EVALUATION OF REGENERATION RESPONSE IN TISSUE CULTURE OF SELECTED GROUNDNUT (*Arachis hypogaea L.*) VARIETIES ADAPTED TO EASTERN AND SOUTHERN AFRICA

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DECLARATION

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

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DEDICATION

I gratefully dedicate this thesis to my beloved husband Francis Mukiri and my children Clifford and Chiku for their undying love, encouragement support and prayers throughout my journey towards achieving this goal.

This thesis is dedicated in all sincerity and respect to my late dad, Sammy and my mum, Grace for their love, support and encouragement through all my education.

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ABBREVIATIONS AND ACRONYMS

ABSF	-	African Biotechnology Stakeholders Forum
ANOVA	-	Analysis of Variance
BAP	-	6-Benzyl amino purine
CO_2	-	Carbon (IV) dioxide
DNA	-	Deoxyribonucleic acid
2, 4-D	-	2, 4-Dichlopheoxyacetic acid
GM	-	Germination Media
GRD	-	Groundnut rosette disease complex
GRAV	-	Groundnut rosette assistor virus
GRV	-	Groundnut rosette virus
HCl	-	Hydrochloric acid
HEPA	-	High efficiency particulate air
IAA	-	Indole-3- acetic acid
IBA	-	Indole-3- butyric acid
ICGV	-	ICRISAT groundnut variety number
ICRISAT	-	International Crops Research Institute for the Semi-Arid Tropics
ILRI	-	International Livestock Research Institute
IPCV		Indian peanut clump virus
KARI	-	Kenya Agricultural Research Institute
MS	-	Murashige and Skoog Media
NAA	-	Naphthalene Acetic Acid
NaOCl	-	Sodium Hypochlorite
NaOH	-	Sodium hydroxide
PABA	-	Paramino Benzoic Acid
PGRS	-	Plant Growth Regulators
pH	-	Potential Hydrogen
Psi	-	Pounds per square inch
RIM	-	Root Induction Media
SatRNA	-	Satellite Ribonucleic Acid
SEM	-	Shoot Elongation Media
SIM	-	Shoot Induction Media
SSA	-	Sub-Sahara Africa Authority
TSWV	-	Tomato spotted wilt virus
TDZ	-	Thiadiazuron

ABSTRACT

Cotyledon explants from mature groundnut seeds (*Arachis hypogaea* L.) adapted to eastern and southern Africa, were optimized to obtain adventitious shoot buds with high frequencies. This was done using commercial bleach (jik, active ingredient sodium hypochlorite (3.5% v/v NaOCl) at different concentrations and exposure time as compared to 0.1% mercuric chloride. Results showed that 0.525% NaOCl and 1.050% NaOCl concentrations within an exposure time of 10-20 minutes were the optimum active range in all the varieties provided. There was constant contamination of CG-2 even after repeated sterilization indicating endogenous contamination. This showed that surface sterilization was not sufficient to sterilize it. While using mercuric chloride treatment, Chalimbana variety recorded the highest percentage of surviving explants (100%) and CG-2 had the lowest (82%). Regeneration experiments performed using already established optinum NaOCl sterilant concentration in each variety as compared with 0.1% mercuric chloride for 10 minutes (sterilant). Cotyledon explants were cultured in shoot induction medium (with BAP and 2, 4-D), shoot buds started to form along the proximal cut end of the cotyledon since this was the area of high regeneration potential due to presence of high number of meristematic cells. Well-formed shoots transferred to shoot elongation medium (SEM) elongated well in the presence of BAP growth regulator and later developed multiple shoots of about 4-8 shoots per explant. In SEM tubes, growth of all the varieties was rapid and prolific. Results showed that explants regenerated rapidly and with vigour. There was interaction ($p>0.05$) between variety and treatments in percentage regenerated plants. For example, JL-24 variety regenerated better using NaOCl than mercuric chloride. In terms of rapid growth and number of plants produced using NaOCl treatment, JL-24 variety was the best followed by CG-2, the least was ICGV-12991. Using mercuric chloride JL-24 regenerated the most plants followed by ICGV-90704 while the least was CG-2. All the groundnut varieties introduced into tissue culture in this study responded well, acclimatized, produced normal flowers and seedpods within 4 months. The cotyledon regeneration protocol proved to be a good regeneration system using both NaOCl and mercuric chloride sterilants. Of the selected African adapted varieties evaluated in this study ICGV-90704 performed best using mercuric chloride while CG-2 using NaOCl was the best. These are the varieties recommended for transformation, which could be one of the possible methods to offer resistance to viruses and this should have impact on crop improvement on productivity and food security, especially in the semi-arid tropics.

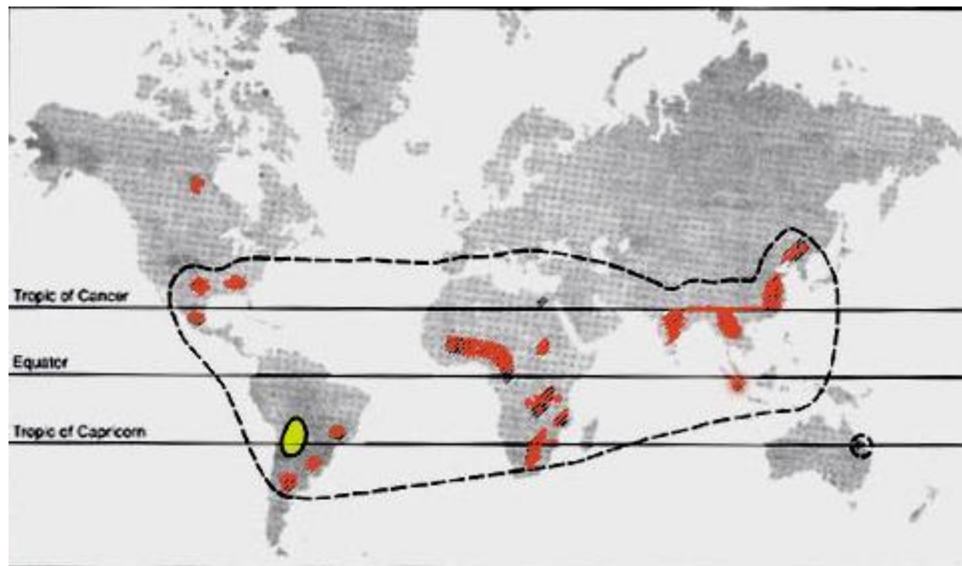
CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND TO THE STUDY

Groundnuts (*Arachis hypogaea* L.), also known as peanuts or monkey nuts belong to the family Fabaceae. This also includes soyabeans, cowpeas and pigeon peas. It originated in South America and was probably domesticated in the valley of the Paraguay and Parana rivers (Savage and Keenan, 1994). They appear to have been widely distributed in the West Indies. In the 16th century the Portuguese took them from Brazil to West Africa (Smith, 1995). The areas of greatest production in the world are China, India, U.S.A and Africa. In West Africa, Nigeria, Senegal and Gambia are the leading producers (Smith, 1995) (Figure 1).

Figure 1: Map of origin and areas where groundnut is an important crop.
Source:http://wikipedia.org/wiki/peanut.



After the Second World War, hungry Europe was in a hurry to win and develop new sources of food and raw materials. For example, Britain shortage of cooking fat in 1946, discovered that the great new source was in East Africa (British Groundnut Scheme in Tanzania) (then Tanganyika), Zambia (then Rhodesia) and Kenya, this was for large scale mechanized production, and this was how groundnut culturing was established in East Africa (Khonga, 2008).

Groundnuts are dicotyledonous and grow 30 to 50 cm tall, with a well developed tap root system and numerous lateral roots (Onwuema, 1979). Root nodules are present on the taproots. The leaves are opposite pinnate with four leaflets (two opposite pairs with no terminal leaflets). Flowers arise in the leaf axils at the base of the stem and have a typical leguminous flower structure with five united sepals and five yellow petals (Savage and Keenan, 1994) (Plate 2). Immediately after fertilization the region behind the ovary begins to elongate and grow downward into the soil. This is referred to as ‘pegging’. In the soil it matures into an indehiscent pod with one to four seeds. The seeds have a thin seed coat and contain no endosperm with two massive cotyledons. Its genome is made up of 40 chromosomes referred to as an allotetrapoid, meaning ‘from two different species’ (Stalker and Moss, 1987).

Plate 1: Generalized structure of a groundnut plant, showing various external features. Source:http://wikipedia.org/wiki/peanut.

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Seeds germinate about 5 days after planting and flowering occurs in one and half months. The crop is annual, normally ready for harvest in 4 months time. It grows best in light sandy, loamy soil. They require up to 5 months of warm weather and seasonal rainfall of 500-1000 mm (Smith, 1995).

Groundnuts have four major cultivar groups distinguished by branching habit and branch length. They are Spanish, Virginia, Valencia and Runners types. The small Spanish types are grown in South Africa and in Southwestern and Southeastern, United States. They are

large seeded, higher yielding, more disease resistant cultivars with higher oil content, compared to other varieties. Virginia types are large seeded and grown in Virginia, North Carolina and Tennessee in the U.S.A. They are increasing in popularity, due to demand for large peanuts for processing. Valencia types are coarse with heavy reddish stems and large foliage. They are grown on a small scale in Mexico. It is the preferred type for boiled groundnuts as they have the best flavour. Runner's types are found in Georgia, Alabama, Florida and South Carolina. These cultivars have good flavour, better roasting characteristics and higher yields compared to Spanish types (Onwuema, 1979). Specific cultivar groups are preferred for particular uses because of differences in flavour, oil content, size, shape and diseases resistance (Butterworth, 2004).

