corroborates that of Gagliardi *et al*. (2002), who observed no survival of non-dehydrated and cryopreserved embryonic axes of *Arachis* species. Lack of germination of the non-desiccated plant material has

also been reported in other crops (Azimi, *et al*., 2005). Similarly, embryonic axes desiccated for 1 hr also failed to survive the cryogenic treatment. This could be attributed to the insufficient reduction of cellular water content at this period which could have caused the formation of lethal ice crystals that damage the cells during liquid nitrogen storage or thawing. Halmagyi *et al*. (2010) reported that insufficient dehydration of explant before freezing can cause the formation of ice crystals during freezing or warming leading to the destruction of cellular structures and death of the explant. Desiccation of embryonic axes from 2 hr to 5 hr rapidly caused significant reduction in moisture content. This greatly improved the survival and shoot formation of cryo-preserved embryonic axes with the highest survival (96.67% - 100%) and subsequent shoot formation (91.67% - 96.67%) obtained at an average moisture content of 17% after 4 to 5hr desiccation rates. Ozudogru *et al.* (2009) reported 100% germination in embryonic axes of groundnut when moisture content was reduced from 25% to 8.5% which was achieved at 2.5hr desiccation time. While Gagliardi *et al*. (2002) reported 80% shoot development at 18% moisture content after 1hr desiccation rate in *Arachis* species. Maximum survival of 100% was also reported in zygotic embryos of jatropha desiccated to 9.4-9.7% MC before liquid nitrogen storage (Prada *et al.,* 2015). The improved survival and shoot formation with decreasing moisture content could be also be due to the increased accumulation of sugars during drying. Percy *et al*. (2001), reported that accumulation of sugars during desiccation may serve to maintain cellular integrity by osmotically decreasing cell volume, or act directly to protect by stabilization of membranes. It is also possible that the improved survival and shoot formation was due to the probable accumulation of ABA in the desiccated embryos. ABA has been reported to promote desiccation tolerance in the mature embryo through the synthesis of late embryogenesis abundant (LEA) proteins encoded

by mRNA (Taiz and Zegler, 2003). ABA has also been implicated in cold acclimation in plants (Xue-Xuan *et al*., 2010).

Results obtained from this study showed significant differences among the varieties evaluated, SAMNUT 23 embryonic axes recorded the lowest moisture content while the highest survival and shoot formation was observed in SAMNUT 22 and SAMNUT 23. These observed differences could be due to genotypic influence, as tissue culture response in groundnut is strongly influenced by the plant genotype (Mroginsky *et al*., 1981; Banerjee *et al*., 2007). Genotypic influence has also been reported in cryopreserved embryonic axes of maize (Usman and Abdulmalik, 2010).

There was a significant interaction between the varieties and time of desiccation for moisture content, survival and shoot formation of cryopreserved embryonic axes of groundnut. SAMNUT 21 and SAMNUT 23 recorded very low moisture content from 1hr desiccation time while SAMNUT 10 and SAMNUT 22 recorded very low moisture content from 2hr desiccation. Significantly high survival rates were observed in SAMNUT 22 and SAMNUT 23 from 2hr desiccation time, in SAMNUT 21 from 2hr while in SAMNUT 10 3hr desiccation time. As regards to shoot formation rate the highest value was observed in SAMNUT 10 at a 5hr desiccation time, in SAMNUT 21 from 4hr desiccation time while in SAMNUT 22 and SAMNUT 23 from 3hr desiccation time. This shows that the varieties attained low moisture contents at different times, which also influenced their survival and shoot formation. These could be attributed to the differences in the genotypic constitution of the groundnut varieties. Wen and Song (2007) reported that, survival and the emergence of post-thaw embryos were closely related to their moisture contents prior to freezing.

