A Short Review and preliminary study on peanut (*Arachis hypogaea* L.) tissue culture

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR
THE BACHELOR OF SCIENCE IN BIOTECHNOLOGY

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December, 2018

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Dedicated to

my friends and family

DECLARATION

I hereby declare that the research work embodying the results reported in this thesis title entitled **'A Short Review and preliminary study on peanut (*Arachis hypogaea* L.) tissue culture'** submitted by the undersigned has been carried out under supervision of Dr. Aparna Islam, Professor, Biotechnology Programme, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

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ACKNOWLEDGEMENT

First of all, I am thankful to Almighty Allah for giving me the strength and patience that allowed me to complete this dissertation.

I am grateful to my parents and family for supporting and encouraging me in every stage of my life. They have always supported me in my studies and provided me with the best possible academic environment. I am thankful to my sister for supporting me through thick and thin and inspiring me to work hard.

My sincere acknowledgement and gratitude goes to Professor A. A. Ziauddin Ahmad, former Chairperson, Department of Mathematics and Natural Sciences, Brac University, for being a role model and steering me into the right direction. I thank Professor Yusuf Haider, Chairperson, Department of Mathematics and Natural Sciences, Brac University, for allowing me to carry out my research work in a disciplined way.

This dissertation would not be possible without my supervisor, Dr. Aparna Islam, who inspired me to work in this field. Thanks to her wise insights and constructive criticisms, I was able to successfully complete my research. She was always there when I needed help or guidance.

This research could be conducted smoothly because of the teachers, seniors, teaching assistants and laboratory assistants. I express my sincere gratitude to Kashmery Khan, Shahana Chowdhury, Asma Afzal and Nahreen Mirza for being such great sources of help and support throughout this research period.

I want to take this opportunity to thank Shabnam Syeed, Nadia Obaid and Promi Tahsin for always being there and providing assistance when needed. Special thanks to Samia Afroz for being the best lab partner I could ask for. Last but not the least, I would like to express my infinite appreciation and gratitude to Sinjan Saadat for supporting me no matter what, for providing the motivation I needed to work hard and for being a great friend during stressful times.

(Ashfia Nahreen)

December, 2018

ABSTRACT

Peanut (*Arachis hypogaea* L.) belongs to the family leguminosae and is one of the most important oil seed crops in the world. Peanut seeds contain high seed oil, proteins, amino acids and vitamin E. However, this crop faces several yield constraints like biotic and abiotic stresses. With the climate change, salt water intrusion to inland soil and loss of cultivable land makes it critical to develop a high yielding variety of peanut with salt tolerance. This can be achieved by utilizing biotechnology processes. A prerequisite for the establishment of a genetic transformation protocol is an efficient and reproducible protocol for the *in vitro* regeneration of peanut. For this, an extensive study of the existing *in vitro* regeneration protocols was performed. In light of these studies, the role and interaction of decapitated embryo explants in different combinations and concentrations of growth hormones of two farmer popular varieties was investigated. Among the various tested combinations of BAP and Kn in MS medium, the best combination was 3 mg/l BAP and 1 mg/l Kn for BINA Chinabadam 2 and 3 mg/l BAP and 0.5 mg/l Kn for BINA Chinabadam 4. Decapitated whole embryo proved to be a better explant than decapitated half embryo for both varieties. The highest percentage of shoot regenerating explants was seen in two days old decapitated whole embryo (90%) of BINA Chinabadam 2 and the highest average number of shoots was seen in two days old decapitated whole embryo of BINA Chinabadam 4 (5.10). Two days old explants also responded better in culture medium than old day old explants.

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LIST OF ABBREVIATIONS

The following abbreviations have been used throughout the text.

BAP	6-Benzylaminopurine
Kn	Kinetin
TDZ	Thidiazuron
NAA	Napthalene acetic Acid
IAA	Indole-3 Acetic Acid
IBA	Indole-3 Butyric Acid
2, 4- D	2,4-Dichlorophenoxyacetic acid
Pic	Picloram
2, 4, 5 T	2,4,5-Trichlorophenoxyacetic acid
GA_3	Gibberelic Acid
ABA	Abscisic acid
MS	Murashige and Skoog (1962) medium
HCl	Hydrochloric Acid
$AgNO_3$	Silver Nitrate
NaOH	Sodium hydroxide
BINA	Bangladesh Institute of Nuclear Agriculture
mg	Milligram
gm	Gram
kg	Kilogram
ml	Millilitre
l	Litre
cm	Centimetre

Chapter – 1: Introduction

CHAPTER 1

INTRODUCTION

Peanut (*Arachis hypogaea L.*) is an annual grain legume crop of the genus Arachis. It belongs to the largest plant family Fabaceae and is also known as groundnut.