Groundnut is cultured in the semi-arid tropical and sub-tropical regions of nearly 100 countries on six continents between 40° N and 40° S (Naidu *et al.,* 1999). It is the World's 13th most important food crop, 6th most important source of edible oil and 3rd most important source of vegetable protein. For people in many developing countries groundnuts are the principal source of digestible protein, oil and vitamins. The seeds contain 24-34% protein and 47-50% oil (FAO, 1997). It is an important crop for household consumption and sale to rural and urban populations. In many countries groundnut cake, made from the straw and stems, is used as livestock feed (Smatt, 1994). As a legume, groundnut improves soil fertility by fixing nitrogen. Groundnuts also contribute significantly to food security for smallholder farmers of semi-arid cereal cropping systems therefore it is an important crop in sub-Sahara Africa (SSA) (Smatt, 1994).

Groundnuts require few inputs, making them appropriate for culturing in low-input agriculture systems by smallholder farmers who culture plots of less than one hectare and grow different crops, often in mixtures (Naidu *et al.,* 1999). There are several constraints to the productivity of groundnuts that result in substantial economic losses annually. They include fungal diseases, viral diseases and aflatoxins (Sharma and Ortiz, 2000). The most destructive virus disease that affects groundnuts in Africa is groundnut rosette disease (GRD). Outbreak of GRD is sporadic and unpredictable and can result in yield losses of up to 80% (Subrahmanyam *et al*., 2001). This disease is transmitted by the aphid (*Aphis craccivora*). Other viral diseases include bud necrosis disease caused by tomato spotted wilt virus (TSWV), (Yang *et al*., 1998) and clump disease caused by Indian peanut clump virus (IPCV), although these do not pose problems in Africa (Sharma and Anjaiah, 2000).

A very serious post-harvest constraint of groundnuts is aflatoxins. Invasion of seeds by toxigenic strains of the fungus *Aspergilllus flavus* and consequent aflatoxin contamination is a continuing serious problem in most countries where groundnuts are cultured (Mehan and McDonald, 1981). The fungus *A. flavus* attacks stored seeds and also has been found on living plants. It produces aflatoxin which can be lethal to birds and mammals feeding on infected seed (Mehan and McDonald, 1981).

Other fungal diseases include early and late leaf spot diseases caused by *Cercospora arachidicola* and *Cercospora personata*. It is characterized by dark spots surrounded by a yellow ring and is most common on the lower leaves of the plant. It causes defoliation.

The disease is widespread and can be very serious in most groundnut producing countries (Smatt, 1994).

Methods that are available to control diseases in the field usually entail the use of chemicals which are expensive. Use of conventional breeding programmes to confer genetic resistance has limited success to date and there is a need to identify sources of resistance in germplasm (Naidu *et al.*, 1999). In this regard, the International Crops Research Institute for the Semi Arid Tropics (ICRISAT) conducted a screening programme for rosette resistance. However, most of the rosette resistant varieties released to date for many production systems in Africa perform poorly due to inadequacy of rains in most areas. Groundnuts require a long growing season (150-180 days) to attain maturity. This makes them susceptible to drought during the end of the season (Subrahmanyam *et al.,* 2001).

One possible avenue to address the above constraints is genetic transformation for disease resistance. This can broadly be defined as the process of introducing foreign DNA into cells (Torres, 1989). Although several reports and protocols on tissue culture and transformation systems from diverse explants of groundnuts have been published (Cheng *et al.*, 1996, Kanyand *et al.,* 1997, Li *et al.,* 1994, Livingstone and Birch, 1999, Venkatchalam *et al*., 1999,) there has been limited success rates in terms of transgenic plants and their progeny.

1.2 Statement of the problem

Groundnut production in eastern and southern Africa is constrained by several factors which include rosette disease, which can cause yield losses, as high as 80%. Other constraints include aflatoxin production by *Aspergillus*, which is the most serious post-harvest constraint of groundnut and can be lethal to consumers. Genetic engineering has the potential to alleviate some of these constraints of groundnut culturing. However, to date varieties adapted to eastern and southern Africa have not been evaluated for regeneration and genetic transformation potential.

1.3 Hypothesis

The tissue culture protocol developed by Sharma and Anjaiah (2000) is genotype independent and can also be applied to various groundnut varieties adapted to eastern and southern Africa.

1.4 Research objectives

General objective

To evaluate the regeneration response in tissue culture of various groundnut varieties adapted to eastern and southern Africa.

Specific objective

- Determine the optimal level of sodium hypochlorite (NaOCl), the active ingredient of commercial bleach (jik), in all the varieties and its efficiency for sterilization as compared to mercuric chloride.

- Evaluating the regeneration response of the introduced varieties according to the method of Sharma and Anjaiah (2000).
- Identifying varieties that will be suitable candidates for genetic transformation.

1.5 Justification

Groundnut is one of the most important legume crops in the world. It is drought tolerant and is one of the few crops that remain in the field after a prolonged drought. It can be grown in any type of soil with little farm inputs and still give a reasonable yield (Smatt, 1994). The application of biotechnological methods, especially marker assisted selection and genetic transformation for the improvement of groundnuts has been shown to have great potential (Beachy, 1997).

Several protocols have been published (Cheng *et al.*, 1996, Kanyand *et al.,* 1997, Li *et al.,* 1994, Livingstone and Birch, 1999, Venkatchalam *et al*., 1999) but not much success has been achieved in obtaining large numbers of transgenic plants during transformation due to lack of efficient protocols to regenerate whole plats through in vitro regeneration of adventitious shoot buds from the transformed tissues. Stable engineered resistance requires the production of numerous independent transformants to allow the selection of those with the appropriate level of gene expression. In 2000, Sharma and Anjaiah reported an efficient protocol for direct organogenesis of s hoot buds from the embryogenic region of cotyledons, developed at ICRISAT in India. This study proposes to optimize the regeneration protocol using NaOCl sterilant as compared to 0.1% mercuric chloride for 10 minutes (used by Sharma and Anjaiah, 2000), and to evaluate the genotype-independent

nature and efficiency of regeneration of this protocol for locally adapted groundnut varieties from Eastern and Southern Africa.

CHAPTER TWO

LITERATURE REVIEW

2.1 Groundnut utilization and production

Groundnut is an important food security crop for household consumption and also a source of family cash income. This is due to its adaptability to adverse environmental conditions such as drought, ability to grow well in marginal soils and relatively short growing season that allows it to be harvested throughout the year. In addition, as a leguminous plant the harvested leaves and stems can be incorporated into the soil as low input fertilizer (Smatt, 1994). The crop fulfils an important role in providing food security because under adverse climatic conditions and low input regimes, it yields higher amounts of food energy and macronutrients per hectare than any other crop in arid and semi arid regions (Smatt, 1994).

2.1.1 Nutritional value

Groundnut is a rich source of protein, containing the essential amino acids cystine, methionine and lysine for growth and health. It is also a good source of vitamin B3 (niacin) necessary for brain health and good blood circulation (Smith, 1995). Recent research shows that groundnuts contain high concentrations of p-coumaric acid (an antioxidant) and a significant source of resveratrol, a potential anti-aging substance which is also associated with reduced cancer incidence (Butterworth, 2004). It is also a source of coenzyme Q-10 that is used in carbohydrate metabolism. Groundnuts also contain minerals such as calcium which is necessary for the skeleton development and muscle contraction as well as potassium that is required for cell division, protein synthesis,

growth factors and kidney function. It also contains phosphorus for the formation of muscles, skeleton bones, ATP and nucleic acids (FAO, 1997).

2.2 Constraints of groundnut production

Pest and disease control include drying groundnuts properly after harvest, application of insecticide or breeding groundnut cultivars for resistance (Dick, 1987). Most of these methods of control are expensive and use chemicals that might be dangerous to the consumer's health. Some of the resistant varieties released may not be suitable for production systems in Africa. An alternative solution can be to obtain a clean source of explant material which is disease free through tissue culture or that has improved disease resistance built in through genetic transformation (Beachy, 1997).

2.3 Plant tissue culture

Tissue culture is the technique through which a plant part is cultured on a nutrient medium under sterile conditions with the purpose of obtaining growth. Any plant part can theoretically be cultured for example leaf tissues, stem cylinders, auxiliary buds or even single cells. The materials used are routinely referred to as explants (Beachy, 1997). In references to tissue culture the term *in vitro* is very often used, which means in culture while the term *in vivo* means as in a living environment (Torres, 1989). The regeneration of whole organisms depends upon the totipotency of plant cells, in that all plant cells can express the total genetic potential of the parent plant given the correct stimuli (Sugiyama, 1999).

2.3.1 Application of plant tissue culture

The controlled conditions under which tissue culture is performed make these techniques indispensable for biochemical, cytological and physiological studies such as photosynthesis, differentiation and growth (Lindsey and Jones, 1996).