5.4 Cryopreservation of embryonic axes of groundnut (*Arachis hypogaea* L.) by vitrification.

One of the keys to successful cryopreservation by vitrification is the careful control of dehydration and prevention of injury by chemical toxicity. Optimization of the time of exposure to PVS2 is important for producing a high level of shoot formation after vitrification. In this study, we were able to obtain higher survival and shoot formation in embryonic axes dehydrated with PVS2 for 2hr. This result is in agreement with that observed by Ozudogru *et al*. (2009), who observed very high survival rates at 2hr time of dehydration in PVS2 in embryonic axes of groundnut. Very high survivals were also reported at 1 to 2 hr for embryonic axes of groundnut (Gaglardi *et al*., 2002). High survival of 70% was also observed when embryos of jatropha were pretreated with 0.3 M sucrose and 60% PVS2 before liquid nitrogen storage (Prada *et al.*, 2015). The high survival and shoot formation reported here could be attributed to sufficient dehydration. Since only after sufficient dehydration that the cell content will be able to vitrify upon rapid cooling in liquid nitrogen without forming lethal ice crystals and hence ensuring high survival. According to Volk and Walters (2006), the three mechanisms by which PVS2 aids cryoprotection are by replacing cellular water, changing the freezing behaviour of water remaining in the cells and impeding water loss during air drying. In addition to their physical protective properties, cryoprotectants also impart additional protection against cryoinjury as they can stabilize proteins and membranes and act as antioxidants (Fuller, 2004). The poor survival and shoot formation observed at 1h time of exposure to PVS2 could be attributed to the insufficient dehydration of the embryonic axes. While the drastic decline in both survival and shoot formation rates observed from 3 to 5 h dehydration period could be due to the toxicity of the PVS2.

Chemical toxicity of PVS2 usually increases with time and causes a decrease in survival rates (Yamuna *et al*., 2007).

The poor performance of SAMNUT 10 and 21 compared to the other varieties could be attributed to the sensitivity of these varieties to the PVS2 treatment. Takagi (2000) reported that sensitivity to dehydration of vitrification solution varies among species and even within cultivars. Genotypic influence has been reported in vitrified embryonic axes of maize (Usman and Abdulmalik, 2010), shoot tips of grape (Matsumoto and Sakai, 2003) and potato (Panta *et al*., 2015).

A significant interaction was observed between the variety and dehydration time in PVS2. Maximum shoot formation was observed in SAMNUT 10, SAMNUT 21 and SAMNUT 23 after 2hr dehydration in PVS2. On the contrary, in SAMNUT 23 this was obtained after 3hr dehydration in PVS2. This suggests that vitrification technique is variety-dependent with each variety requiring different dehydration time in PVS2. The high post thaw survival and shoot formation recorded in this study could also have been enhanced by the preculturing of the embryonic axes on high sucrose medium and treating with loading solution before exposure to PVS2. Matsumoto *et al*. (1994; 1995) reported the positive effect of preculturing on high sucrose media and of loading treatment on post thaw survival.

5.5 Cryopreservation of embryonic axes of groundnut (*Arachis hypogaea* L.) by encapsulation-dehydration.

The optimizations of the pretreatment procedure and of sample residual moisture content are key factors for successful application of encapsulation-dehydration

(Gonzalez-Benito *et al*., 1998). Removal of most or all freezable water from cells causes vitrification of internal solutes during rapid exposure to liquid nitrogen, thereby avoiding lethal intracellular ice crystallization (Engelmann, 1997). High moisture content has been reported to cause the formation of extra and intracellular ice crystals (Moges *et al*., 2004). In our study, the highest survival (73%) and shoot formation (63%) of encapsulated cryopreserved embryonic axes of groundnut was obtained after 6hr air dehydration to moisture content of 20%. This result shows that a reduction in freezable intracellular water is an important requirement for successful cryopreservation. Paul *et al.* (2000) reported that dehydration to a 21% water content resulted in 83.7% recovery of encapsulated-dehydrated cryopreserved shoot tips of apple. Similarly, Soliman (2013) obtained the highest survival (74.5%) and regrowth (71.8%) of encapsulated cryopreserved shoot tips when precultured for two days in medium containing 0.75 M sucrose and dehydrated for 2 hr to 19.55% moisture content. The high survival and shoot formation recorded in our study could have also been enhanced by Preculturing in 0.75M sucrose for 72hr. Positive effects of extending preculture duration for four days in 0.75M sucrose were reported for *Dioscorea floribunda* (Mandal and Sangeeta 2007).