Plant tissue culture is a practice used to propagate plants under sterile conditions, often to produce clones of a plant. Different techniques in plant tissue culture may offer certain advantages over traditional methods of propagation (Fosket, 1994, Abel *et al.,* 1986, Hu and Wang, 1983, Harris and Oparka, 1994, George and Hall, 2008) including:

- The production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits
- To quickly produce mature plants.
- The production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds.
- The regeneration of whole plants from plant cells that have been genetically modified.
- The production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens.
- The production of plants from seeds that otherwise have very low chances of germinating and growing e.g. orchids and nepenthes.
- To clean particular plant of viral and other infections and to quickly multiply these plants as 'cleaned stock' for horticulture and agriculture.

The greatest value of this technology of tissue culture lies not so much in their application to mass clonal propagation but rather in their role underpinning development and application in plant improvement, molecular biology and bioprocessing, as well as their importance in research (George and Hall, 2008).

2.3.2 Tissue culture media

An explant requires a nutrient medium for growth. A nutrient medium is a watery solution of all the substances in the correct quantities relative to one another that the explant requires for growth (Murashige and Skoog, 1962). The type of tissue culture medium selected depends on the species to be cultured. Some species are sensitive to high salt concentrations or have different requirements for Plant Growth Regulators (PGRs). The type of organ cultured is important, for example, roots require the presence of auxins in the medium (Roberts *et al*., 1990).

Most plant tissue culture media are readily available in the market. Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) is the most commonly used basic tissue culture medium for plant regeneration from tissues and callus. It was originally developed for tobacco. Others include, but are not limited to, Linsmaier and Skoog medium (Linsmaier and Skoog, 1965), Gamborg's B5 medium (Gamborg *et al*., 1968) which was originally devised for soybean callus cultures, White's medium (White and Woofe, 1963), Schenk and Hildebrand medium (Schenk and Hildebrandt, 1972) for monocots and dicots and Nitsch medium (Nitsch and Nitch, 1969). Most of these media are available commercially but they can also be prepared in the laboratory from stock solutions. Tissue

culture media consists of 95% water, macroelements, microelements, PGRs, vitamins, sugars and amino acids. The different media differ in the concentrations of the various constituents and the source of the minerals.

2.3.2.1 Inorganic mineral elements

Plant tissues growing *in-vitro* require a combination of macroelements and microelements. Their concentrations are species dependant. Macroelements are classified as inorganic mineral elements and include nitrogen, potassium, magnesium, calcium, phosphorus and sulphur (Dodds and Roberts, 1985). Microelements include cobalt, iron, manganese, molybdenum, copper and zinc. Microelements are included in the medium in low concentrations whereas macroelements are needed in much higher concentrations (Dodds & Roberts, 1985).

2.3.2.2 Organic compounds

The most commonly used organic compounds include sugar, myoinositol, glycine, nicotinic acid, pyridoxine and thiamine. Sugar is a very important part of any nutrient medium as it serves as carbon source and its addition is essential for *in vitro* growth and development of the culture (Murashige and Skoog, 1962). Plant cultures are unable to photosynthesize effectively for a variety of reasons including insufficient organized cellular and tissue development, lack of chlorophyll, limited gas exchange, CO_2 in the tissue culture vessels and less than optimum environmental conditions (Skoog and Miller, 1957).

The most commonly used carbon or energy source is sucrose, since this sugar is also synthesized and transported naturally by the plant. Other sugars that may be used include glucose, fructose, sorbitol and maltose. Inositol, a sugar alcohol, is also added to most plant culture media as a carbon source. It can significantly improve *in vitro* response and its effect on growth is significant. It is also important in the synthesis of phospholipids and cell wall pectins (Schenk and Hildebrandt, 1972).

Another organic medium component is vitamins. These are part of enzymes, or cofactors for essential metabolic functions. The most essential vitamin in culture is thiamine (Vitamin $B_{1)}$ at 0.1-5.0 mg/l. It is involved in carbohydrate metabolism and biosynthesis of some amino acids. Nicotinic acid or niacin (Vitamin B_3 or PP) forms part of respiratory co-enzymes at the same concentration as thiamine. Pyrodoxine (Vit B_6), folic acid (Vit M), riboflavin (Vit B_2), ascorbic acid (Vit C), pantothenic acid (Vit B_5) acid, tocopherol (Vit E) and paramino benzoic acid (PABA) are also commonly used in tissue culture media (Caponetti, 2000). Amino acids are also sometimes used in cell culture media for example l-glutamine, asparagine, serine and proline. They are used as sources of reduced organic nitrogen for example, maintaining somatic embryogenesis (Baraun, 1998).

2.3.2.3 Plant Growth Regulators (PGRs).

PGRs regulate the initiation and development of shoots and roots on explants and embryos on semi-solid medium or in liquid medium cultures. They also stimulate cell division and expansion (Tahimoto and Harada, 1984). They exert dramatic effects depending on the concentration used, the target tissue and their inherent activity. The

most important PGRs used in tissue culture are auxins and cytokinins (Gaspor *et al.,* 1996). Auxins play a role in many developmental processes, including cell elongation and swelling of tissues, apical dominance, adventitious root formation and somatic embryogenesis (Blakesley and Chaldecott, 1993). Generally, when the concentration of auxin is low, root intiation is favoured. When the concentration of auxin is high, callus formation occurs (Link and Eggers, 1947). Examples of synthetic auxins include 2, 4-dichlo-phenoxyacetic acid (2,4-D) and naphthalene-acetic acid (NAA). Naturally occurring auxins include indoleacetic acid (IAA) and indolebutyric acid (IBA). Cytokinins promote cell division and stimulate intiation and growth of shoots *in vitro*. The cytokinins most commonly used are zeatin, kinetin, benzyladenine (BA) and thiadiazuron (TDZ) (Baraun, 1998). In higher concentrations (0.1 to 10 mg/l), cytokinins induce adventitious shoot formation but inhibit root formation (Baraun, 1998).

2.3.2.4 Gelling Agents

Media for plant cell culture *in vitro* can be used in either liquid or solid forms, depending on the type of culture being grown. For any culture types that require the plant cells or tissues to be grown on the surface of the medium, it must be solidified (gelled). Agar, produced from seaweed is the most common type of gelling agent, and is ideal for routine applications (Murashige and Skoog, 1962). However, because it is a natural product, the agar quality can vary from supplier to supplier and from batch to batch. For demanding applications a range of purer (more expensive) gelling agents are available. Purified agar or agarose and a variety of gellan gums, can be used (Sugiyama, 1999).

2.3.3 Sterilization

Plants are invariably infested externally with fungi, bacteria, yeasts and animal pests. These organisms may be present on all external surfaces and are also likely to find their way into small crevices, such as bud scales. For the great majority of plant cultures, superficial contamination needs to be controlled before the explants are introduced into the medium (George and Sherrington, 1984). The steps which are necessary for effective decontamination will depend on the nature of the plant material and kind of explants which are to be taken from it. Different chemical substances can be used for sterilization. The chemical agents used for sterilization must have certain features like antimicrobial activity, lack of toxicity, ability to penetrate and activity at ordinary temperature. Explants used in tissue culture like seeds, tissue pieces, excised embryos, pollen grains etc. are treated with disinfectants like hydrogen peroxide, sodium hypochlorite, ethyl alcohol, mercuric chloride etc. to remove different forms of microorganisms (surface sterilization). Inoculation tools like forceps, scalpels, and needles are autoclaved and surface sterilized by dipping in alcohol followed by flame sterilization (Caulo, 2000).

Methods of sterilization may differ for different plant species. Groundnut pods can be scrubbed with soap and water before sterilization. Rinsing with running tap water for some time (about half to one hour) can also be used (Traore *et al.,* 2005). The next most common sterilant is mercuric chloride. In view of the high mammalian toxication of this compound and the environmental hazard of mercury products it should be used with caution (George and Sherrington, 1984). Therefore mercuric chloride is seldom used as a plant sterilant today, as it is dangerous to use, and is difficult to dispose. Organomercuric

compounds can also be used for sterilization purposes, but they are environmentally unacceptable and not recommended (Powling and Hussey, 1981).

2.4 Regeneration

All normal living cells within the plant body possess the potential capacity to regenerate the entire organism i.e. totipotency. Regeneration occurs when pieces of tissue (explants) are isolated and grown aseptically in suitable growth media and conditions (Venkatchalam *et al.,* 1999). Tissue culture and plant regeneration are an integral part of most plant transformation protocols. Most methods of plant transformation applied to genetically modified crops require that a whole plant is regenerated from isolated plant cells or tissue which has been genetically transformed. This regeneration is conducted *in vitro* so that the environment and growth medium can be manipulated to ensure a high frequency of regeneration. In addition to a high frequency of regeneration, the regenerable cells must be accessible to gene transfer by whatever technique is chosen. The subsequent regeneration step is often the most difficult step in plant transformation studies (George and Hall, 2008).

The key to success in integrating plant tissue culture into plant transformation strategies is the realization that a quick and efficient regeneration system preferably from single cells must be developed (Livingstone and Birch, 1999). However, the system must also allow high transformation efficiency from whichever transformation technology is adopted.

There are two regeneration methods widely used in plant transformation studies i.e. somatic embryogenesis and organogenesis (Venkatchalam *et al.,* 1999). Somatic embryogenesis is where embryo-like structures which can develop into whole plants analogous to zygotic embryos are formed from somatic tissues (Gray and Purohit, 1991). These somatic embryos can be produced either directly or indirectly.

Organogenesis relies on the production of organs, either directly from explants or indirectly from a callus. Alternatively, axillary bud formation and growth can also be used to regenerate whole plants from some types of tissue culture (Sugiyama, 1999). Organogenesis is therefore the appearance of a shoot and a root followed by the vascularisation of cells in between, leading to root-shoot connection and eventually a whole plantlet (Christianson and Warnick, 1988). Both root and shoot formation are facets of the same process since the same meristem or primodium can be inducted to form a root or a shoot if the appropriate hormone control is achieved. Generally a high auxin: cytokinin ration favours root growth, while a low auxin: cytokinin ratio favours shoot growth (Bronner *et al.,* 1994). In particular, it is the auxin to cytokinin ratio of the medium that determines which developmental pathway the regenerating tissue will take (Sugiyama, 1999).

ICRISAT researchers, by manipulating various factors affecting tissue culture such as culture media constituents and explants were able to develop an efficient tissue culture protocol based on cotyledon explants from mature seeds (Sharma and Anjaiah, 2000). This protocol so far has worked with most Spanish type cultivars in India. For example

the *Agrobacterium* mediated genetic transformation of groundnuts, was applied successfully to JL-24 variety with the Indian peanut clump virus (IPCV) coat protein gene. The potential of this development to genetically transform groundnut to obtain resistance to viruses and other biotic constraints have potential positive impact on crop productivity (Eapen and George, 1994).

2.5 Temperature, light and humidity

The normal temperature in the growth chamber in tissue culture work is 25°C±2°. It is important to have good air circulation in the growth chamber or cabinet. An air conditioner is normally used for this purpose (Vasil, 1984).

Most *in vitro* cultures require a light intensity of 3000-10000 lux for adequate growth but some plants may require light intensity of above or below this limit and some plants may require low light for the first stage of development. Usually a regime of 16 hours light and 8 hours dark is ideal, but the regime may change depending on the plant or stage of growth (Skoog and Miller, 1957). Light is best provided by cool white fluorescent tubes. Overheating is sometimes a problem but ballasts or chokes kept outside the growth room, may reduce the overheating (Vasil, 1984).

In most cases humidity is not a problem because the relative humidity of the microenvironment within the culture vessel is approximately 100%. However, where the atmosphere is dry, moisture loss from the culture medium may occur. To prevent this, the culture tubes or plates should be sealed with a layer of parafilm (Vasil, 1984).

2.6 Laminar flow cabinet and growth room sterilization

Transfer of explants to the tissue culture medium is done in a laminar flow cabinet. Such a hood is equipped with positive pressure ventilation and bacteria-proof high efficiency particulate air (HEPA) filters (Vasil, 1984). The constant flow of bacteria- and fungal spore-free filtered air prevents particles from settling on the working area, which must be kept clean and disinfected (Kurtz *et al.,* 1991).

All the tools used in the hood, such as forceps, spatulas, scalpels, blades, scissors and Petri dishes must be sterile. To avoid contamination from the laminar flow cabinet, the internal surface of the cabinet should be disinfected with 70% ethanol and dettol at least once a week. Before and between work sessions, the working surface should be wiped with 70% ethanol. The doors and windows of the growth chamber in a tissue culture laboratory should be kept closed to prevent turbulence and contamination by insects and aerosols. Contaminated materials should not be taken into the cabinet. Persons working in this area should wear clean protective clothing such as a laboratory coat all the times. Hands and arms should be scrubbed before commencing work. All glassware, instruments and left-over plant materials should be removed soon after (Harris and Oparka, 1994). To reduce contamination in the growth room, contaminated cultures should be removed regularly and the room should be disinfected. When there are no cultures in the room, the growth chambers may be fumigated with ethanol (Torres, 1989).

2.7 Hardening of plantlets.

Hardening is the process of gradually acclimatizing plants to less humid conditions that is experienced in tissue culture or during rooting (Kyte, 1987). Before a plant can be transplanted it should be in the correct physiological state. Some plants may need roots to survive the transplantation and although their roots are not fully functional they usually recover faster than plants that still have to form roots. Successful hardening of *in vitro* plants, entails simulating the growth conditions that the plant was used to in the culture vessel in the glass house (DeFossard, 1976). As plants get acclimatized to green house conditions the humidity can be lowered and the light intensity increased. The best time of the year to harden plants off is the time when temperature and humidity can best be controlled (Pierik, 1987).

2.8 Photographic methods for plant cell and tissue culture.

Direct visual observations are important in plant tissue culture work unlike other disciplines, such as molecular biology and virology. Scientific research using tissue culture demands that experimental methods and results be documented through photography and repeated, so that they can be confirmed and built upon by others (Shaw and Rawlings, 1994). With *in vitro* culture, the confirmation that the basic experimental response has occurred is based primarily on visual proof (Harris and Oparka, 1994). For example, whether or not cultured tissues undergo regeneration, is confirmed by visualizing the presence of emerging shoot buds or organs.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Introduction

To evaluate the regeneration in tissue culture of groundnuts adapted to Eastern and Southern Africa, experiments were carried out between March 2007 and September 2008 under controlled environment conditions in a growth room. Later plantlets were transplanted in to pots and then grown in the greenhouse.

The whole study was carried out at the tissue culture laboratory of the Kenya Agricultural Research Institute (KARI) Biotechnology Centre, Nairobi and at International Livestock Research Centre (ILRI) Nairobi sterilization using mercuric chloride was carried out there as there was an available waste disposal by use of electrolysis. It was divided into two parts. The first part was concerned with optimization of the sterilization using commercial bleach (NaOCl active ingredient) as compared to 0.1% mercuric chloride for 10 minutes in order to establish explants free from contamination in tissue culture. The second part was concerned with the evaluation of regeneration potential according to the method of Sharma and Anjaiah (2000) by comparing the regeneration response of explants treated with sodium hypochlorite as compared those treated with 0.1% mercuric chloride as used by Sharma and Anjaiah, 2000.

3.2 Plant Material and Explants

Seeds of six groundnut varieties adapted to Eastern and Southern Africa, were used in this study. Five of these, ICGV-12991, ICGV-90704, JL-24, ICGV-99568 and Chalimbana were obtained from the ICRISAT groundnut breeding station in Malawi. The

sixth variety, CG-2, was obtained from a Kenyan seed company Leldet Ltd (Nakuru).

Variety ICGV-12991 is a short duration, drought tolerant, Spanish type groundnut variety with a high level of field resistance to groundnut rosette disease (Naidu *et al.,* 1999). It was originally collected from a farmer's field in South India and was introduced in a germplasm screening program for resistance to groundnut rosette and early leaf spot diseases in Malawi. It was extensively tested and distributed by the national programs of this country. This variety has a branching pattern with primary and secondary branches. It produces small two seeded pods with thin shells and a slight but medium constriction. It has no seed dormancy. The oil content of this variety is 43% and 27% being protein. It is an early maturing plant, within 130 to 140 days depending on its location (Deom *et al*., 2006).

Variety ICGV-90704 is a high yielding, medium duration, Virginia type groundnut variety. It is resistant to groundnut rosette virus (GRV) but susceptible to aphids, the vector for GRV transmission (Subrahmanyam *et al.*, 2000). It was developed by ICRISAT following repeated bulk selections for rosette disease resistance (and other desirable characters) using the infector row technique (Nigam and Bock, 1990). It has a semi-erect growth habit with alternate branching pattern with oval dark green leaves. Pods are moderately constricted with deep brown colored seeds. Seeds exhibits dormancy period. The oil content of this seeds is 45% to 48%. It matures in 130 to 140 days depending on the location (Monyo, 2008).

Variety Jl-24 is a Spanish variety, early maturing and high yielding commercial cultivar although it yields less compared to ICGV-12991 (Naidu *et al*., 1999). This variety was used as a control variety in the current study because it is a variety that ICRISAT scientists often use in India for transformation experiments.

Variety ICGV-99568 is a Spanish variety, early maturing and GRD resistant. It was released in Malawi in the late 1980s by ICRISAT (Nigam and Bock, 1990).

Chalimbana is a local variety in Malawi. It is highly susceptible to rosette disease (Monyo, 2008).

Variety CG-2, received from Leldet Company, is a Kenyan variety and was collected from farmers and not from a breeding station. It has a small two seeded cotyledon pod.

3.3 Preparation of MS nutrient medium

3.3.1 Stock solutions of the PGRs

For the preparation of BAP and NAA stock solutions of 0.5 mg/ml, each was first dissolved in a few drops of 0.1 M NaOH while swirling. Distilled water was used to top up to final volume of 10 ml. Stock concentration of 0.5 mg/ml 2,4-D, was dissolved in 3 drops of ethanol and then slowly topped up to a volume of 10 ml with distilled water. The stock solutions were stored in the refrigerator (4°C) in labelled tubes (Appendix 1).