Among the varieties evaluated SAMNUT 23 and SAMNUT 22 produced the highest survival rates compared to the other varieties. Similarly, significant variation was also observed among the varieties evaluated in terms of shoot formation and moisture content with SAMNUT 23 producing the highest shoot formation of 61.3% at the lowest moisture content of 33.9%, followed by SAMNUT 22. The better performance of these two varieties over the other varieties could be attributed to genotypic influence and to the low moisture content of their embryonic axes, which improved their survival

and subsequent shoot formation by preventing the formation of lethal inter- and intra-cellular crystallization during cryopreservation. Alla-N'Nan *et al*. (2014) reported genotypic differences among coconut accession cryopreserved by encapsulation-dehydration technique.

A significant interaction was observed between variety and dehydration time on survival and shoot formation of cryopreserved encapsulated embryonic axes of groundnut. Maximum survival and shoot formation rate was observed in all the varieties with the exception SAMNUT 23 at a 6hr dehydration time while in SAMNUT 23 from 4hr dehydration time. These results showed that the survival and shoot formation of the cryopreserved encapsulated embryonic axes was strongly influenced by the variety and the dehydration time, which could be due to differences in the moisture content of the seeds of the varieties.

5.6 Cryopreservation of embryonic axes of groundnut (*Arachis hypogaea* L.) by encapsulation-vitrification.

The critical factor determining the success of cryopreservation by encapsulation-vitrification is the exposure time in the PVS2, longer exposure of plant material in the PVS2 could cause a drastic decline in both survival and subsequent regeneration. Therefore, it is important to optimize the exposure time in PVS2 for successful cryopreservation of plant material. In our study survival rate of encapsulated embryonic axes of groundnut was very low reaching a maximum (48%) after 1hr dehydration in PVS2. This result contradict several reports that indicated the encapsulation-vitrification technique produces high recovery and shoot formation from cryopreserved shoot tip or meristem in numerous plant species of both tropical and temperate origins

(Hirai and Sakai, 1999; Moges *et al*., 2004). The low survival rate observed in our study could be due to the toxicity of the PVS2. The PVS2 is characterized by its high chemical toxicity, because it includes the permeating cryoprotectants DMSO and ethylene glycol (Kim *et al*., 2009). Exposing cells to high concentrations of cryoprotective additives is potentially injurious. This is due to the phytotoxic effects of individual components or their combined osmotic effects on cell viability (Towill and Jarret, 1992; Sakai, 2000). Suggesting the need to optimize the preculturing phase with the view to enhance survival and regenerate true plants. The precultured treatment is used to increase tolerance of explants to dehydration and subsequent freezing in liquid nitrogen. Preculturing of explants in media enriched with high sucrose concentrations has been reported to increase the total soluble protein and sugar contents in the treated tissues. An increase in the protein level can be considered as one of the earliest physiological responses of osmotically stressed cells, and could be related to the improvement of the freezing tolerance of tissues (Wang *et al*., 2004).

Among the varieties evaluated SAMNUT 22 and SAMNUT 23 comparably produced the highest survival rates. The high survival recoded by these two varieties could be attributed to their better tolerance to the PVS2 compared to the other varieties and to genotypic influence. Generally, the tissue culture response in the groundnut is highly genotypic dependent (Banerjee *et al*., 2007; Nazir *et al*., 2011).

5.7 Effect of sucrose and mannitol concentrations, varieties and conservation period on survival rates of slow growth maintained cultures.

The maintenance of shoot cultures is obtained through the slowing down of cell metabolism, as a consequence of shoot growth. The use of osmotically active

compounds, such as sucrose and mannitol can interfere on shoot growth and influence markedly the maximum storage time (Scherwinski-Pereira *et al*., 2010). In this study the effect of different sucrose and mannitol concentrations, on survival of cultures was observed to be significant. Results revealed that the highest survival (33.8%) was obtained on MS medium supplemented with 10g/L sucrose and 20g/L mannitol while the lowest survival percentage (25.0%) was observed in the control medium (Table 4.16). Sarkar and Naik (1998) reported that 20 or 40 g/L mannitol in combination with sucrose could enhance survival of *in vitro* conservation of potato. Charoensub and Phansiri (2004) also reported that the addition of 20 g/L mannitol to leadwort culture medium prolonged subculture time to 8 months.

As for the effect of variety on survival of cultures, results showed that the highest survival (33.1%) was recorded by SAMNUT 23, followed by SAMNUT 22 (29.4%). The observed difference could be attributed to genotypic influence. Wilkins *et al*. (1988), reported that slow growth storage methods are extremely variable and genotype dependent. Ahmed and Anjum (2010) also observed significant genotypic difference in terms of survival in pear.

With regard to the effect of different conservation periods, results in indicate that the highest survival (73.8%) was obtained from cultures conserved for 3 months, followed by survival rates (45.0%) obtained from 6 months conservation period. Cultures maintained for 9 months and 12 months, however, failed to survive. The decline after 3 months and none survival after 9 months to 12 months could be attributed to the decrease in nutrients and increased in osmotic stress. Lack of survival could also be due the depletion of oxygen concentration in the nutrient medium which could have led

to anaerobic respiration. Plant tissues when subjected to hypoxic (low) or anoxic (zero) concentration of ambient oxygen, they are forced to carry out anaerobic respiration. Anaerobic respiration could lead to lactate accumulation which promotes acidification of the cytosol (Taiz and Zegler, 2003). Maximum survival and healthy shoot cultures in the first 3 months of conservation and decreased in survival after 6, 9 to 12 months have been reported in grape (Hassan *et al*., 2014) and in globe artichoke (Shawky and Aly, 2007).

A significant interaction was observed between the varieties and conservation period on survival rates of cultures. Maximum survival was observed in all the varieties at 3 months conservation period while at 6 months conservation period SAMNUT 23 was only significantly different from SAMNUT 21. Ahmed and Anjum (2010) also observed a significant interaction between variety and storage period in pear, with a maximum survival rate obtained with 3 months storage period in the varieties.

5.8 Effect of sucrose and mannitol concentrations, varieties and conservation period at shoot height.

Shoot height was significantly retarded with decreasing concentration of sucrose and increasing concentration of mannitol in the conservation medium. The tallest shoots (7.8cm) were obtained in the control medium. Whereas medium supplemented with 10g/L sucrose and 20g/L mannitol recorded the lowest shoot height (3.0cm). This could be due to increased osmotic stress imposed by the osmoticum in the culture medium and reduced nutrients. Osmotic regulators act as growth retardants by causing osmotic stress to the material under conservation. When added to the culture medium, these carbohydrates reduce the hydric potential and restrict the water availability to the

explant (Shibli *et al.* 2006) thereby reducing the optimal turgor pressure needed for cell division and inhibit growth (Tahtamouni *et al*., 2001). Minoo *et al.* (2006) also observed significant decrease in shoot height of vanilla with increased concentration of mannitol and reduced sucrose in culture medium.

CHAPTER SIX

6.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

Experiments were carried out between 2009 and 2013 in the Biotechnology laboratory of IAR Ahmadu Bello University Samaru, Zaria (11°11´N, 7°38´E; 686m above sea level) to develop an efficient regeneration protocol for groundnut also to establish efficient cryopreservation and slow growth protocols for conservation of groundnut germplasm. Four IAR groundnut varieties; SAMNUT 10, SAMNUT 21, SAMNUT 22 and SAMNUT23 were used in the study. Explants used in the study were embryonic axes which were obtained from seeds under aseptic condition. The experiments were factorial and treatments were laid out in a Completely Randomized Design replicated three times. In the regeneration experiment embryonic axes were cultured on MS media supplemented with varying concentration of BAP (0, 5, 10, 15, 20 and 25 mg/L BAP) for shoot initiation and multiplication. Results showed that shoot regeneration occurred directly without an intervening callus phase in all treatments, with maximum shoot multiplication obtained from MS medium supplemented with 15 mg/L BAP.