3.3.2 Step by step medium preparation

Murashige salts (MS salts) consists of macro and microelements already made (Duchefa), exactly 4.42 grams, was measured as indicated in Appendix 2 and mixed in a 1 litre beaker half filled with distilled water. The carbon or energy source (sucrose) was added at a concentration of 3% (w/v). The appropriate amount of PGRs (for example, 9 ml BAP, 4.5 ml 2,4-D of stock concentration 0.5 mg/ml (already established in the protocol) was measured as indicated in Appendix 2 and mixed in a beaker containing 500ml of distilled water. The beaker was placed on a magnetic stirrer so that when different components were added they were thoroughly mixed. The pH of the medium was adjusted to 5.8. This was done using 0.1 M NaOH (base) or 0.1 M HCl (acid). The final media volume which was 1 litre was topped up using distilled water. The correct amount of agar (0.8% w/v), (the gelling agent) was weighed and added to the medium in the beaker while still stirring. The media was placed in the autoclave at 121°C for 15 minutes sterilization at 1.06 kgcm^{-2} pressure. After the media was ready, pressure was lowered by releasing it, temperature was cooled to a point enough to handle (40°C), and it was removed from the autoclave and was cooled inside a sterile hood. While still warm, the medium was dispensed into pre-sterilized petri dishes (15mm height by 100mm diameter) for SIM or autoclaved tubes for SEM and RIM media. They were then capped, stored while slanting (SEM and RIM media) ready for use.

3.3.3 Sterilization of equipment

Tools and glassware were washed immediately after use in warm soapy water and 1% sodium hypochlorite and then dried on a rack. Before autoclaving they were wrapped in

heavy duty aluminium foil and autoclaved at 121°C and 1.06 $kgcm^{-2}$ pressure for 15 minutes. When the equipments were cooled to 40°C (enough to handle), they were moved to the sterile transfer hood for offloading.

3.3.4 Procedure for maintaining aseptic conditions

A transfer hood is equipped with positive ventilation and high efficiency particulate air (HEPA) filters. Air is forced into the cabinet through a dust filter and a HEPA filter, and is then directed either downward or outward over the working surface at a uniform rate. The filtered air prevents bacteria and fungal spores and non filtered air and particles from settling on the working area which must be kept clean and disinfected when the hood is operational. The transfer hood was turned on 30 minutes prior to the work in the hood so that the positive pressure was maintained. This ensured all the air that passed over the work surface was sterile. All the windows in the tissue culture laboratory and air conditioning vents were closed to prevent air flow coming out from the hood. The interior of the transfer hood was sprayed with (70% v/v) ethanol and frequently swabbed with cotton wool soaked in 70% ethanol before and during the inoculation process. To maintain sterility of the interior all the working tools placed in the hood were sprayed with 70% ethanol. Before the inoculation process hands were washed thoroughly with soap and water and sprayed with 70% ethanol. During the entire work period, caution was taken not to obstruct the air flow across the surface coming from the filter unit.

Sterilization of forceps and blades was done, before opening glass containers and culture vessels by dipping them into a jar containing 70% ethanol and then passing them over a

flame inside the hood. When the flame had consumed the alcohol the tools were allowed to cool on the rack until when ready for use.

3.4 Tissue culture methodology

3.4.1 Optimization of sterilization of explants and *in vitro* establishment.

Mature seeds that were removed from pods which were stored at 4°C. They were allowed to adjust to room temperature for 10 minutes prior to sterilization and *in vitro* culturing. Seeds that were in good condition and with intact seed coats were selected (from the rest of the pool of seeds) randomly per variety and washed and rinsed three times thoroughly with sterile distilled water. They were surface sterilized with 70% ethanol for five minutes, followed by sterilization with varying concentrations of commercial bleach (3.5% v/v, NaOCl active ingredient) as specified below.

The first set of sterilization experiments involved a wide range of NaOCl sterilant concentrations from 0.0175% w/v overnight, 0.3250% w/v overnight, 0.5025% w/v for 10 and 20 minutes, 1.050% w/v 5, 10, 15, 20 and 30 minutes and exposure times as optimized by other protocols (Christian and Warnick, 1988), that used commercial bleach as a sterilant, to establish the best treatments per variety. There was three replicas each NaOCl concentration per variety.

The second sterilization experiment consisted of three repetitions using Mercuric chloride (0.1%) sterilant for 10 minutes (Sharma and Anjaiah, 2000) for each of the six varieties. These two sterilants (NaOCl, mercuric chloride) were used to compare survival of

explants in all the varieties. Each Petri dish contained seven explants of each variety.(Sharma and Anjaiah, 2000 only used mercuric chloride but did not use sodium hypochlorite a less toxic, available and affordable sterilant).

Tween 20, a detergent that acts as a wetting agent to break the surface tension, was also added to each sterilant and seeds. The mixture was swirled for different periods of time depending on the treatment and repeated with each of the different varieties. In all cases, double sterilized water was used after the sterilization step to rinse three times the seed until it was completely clear and free of foam. The seeds were then soaked in sterile water for 2 hours. The seed coat of each soaked seed was removed using a long pair of sterilized forceps to hold the seed in position and a small pair of sterilized forceps with curved ends to pinch the seed coat and tear it off. Cotyledons were separated from each other using a pair of sterilized forceps. The embryo was scooped out with the blunt side of the scalpel blade, avoiding making any cuts in the embryo area of the cotyledon. Then each cotyledon was split lengthwise into two by a single cut that did not cut through the embryo region and each of these halved cotyledons was treated as an explant. During this experiment, when a CG-2 variety was found to be difficult to sterilize, its explants were further treated by an additional soaking in 0.175% NaOCl for 5 minutes after seed coat removal to reduce contamination.

3.4.2 Culturing of the explants for regeneration

After sterilization, cotyledon explants were cultured on shoot induction media (SIM) to evaluate regeneration (Plate 3). SIM consisted of 4.42 grams MS (Murashige and Skoog, 1962) salts supplemented with B5 vitamins, 20 µM BAP, 10µM 2, 4-D (Sharma and

Anjaiah, 2000), 3% (w/v) sugar and 0.8% (w/v) agar at pH 5.8 (Appendix 2).

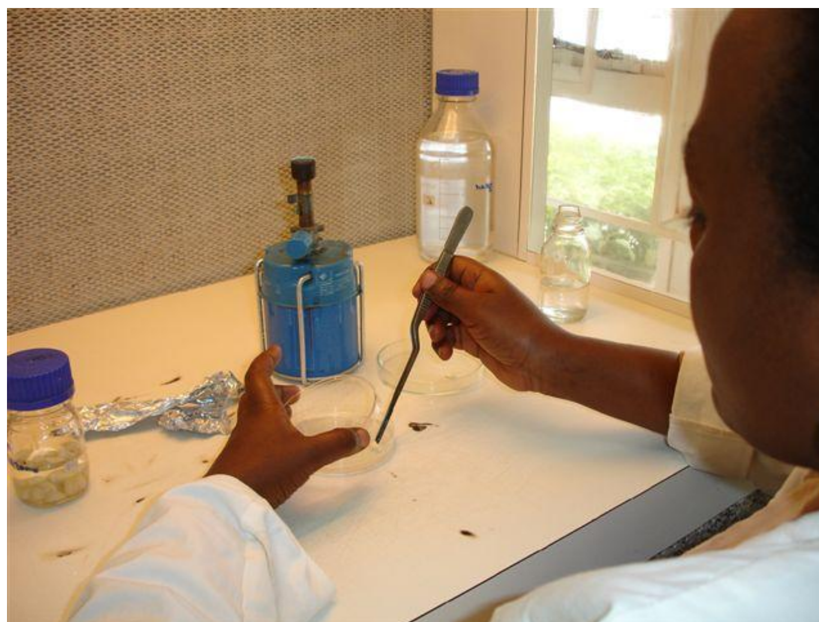


Plate 2: Preparation of explants for culture in SIM.

Each half cotyledon was cultured, cut surface in contact with the medium, and was considered as one explant. Each treatment (NaOCl and mercuric chloride) was repeated three times. Within 28 days of culture, shoot buds started to form. Shoots were transferred to fresh media every 28 days, cutting away any browning tissues and callus. Well formed shoots were separated from the explant and transferred to shoot elongation media (SEM) consisting of MS salts supplemented with B5 vitamins, 2 µM BA from the protocol), 3% (w/v) sucrose and 0.8% (w/v) agar that were dispensed in test tubes (Appendix 2). Well elongated shoots (about 3cm long) were transferred to root induction media (RIM), consisting of MS salts supplemented with B5 vitamins and 5µM NAA (from the protocol), 3% (w/v) sucrose and 0.8% (w/v) agar that were dispensed in test tubes (Appendix 2). Explants were cultured at 26^0C in a growth room under a 16 hours

photoperiod, 8 hours dark cycle provided by cool florescent lamps (Philips, 30 watts).

3.4.3 Hardening of i*n vitr*o regenerated plants in a greenhouse

Rooted shoots from the RIM which had formed enough strong roots (6-8 roots) for planting were washed thoroughly with sterilized water to remove agar and then planted in sterile soil (autoclaved sand and soil mixture 1:1) in small pots measuring 100 mm diameter by 90 mm perforated at the base. The pots were allowed to stand in distilled water and covered with non perforated plastic bags. After a day a small cut was made from the corner of the plastic bag, to reduce humidity. If the plant seemed to be coping well a second cut was made after a 2 days. Subsequently, the plastic bags were removed completely (after 10 days) and once the plants were well established, they were transferred to bigger pots (300 mm diameter) in the greenhouse where they were watered daily for further development.