Among the groundnut varieties evaluated SAMNUT 23 and SAMNUT 22 proved to be the most responsive to *in vitro* regeneration in terms of vigour and multiplication potential with 4.7-4.8cm shoot height and 4.6-5.1 shoots/explant. For *in vitro* rooting microshoots of 3cm length were subcultured on MS media supplemented with 1.0 or 1.5 mg/L NAA and compared with a hormone free medium (control). Rooting was significantly influenced by the presence and concentration of NAA. Microshoots subcultured in medium supplemented with 1 mg/L NAA rooted within 2weeks of subculture, producing the highest root-induction frequency (82%). While the hormone

free medium recorded the longest roots (7.4cm). Significant genotypic effect was also observed, with SAMNUT 22 having more number of roots/plantlet and root-induction frequency (60%).

Cryopreservation, the storage of biological materials in liquid nitrogen (LN), is a useful method for long term conservation of plant germplasm. In this study four commonly used cryopreservation techniques were experimented on the groundnut varieties i.e. desiccation, vitrification, encapsulation-dehydration and encapsulation-vitrification techniques.

In the desiccation technique embryonic axes were desiccated in the laminar hood for 0, 1, 2, 3, 4 and 5 hr prior to LN storage. The highest survival ranged from 97% - 100% and shoot formation of between 92% and 97% of cryopreserved embryonic axes were obtained at an average moisture content of 17% after 4 - 5 hr desiccation. Among the varieties evaluated, SAMNUT 23 and SAMNUT 22 recorded the highest survival of 65% and shoot formation of 57-60% respectively. A significant interaction was also observed between the varieties and desiccation time for moisture content, survival and shoot formation rate. Maximum survival and shoot formation was observed when embryonic axes were dehydrated to 17-19% MC prior to LN (-196°C).

For vitrification technique, embryonic axes were dehydrated in plant vitrification solution (PVS2) for 0, 1, 2, 3, 4 and 5hr prior to LN storage. Results show that survival and shoot formation of cryopreserved embryonic axes were significantly influenced by the dehydration duration in PVS2 with embryonic axes treated for 2 hr recording the highest survival (70%) and shoot formation (65%). Among the groundnut varieties

evaluated, SAMNUT 22 and 23 gave the highest survival (74 and 76%) and shoot formation (72 and 73%) after cryopreservation. A significant interaction was observed between the variety and dehydration time in PVS2 on survival and shoot formation. Maximum shoot formation was observed in SAMNUT 10, SAMNUT 21 and SAMNUT 23 after 2 hr dehydration in PVS2. In the case of SAMNUT 22 this was achieved after 3hr dehydration in PVS2.

In encapsulation-dehydration embryonic axes were encapsulated in Na-alginate precultured on 0.75M sucrose for 72hr before desiccation under the sterile air of laminar hood for 0, 2, 4 and 6hr before LN storage. The highest survival (73%) and shoot formation (63%) of encapsulated cryopreserved embryonic axes of groundnut was obtained after 6hr air dehydration to moisture content of 20%. Among the varieties evaluated SAMNUT 23 recorded the highest survival of 67% and shoot formation of 61% respectively.

In the encapsulation-vitrification technique, encapsulated embryonic axes were dehydrated in PVS2 for 0, 1, 2 and 3hr before LN storage. Maximum survival rate of cryopreserved encapsulated embryonic axes was 48% obtained after 1hr dehydration in PVS2. Among the varieties evaluated SAMNUT 22 and SAMNUT 23 comparably produced the highest survival rates of 44% and 42% respectively.

A slow growth experiment was conducted with four conservation media (MS medium+30g/L sucrose, MS medium+20 g/L sucrose +10 g/L mannitol, MS medium+15 g/L sucrose +15 g/L mannitol and MS medium+10 g/L sucrose +20 g/L mannitol) and for four conservation periods (3, 6, 9 and 12 months). Results revealed

that the highest mean value of survival rate (34%) was obtained on MS medium supplemented with 10 g/L sucrose and 20 g/L mannitol. As for the effect of variety on survival of cultures, results showed that the highest survival (33%) was recorded by SAMNUT 23. With regard to the effect of different conservation periods, the highest survival (74%) was obtained from cultures conserved for 3 months, followed by survival rates (45%) obtained from 6 months conservation period. Cultures maintained for 9 and 12 months, failed to survive. The results also revealed that the lowest shoot height (3.03cm) was obtained on MS medium supplemented with 10g/L sucrose and 20g/L mannitol.

6.2 Conclusions

I. All the varieties evaluated in this study proved to be responsive to *in vitro* regeneration and *in vitro* conservation indicating their amenability to tissue culture.

II. Results obtained from this study showed that for regeneration of groundnut through direct organogenesis, MS media supplemented with 15mg/L BAP is optimum for shoot initiation and multiplication while MS media supplemented with 1mg/L NAA is optimum for *in vitro* rooting of groundnut.

III. Results obtained from the cryopreservation shows that in the desiccation technique, dehydration of embryonic axes to 17 -19% MC prior to LN (-196°C) storage was found to be suitable for cryopreservation of the groundnut varieties. With regards to the vitrification technique, maximum shoot formation was observed in SAMNUT 10, SAMNUT 21 and SAMNUT 23 after 2 hr dehydration in PVS2. In the case of SAMNUT 22 this was achieved after 3hr

dehydration in PVS2 before LN storage. In the ED technique, maximum survival and shoot formation was achieved by dehydration of encapsulated embryonic axes of groundnut to 20% MC before LN (-196°C) storage. While in the EV technique dehydration of encapsulated embryonic axes for 1hr was found to be sufficient for maximum survival in groundnut.

IV. Results obtained from the slow growth experiment show that MS medium supplemented with 10g/L sucrose and 20g/L mannitol effectively reduced shoot growth and produced the highest survival after 3 months conservation period.

6.3 Recommendations

I. The regeneration protocol developed in this study, in addition to being used for *in vitro* conservation could also be utilized for genetic transformation of groundnut.

II. In view of the high survival and shoot regeneration obtained in the desiccation, vitrification and encapsulation-dehydration techniques, these techniques are hereby recommended for the cryopreservation of groundnut.

III. In the encapsulation-vitrification technique, although low survival was recorded with no shoot regeneration achieved. But the potential is there, if further experimentation will be carried out to improve on the protocol. Most especially in the preculturing phase. This could enhance tolerance to PVS2 thereby, improving the survival rates and regeneration of true plants.

IV. In the slow growth technique, medium supplemented with 10g/L sucrose and 20g/L mannitol could be used for the medium-term conservation of groundnut with subculturing intervals of three (3) to six (6) months.

V. Although no morphological difference was observed between the plants regenerated from *in vitro* conserved cultures and that of the parent plants, further studies are recommended to ascertain the genetic fidelity of these materials using molecular techniques.

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APPENDICES

Appendix 1: Analysis of variance for effect of BAP concentration and variety on shoot height and multiplication potential in embryonic axes groundnut.

Source of variation	*Degree of freedom*	*Mean Square*		
		Shoot height (cm)	Mean number of shoots/explant	Mean number of axillary branches
BAP treatment	5	58.45**	60.99**	10.57**
Variety (V)	3	2.18**	5.97*	0.74*
BAP x V	15	0.27	2.06	0.13
Error	24	0.29	1.85	0.19

**P≤0.01

Appendix 2: Analysis of variance for effect of NAA concentrations and variety on root induction in microshoots of groundnut.