3.5 Data collection and analysis

Collection of data commenced two weeks after first introduction of the explants into tissue culture and was repeated after every 14 days. The following growth characteristics of plantlets were recorded for all treatments;

- Total number of explants greening (positive development)
- Total number of explants that formed callus (positive development)
- Total number of explants with no reaction (negative development)
- Total number of contaminated explants (negative development)
- Total number of explants with shoots and shoot buds (positive development)

- Total number of plantlets produced (positive development)
- Rooting explants

Contamination of explants was monitored throughout the culturing and contaminated cultures were discarded immediately they were observed. Survival was determined by counting the number of surviving explants (greening, with callus, with shoot buds forming) compared to the total number cultured for each treatment. The number of shoots that were formed per explant was determined by counting at fortnight intervals. Root initiation in each variety was also recorded. The establishment of *in vitro* plantlets in the greenhouse was determined by counting the plants that survived until full establishment was achieved. Means within column were separated using Student Newman Keuls. Analysis was done using the ANOVA procedure.

CHAPTER FOUR

RESULTS

4.1 Optimization of surface sterilization of explants and *in vitro* establishment.

4.1.1 Effects of using different concentrations of NaOCl on the survival of explants.

The number of explants that survived in each variety was recorded within 3 to 28 days after culturing in SIM. Survival percentage rate with wide range of NaOCl concentration in all the varieties ranged between 0-100 percent.

Treatments such as 0.175% and 0.350% overnight recorded low rate of surviving explants (0-20%). Concentrations that had most number of surviving explants included 0.525% and 1.050%, of NaOCl for 10 to 20 minutes with each variety recording highest surviving explants of 60-100% (Table1). This showed that 0.525% and 1.050% were more effective treatments.

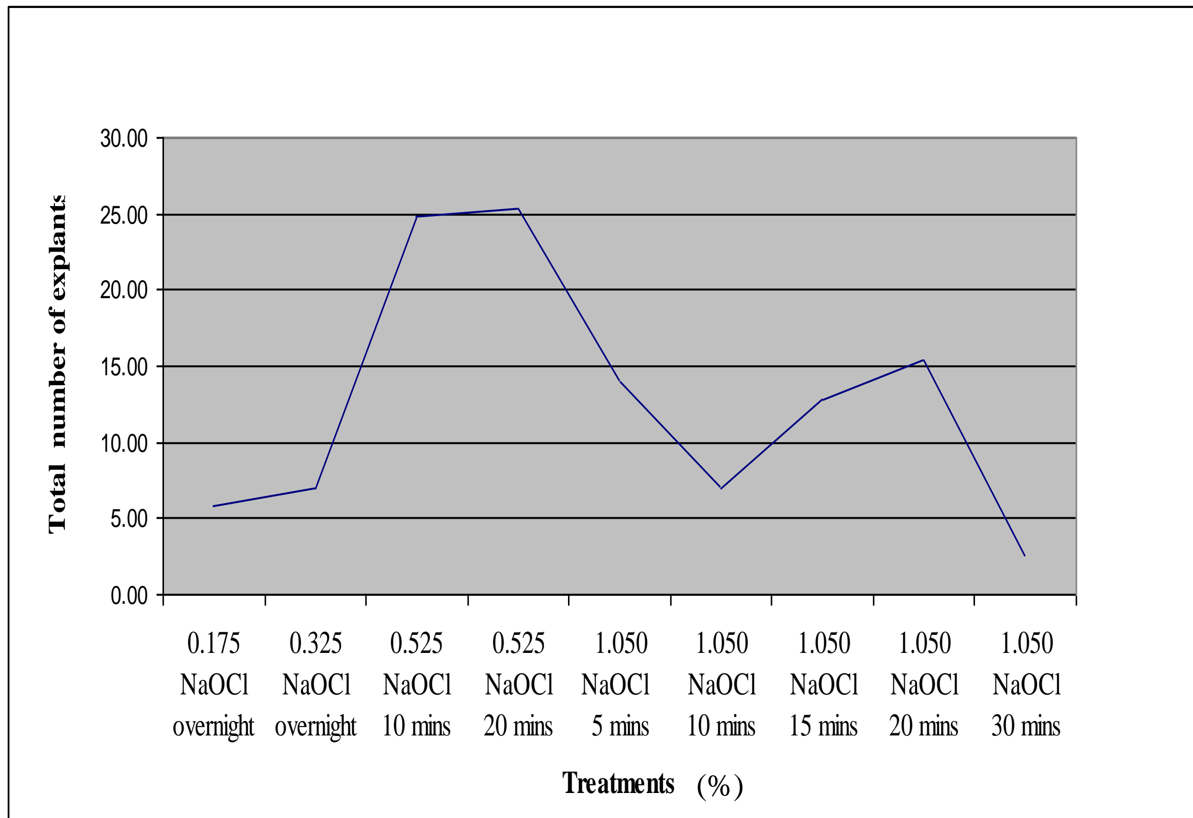
ANOVA revealed significant difference s among the number of explants that survived the various NaOCl treatments (F=3.94; df=8, 40; p=0.002). Exposure time also affected the ability of the explants to survive. However there was no significant difference in the number of explants that survived among varieties (F=0.39; df=5, 40; p=0.005)

Table 1: Percentage survival of explants per variety after treatment with NaOCl at various concentrations and durations (Means were separated using Students Newsman Keuls)

Treatment	**Variety**					
NaOCl (v/v)	ICGV-12991	ICGV-90704	JL-24	ICGV-99568	Chalimbana	CG-2
0.175% overnight	0d	60b	20cd	0d	20cd	0d
0.350% overnight	40c	0d	20cd	0d	20cd	40c
0.525% 10 minutes	0d	86ab	40c	100a	100a	100a
0.525% 20 minutes	83ab	83ab	83ab	49c	40c	57b
1.050% 5 minutes	40	60b	80a	40c	20cd	0d
1.050% 10 minutes	37	17d	6d	20cd	40c	0d
1.050% 15 minutes	60b	60b	100a	63b	0d	0d
1.050% 20 minutes	80ab	40c	0d	63b	20cd	60b
1.050% 30 minutes	6d	9d	0d	6d	11d	11d

The optimal concentration in each variety was based on explants that showed the highest rate of survival or had the least amount of contamination. The optimal NaOCl concentration for most of the varieties was therefore either 0.525% at10 to 20 minutes or 1.050% at 15 and 20 minutes (Table 1). Numbers with the same letters are not significantly different.

Figure 1: Cumulative effect of wide range of NaOCl treatment on total number of explants that survived in all the varieties.



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The figure with the highest number of explants surviving indicated the optimal NaOCl treatment for each variety and these were used in subsequent regeneration experiments. There was a significance difference between treatments (p=0.05). Based on these results, the best NaOCl treatment for each variety was established. The optimal treatment for each variety was

i) ICGV-12991; 1.050% applied for 20 minutes

ii) ICGV-90704; 0.525% applied for 10 minutes

iii) JL-24; 1.050% applied for 15 minutes

iv) ICGV-99568:1.050% applied for 20 minutes

v) Chalimbana; 0.525% applied for 10 minutes

vi) CG-2; 1.050% applied for 20 minutes

4.1.2 Sterilization treatment using 0.1% mercuric chloride for ten minutes.

The survival rate of explants from all varieties was much higher following a mercuric chloride treatment as compared to NaOCl treatment. Chalimbana variety recorded 100% and CG-2 exhibiting the lowest at 82% (Table 2).

Table 2: Explant survival (%) following surface sterilization with 0.1% (w/v) mercuric chloride.

Variety	Total number of explants	Surviving explants (%)(±SE)
ICGV-12991	35	89±2
ICGV-90704	35	94±1
JL-24	35	94±1
ICGV-99568	35	94±2
Chalimbana	35	100±1
CG-2	35	82±2

Results represent the average of 3 experiments. There was no significant difference using 0.1% mercuric chloride between varieties ($p=0.05$).

For the optimization of sterilization of explants and *in vitro* establishment regardless of the sterilant, the responses measured were;

i) No reaction .These were explants that showed no physical damage after culturing on SIM

ii) Survived explants. These were explants that turned green, produced callus or developed shoot buds. The type of callus produced was important as shoot buds formed mainly from hard, compact callus and not from white, soft callus.

iii) Contaminated explants. The pathogenic contamination was either bacterial or fungal.

Plate 3: Plates to show general response of explants in tissue culture

a) Explants of CG-2 with 1.050% NaOCl for 20 minutes and a further second sterilization treatment (due to persistent contamination) after seed coat removal with 0.175% NaOCl for 5 minutes, exhibiting no reaction after 28 days' of culture in SIM.

Plate 3b) Greening explants from these Chalimbana explants treated with 0.525% NaOCl for 10 minutes, after 28 days of culture in SIM.

Greening explants are those explants that responded by acquiring a green colouration and shoot bud formation at proximal cut end of the explant (Plate 3b).

Plate 3c) Callus formation observed on ICGV-90704 explants treated with 0.525% NaOCl applied for 10 minutes, after 28 days in culture in SIM.

Plate 3d) Bacterial contamination (indicated by colonies) of CG-2 explants treated with 1.050% NaOCl for 20 minutes and additional treatment with 0.175% NaOCl for 5 minutes after seed coat removal**.** This is a negative response.

Contaminated explants were observed, at least 3 days after incubation. The contamination was bacterial as it had milkish colonies (Plate 3d). These explants were discarded immediately after recording.