Source of variation	*Degree of freedom*	*Mean Square*		
		Root induction frequency (%)	Mean number of roots/plantlet	Root length (cm)
NAA treatment	2	16727.87**	235.19**	144.28**
Variety (V)	3	410.30**	13.11*	32.62**
NAA x V	6	141.84*	10.42*	14.85**
Error	24	44.00	3.33	0.14

**P≤0.01

Appendix 3: Analysis of variance for moisture content, survival and shoot formation of cryopreserved embryonic axes of groundnut using desiccation technique.

Source of variation	*Degree of freedom*	*Mean Square*		
		Moisture content (%)	Survival (%)	Shoot formation (%)
Desiccation rate (D)	5	212.11**	26319.40**	23307.31**
Variety (V)	3	33.20**	846.65**	803.35**
D x V	15	10.01**	240.24**	144.24**
Error	48	3.25	32.81	10.21

**P≤0.01

Appendix 4: Analysis of variance for survival and shoot formation of cryopreserved embryonic axes of groundnut using vitrification technique.

Source of variation	Degree of freedom	Mean Square	
		Survival (%)	Shoot formation (%)
Dehydration in PVS2	5	3839.17**	3648.06**
Variety (V)	3	15586.57**	14871.76**
PVS2 x V	15	615.46**	653.98**
Error	48	66.67**	48.96**

**P≤0.01

Appendix 5: Analysis of variance for survival and shoot formation of cryopreserved embryonic axes of groundnut using encapsulation-dehydration technique.

Source of variation	Degree of freedom	Mean Square	
		Survival (%)	Shoot formation (%)
Dehydration time (D)	4	13156.67**	11926.67**
Variety (V)	3	2708.89**	2851.11**
D x G	12	458.89**	451.11**
Error	40	60.00	40.00

**P≤0.01

Appendix 6. Analysis of variance for moisture content of cryopreserved embryonic axes of groundnut using encapsulation-dehydration technique.

Source of variation	Degree of freedom	Mean
Dehydration time (D)	4	2765.65**
Variety (V)	3	74.24**
D x V	12	22.58
Error	40	5.98

**P≤0.01

Appendix 7: Analysis of variance for survival of cryopreserved embryonic axes of groundnut using the encapsulation-vitrification technique.

Source of variation	Degree of freedom	Mean Square
		Survival (%)
Dehydration in PVS2	4	11756.67**
Variety (V)	3	2317.78**
PVS2 x V	12	334.44*
Error	40	120.00

**P≤0.01

Appendix 8. Analysis of variance for survival under slow growth

Source of variation	*Degree of freedom*	*Mean Square*
		Survival (%)
Treatment (T)	3	420.83**
Variety (V)	3	254.17**
Storage period (P)	3	42012.50**
T x V	9	73.61
T x P	9	287.50**
V x P	9	120.83*
T x V x P	27	58.80
Error	64	31.25

**P≤0.01

BIOGRAPHY

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Previous Qualifications

Institutions attended	Period	Certificate obtained
L.E.A. Primary School Tudun Jukun, Zaria.	1980-1986	Pri. Sch. Certificate
Queen Amina College Kakuri, Kaduna.	1986-1991	Senoir Sec. Sch. Certificate
School of General and Remedial Studies (SGRS), Ahmadu Bello University Zaria	1991-1992	SGRS Result
Ahmadu Bello University, Zaria	1992-1999	B. Agriculture with 2^1
Ahmadu Bello University, Zaria	2002-2006	M.Sc. Crop Physiology

Working Experience

Organization	Period	Position
NYSC, Ezza South Local Government Council, Ebonyi State	2001-2002	Agric. Officer
Niger State Secondary School Education Board	2003-2006	Education Officer
Ahmadu Bello University, Zaria	i. 2006	Assist. Lecturer
	ii. 2006-2010	Lecturer II
	iii. 2010- Date	Lecturer I

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