4.2 Culture of explants for multiplication and rooting.

Explants of JL-24 followed by CG-2 produced highest number of regenerated plantlets using NaOCl as sterilizing agent while JL-24 followed by ICGV-90704 produced regenerated plants using mercuric chloride. Since JL-24 was a control variety in this experiment CG-2 using NaOCl treatment and ICGV-90704 using mercuric chloride treatment were considered to have the best regeneration potential among the locally adapted varieties (Table 3).

The main effect for variety and treatment was not significant ($p>0.05$) in percentage regenerated plants, however, there was a significant interaction ($F_{5,\ 38}=2.52$), $p=0.046$ between the NaOCl and mercuric chloride treatments and varieties. This was with regard to the number of explants that regenerated in JL-24 variety, where NaOCl allowed better regeneration than mercuric chloride. In all the other varieties however, mercuric allowed better initiation of regeneration than NaOCl treatment (Table 3).

Table 3: Comparison of shoot initiation of explants following surface sterilization with either established NaOCl concentration in each variety or mercuric chloride. Means within column were separated using Student Newman Keuls.

Variety	Treatment	No. of explants cultured	% surviving explants with shoots	No of regenerated plantlets per surviving explant (multiple shoots) (±SE)
ICGV-12991	NaOCl	33	6	10±3d
	$HgCl_2$	35	16	31±6ab
ICGV-90704	NaOCl	31	12	12±3cd
	$HgCl_2$	35	39	39±5a
JL-24	NaOCl	31	29	43±26a
	$HgCl_2$	31	28	40±6a
ICGV-99568	NaOCl	32	7	15±4c
	$HgCl_2$	35	17	24±5b
Chalimbana	NaOCl	28	2	15±5c
	$HgCl_2$	35	35	24±6b
CG-2	NaOCl	28	29	19±5bc
	$HgCl_2$	25	20	23±5b

The results also indicated that for explants that were subjected to both of these treatments on the same date and that were subsequently cultured for the same length of time on SIM (as shown in Plate 4a-4d), mercuric chloride sterilant was less harmful to explants than NaOCl.

Plate 4: Comparison between the two sterilants in shoot initiation
a) Comparison of variety JL-24 sterilized with 1.050% NaOCl applied for 20 minutes and the same variety using mercuric chloride sterilant. Both treatments were done and cultured on the same date to compare shoot initiation in SIM.

JL-24-$HgCl_2$ (14.05.2008) JL-24-NaOCl

Plate 4b) Comparison of variety ICGV-12991 with 1.050% sterilized with NaOCl applied for 20 minutes and the same variety using mercuric chloride sterilant. Both treatments were done and cultured on the same date to compare shoot initiation in SIM.

ICGV-12991 $HgCl_2$ (12.05.2008) ICGV-12991 NaOCl

Plate 4c) Comparison of variety ICGV-99568 with 1.050% sterilized with NaOCl applied for 20 minutes and the same variety using mercuric chloride sterilant. Both treatments were done and cultured on the same date to compare shoot initiation in SIM.

ICGV-99568- $HgCl_2$ (14.05.2008) ICGV-99568-NaOCl

Plate 4d) Comparison of variety Chalimbana with 0.525% sterilized with NaOCl applied for 10 minutes and the same variety using mercuric chloride sterilant. Both treatments were done and cultured on the same date to compare shoot initiation in SIM.

Chalimbana- $HgCl_2$ (24.06.2008) Chalimbana-NaOCl

When well formed shoots emerging from the proximal cut ends (Plate 5), they were separated from the rest of the explant and transferred to SEM containing test tubes. These shoot elongated into a single plant (Plate 6) whereas others produced clusters of multiple shoots (Plate 7). Four to eight shoots were recovered from each explant, although over 10 shoots could be recovered if the explants were subcultured on SEM for three subcultures of 2 to 4 weeks on SEM. In a number of cases, the healthy shoots were also contaminated in SEM (Plate 8).

Plate 5: Shoot bud formation in ICGV-99568 (0.525% NaOCl) at the proximal end of explants within 28 days of culturing in SIM on 5.10.2007

Plate 6: Well elongated shoots in SEM medium of ICGV-99568 variety.

Plate 7: In all the varieties clusters of multiple shoots emerged as shoot buds from the single shoot base and later elongated to form a cluster of shoots. An example is illustrated here in ICGV-99568 variety sterilized with established NaOCl concentration.

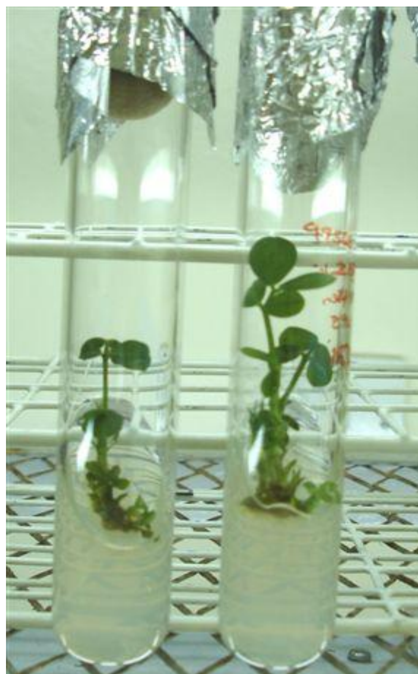

Plate 8: Fungal contamination of shoot explants in SEM containing tubes occurred occasionally in some elongated shoots. This photograph captured an example of such in ICGV-99568 and JL-24 varieties.

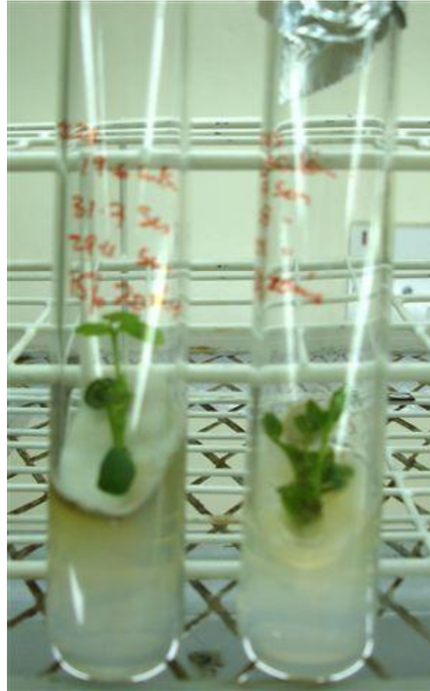

Plate 9: Five varieties showing rooting on RIM**.**

Well elongated shoots of about 3 cm were transferred to RIM for rooting (Plate 9), where healthy roots developed within 14 days in all tested varieties. There was no significant difference among the number of plantlets that rooted (p=0.005). The roots acquired about 3-4 cm length and strong and were transferred outside for hardening process.

In general it took about 110 days to obtain plantlets through tissue culture. In the study presented here, the most challenging step for the successful shoot regeneration was the shoot initiation from the individual explants.

4.3 Hardening of *in vitro* plants in a greenhouse.

All the rooted shoots (<95%, of about 3-5cm tall) survived after transplantation and appeared phenotypically normal. The non-perforated plastic bags that covered the plants in small pots with diameter of 100mm for about 2-3 days (Plate 10a) were to help to acclimatize the plantlets. Later the plantlets were transferred on bigger pots with diameter of 300mm (Plate 10b) where they increased in vigour. In the green house all the plants produced normal flowers and seedpods within four months, and were ready for harvesting (Plate 10c).

Plate 10a) Well rooted plants in small plastic pots, containing sterilized soil and sand mixture in the greenhouse.

Plate 10b) Hardened plantlets growing in the greenhouse. The saucers underneath contained water to be absorbed slowly by the plantlets.

Plate 10c) Mature seed pods harvested after 4 months**.** Each seed pod contained one to two seeds. About 30-40 seedpods were recovered from each plant.

CHAPTER FIVE

DISCUSSION

5.1 Explants preparation, sterilization and establishment *in vitro*.

In this study, a simple and fast protocol has been evaluated for explant sterilization and *in vitro* establishment in tissue culture of groundnuts. Optimization of this protocol was an important aspect in this study for successful regeneration. Effective explants sterilization was therefore necessary for successful tissue culture initiation.

A wide range of NaOCl sterilant was used to determine response of explants to different concentrations and exposure time. Results revealed that there was a significant difference between the numbers of explants that survived among treatments. This shows that these different treatments using NaOCl, affected the ability of the explants to survive. However, there was no significance difference in the number of explants that survived among varieties. This indicated that survival rates after each treatment was not dependent on genotype.

The optimal NaOCl treatment for all varieties was 0.525% to 1.050% within 10-20 minutes. This can be explained by the fact that this concentrations had efficient sterilizing properties as compared to other concentrations provided, and that the active ingredient NaOCl was able to penetrate inner tissues and sterilize them. This is in agreement with research done by (Davies and Dale, 1979). Explants varied considerably in their sensitivity to NaOCl solution. For example, 1.050% NaOCl applied for 30 minutes in all the varieties was not an effective sterilant. This could be attributed to the fact that this

treatment damaged tissues with prolonged exposure of time. Similar results have been reported by Khosh-khui and Sink (1982) in legumes species in which effective NaOCl concentration ranged from 0.350% to 1.050% within exposure duration of 10-20 minutes.

Results revealed that when mild concentrations such as 0.175% and 0.325% NaOCl were used, most explants were contaminated. This could be explained by the fact that the sterilant was not effective in killing the micro organism on the surface or inside the explants as reported by Khosh-khui and Sink (1982). When concentration and duration of explant exposure to disinfectants were low and long the plant tissues were not effectively sterilized and they therefore contaminate or get damaged. However, while using a wide range of NaOCl concentration ICGV-90704 variety emerged with one of the best two treatments as 0.175% NaOCl concentration applied for overnight which is a mild concentration. This could have worked well to that the source of the explants was free from contamination either on the surface or inside the explant.

Variety CG-2 was established to have the lowest number of surviving explants and with the highest number of contaminated explants. This was the reason why it was subjected to a further 0.175% NaOCl treatment applied for five minutes after removal of the seed coat to reduce contamination. The persistent contamination could be due to the source of seed. The CG-2 seeds were collected from farms while the other varieties were collected from the ICRISAT breeding station in Malawi where they undergo various screening procedures (Monyo, 2008).

The responses of explants included no reaction. They were reported to have been 'clean' through out. This was established when these explants were allowed more time to respond and after no positive development was observed. The explants were discarded after two months in culture. Probably, these seeds were still experiencing dormancy state and the factors that were provided in the media or time was not enough to trigger growth response (Vasanth *et al*., 2004).

Callus formation was a positive development towards regeneration although, the type of callus produced (white and soft) was not yielding prolific shoots). Greening, a positive development in regeneration was the first noted stage towards bud formation and eventually shoot emergence. These responses were towards achieving regeneration.

Contamination was a negative response observed in these studies. The various types of contamination observed in these studies included bacteria and fungi. Bacteria are the most frequent contaminants commonly introduced with the explant and are characterized by a slimy appearance and can be of many colours; white, cream, pink or yellow as researched by (George and Sherrington, 1984). The cause of contamination in tissue culture work can be caused by incorrect media preparation, bad aseptic technique and insufficient sterilization of explants material and internal contamination (Pierek, 1987). In these studies contaminations invariably slowed down growth rate and often lead to death of explants. This is the principle behind contamination as phytotoxins are often excreted by the contaminating microorganisms altering the pH of the nutrient medium (Murashige, 1974).

5.2 Culture of explants for multiplication and rooting.

The best performing variety in regeneration using mercuric chloride was JL-24 followed by ICGV-90704, while using NaOCl, JL-24 was the best followed by CG-2. These results show that the control variety JL-24 regenerated well as was expected using both sterilants (Sharma and Anjaiah, 2000). CG-2 variety on the other hand, did not perform well due to high rates of contamination. If a more efficacious sterilization procedure could be found, CG-2 variety might have a good regeneration potential. Variety ICGV-90704 is highly regenerable possibly, due to its big size and clean source.

The significance differences and interactions observed between NaOCl and mercuric chloride treatments in different varieties in culturing could be attributed to the differences in the regeneration potential. This may be due to various factors. Such significant factors include differences in the stage of the cells in the cell cycle as well as the varying ability to transport endogenous growth regulators and metabolic capabilities of the cells (Guerra and Handro, 1988). Explants age and vigour are also factors that must be considered when developing a regeneration protocol. This is because in some species it has been found out that more vigorous plant stock is able to regenerate better than other stocks (Paz *et al.,* 2004).

The comparison between mercuric chloride and NaOCl treatments in terms of regeneration revealed that, mercuric chloride in all the varieties resulted in the highest number of explants that developed shoots. The high concentration of hypochlorite ions in NaOCl produced the bleaching effect. This could have damaged the explants and

weakened the regeneration response. This was the reason why explants sterilized using mercuric chloride had young vigorously growing healthy plantlets that were fast growing as compared to explants sterilized using NaOCl.

A single explant produced many shoot buds from the same area. The emergence of multiple shoot buds from the proximal cut end indicated this was a region of high potential regeneration (Guerra and Handro, 1988) due to presence of highly meristematic cells. Similar results were reported by (Hisajima, 1982), when he found that up to 10 million shoots of almond species could be obtained from a single seed explant in a year after several subcultures. This technique could be initiated from the seeds of many species particularly legumes (Vasanth *et al*., 2004). In this study, the most challenging step was to produce shoots from an explant. This was overcome by embedding the proximal cut end of the explant nutrients at least for the first two weeks of culture initiation because this were regions of high potential of regeneration .

The various fungal contaminations that were recorded in SEM could have resulted from endogenous contamination in the explant and not as a result of surface contamination. The high percentage of plantlets recorded with rooting, indicated that groundnut was an easier to root species, as reported previously (Maria and Alberto, 2000).

5.3 Hardening of *in vitro* plants in a greenhouse

In this study, results indicated that 100% plantlets survived in the greenhouse. All the regenerated plants developed normally by producing flowers and pods within four months and these contained viable seeds. All the plantlets were able to withstand transplantation stress preparing them for exposure to full sunlight, this is in agreement with (Perez and Cerruti, 2008). The extra care taken when young rooted plants were being transferred from culture vessels to the external environment was due to plants growing under low intensity artificial light and high humidity in the growth room as compared to the external environment as researched by (Schielfelbein *et al*., 1997). All the regenerated plants did not show any morphological abnormalities during an observation period of four months in the greenhouse.

CHAPTER 6

CONCLUSION

The regeneration protocol described by Sharma and Anjaiah (2000) is applicable to selected groundnut varieties presented in this study that are adapted to eastern and southern Africa. All the varieties ICGV-12991, ICGV-90704, JL-24, ICGV-99568, Chalimbana and CG-2 responded well in tissue culture with each one of them being regenerable.

It is possible to sterilize groundnut seed explant using NaOCl sterilant. The optimal NaOCl concentration for each variety was ICGV-12991 1.050% applied for 20 minutes, ICGV-90704 0.525% applied for 10 minutes, JL-24 1.050% applied for 15 minutes, ICGV-99568 1.050% applied for 20 minutes, Chalimbana 0.525% applied for 20 minutes and CG-2 1.050% applied for 20 minutes. Any of these varieties would be suitable for transformation studies. However, when using NaOCl as sterilizing agent, CG-2 performed best as found in the results. For better results this study recommends that the source of seed explants for this variety should be free from contamination. Contaminated explants should be disposed without delay by autoclaving to prevent it from spreading to other cultures. Seeds that are obtained from plants through tissue culture regeneration are recommended for this purpose.

This cotyledon regeneration system proved to be an excellent vehicle for production of a large number of groundnut plants over relatively short periods of time. The fact that there was no significance difference in the number of explants that survived and initiated shoots among varieties and that shoot formation was rapid and prolific developing into normal fertile plants indicates the protocol is genotype independent. An adventitious regeneration, rooting and

acclimatization system from cotyledons of *A. hypogaea L* of African origin by Sharma and Anjaiah (2000) has been evaluated and was seen to be efficient. This protocol can therefore be useful for experimental studies in production of clean explants in tissue culture and production of transgenic *A. hypogaea. L* with resistance to viruses and other abiotic constraints such as drought should have tremendous impact on improvement of crop productivity especially in the resource poor agricultural systems of the semi–arid tropics.

RECOMMENDATIONS

All the varieties tested regenerated well from cotyledon explants upon *in vitro* culture and any of the varieties tested in this study will be suitable for future transformation studies with African groundnuts.

Efficient regeneration was also achieved following sterilization with commercial bleach, which can therefore be used for tissue culture purposes, especially when mercuric chloride is not available or when appropriate disposal facilities for mercury-containing waste products are not in place.

An alternative for obtaining uncontaminated explants is to take explants from seedlings which are aseptically grown from surface-sterilized seeds.

This cotyledon regeneration system is efficient can be applied to obtain disease free, large numbers of groundnuts over a relatively short period of 4 months.

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APPENDICES

APPENDIX 1

Growth Regulators (stock concentrations: 0.5mg/ml)

- Benzylaminopurine BAP (Mw 225.26) and Kinetin (Mw 215.22) was dissolved in 0.1M NaOH and made up to 10 ml volume with water.

- 2,4-Dichlorophenoxyacetic acid 2,4-D (Mw 221.04) was made by dissolving in 50% ethanol and made up to 10ml volume with water.

- Naphthalene acetic acid NAA (Mw 186.21) was made by dissolving dilute 0.1M NaOH and made up to 10ml volume with water.

- Each of them was then filter sterilized and then dispensed into eppendorf tubes and stored at -4^0C.

APPENDIX 2

Growth Media

Germination/Shoot Induction Media (SIM/GM)

MS macro and micro salts with B5 vitamins (4.42g)

20µM BAP of 0.5 mg/ml stock concentration

10µM 2, 4-D of 0.5 mg/ml stock concentration

30g sucrose

8 g agar (Difco Bacto Agar)

Adjusted pH to 5.8

Autoclave and pour into sterile petri dishes (25mm by 100mm)

Shoot Elongation Media (SEM)

MS macro and micro elements with B5 vitamins (4.42g)

2 µM BAP of 0.5 mg/ml stock concentration

30 g sucrose

8 g agar (Difco Bacto Agar)

Adjusted pH to 5.8

Autoclave and pour into tubes (15mm by 180mm) and let to settle on a slant

Root Induction Media (RIM).

MS macro micro elements with B5 vitamins (4.42g)

5 µM NAA of 0.5 mg/ml stock concentration

30 g sucrose

8 g agar (Difco Bacto Agar)

Adjust pH to 5.8

Autoclave and pour into tubes (15mm by 180mm) and let to settle on a slant.