Peanut originated from Peru and Brazil, with the earliest archaeological records dating back to 3900-3750 BC (Ozudogru and Lambardi, 2012). Today, peanut is cultivated worldwide in tropical, subtropical regions and warm temperate climates. In 2016 the world peanut production total was approximately 44 million tons with China being the largest producer, followed by USA and India ("FAOSTAT", Date: 2018-11-14).

1.1 Characteristics of peanut plant

Peanut has a special place among domesticated plant species as it produces seeds below the soil and produces flowers above the ground. The plant grows up to 30-50 cm in height and has leaves that are pinnate with four leaflets. The flowers are yellow in colour with reddish veining (Figure 1.1). They are borne in axillary clusters on the stems above ground and last for just one day. One to several flowers may be present at each node and are usually more abundant at lower nodes. The first flowers appear at 4 to 6 weeks after planting and maximum flower production occurs 6 to 10 weeks after planting. After pollination, the flower stalk elongates till the ovary touches the ground. The ovary is pushed underground where the mature fruit develops into a pod. Cells beneath the ovary begin to divide, producing a "peg" that forces the ovary into the ground. As the peg elongates, a cap of cells forms next to the withered style. This cap protects the ovary as it is pushed into the soil. This is similar in function to the root cap at the tip of a root. After the developing ovary has pushed a few centimetres into the soil, downward elongation of the peg ceases. The ripening ovary becomes oriented parallel with the ground surface where it completes its development. The pods mature underground are 3-7 cm long containing 1-4 seeds.

The shape of peanut seeds vary from oblong to nearly round and have a papery seed coat that ranges in colour from whitish to dark purple. The cotyledons provide carbohydrates and protein for the developing embryo until it develops into a seedling with functional roots and photosynthetic leaves. (Figure 1.2) The peanut seeds are very fragile. They will not sprout if the radicle is damaged or if the seed is of a low quality. High quality seeds are produced by storing the seeds in cool and dry conditions with plenty of ventilation and providing protection from pests.

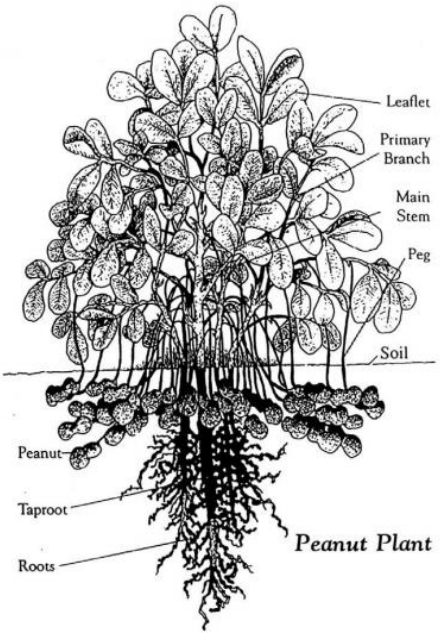

Figure 1.1: A typical peanut plant

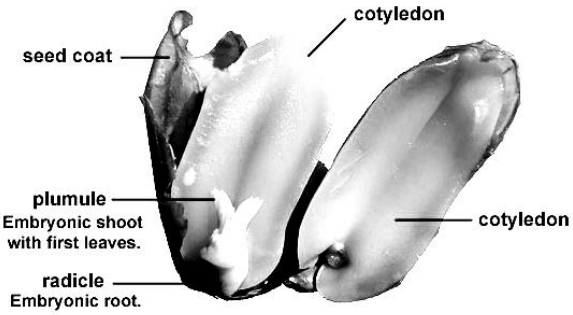

Figure 1.2: Parts of a peanut seed

Peanuts have a high nutritional value and are considered as the most important oilseed worldwide. It contains 36-54% oil, 20-25% proteins and 8-14% soluble sugars. It is rich in vitamin B, vitamin E and amino acids, such as, thiamine, niacin, riboflavin, etc. (Janila *et al*. 2013). Each 100 grams of peanut contains 600 kcal, 50 gram fat, 800 milligram sodium and 10 milligram fibre but no cholesterol.

There are four main varieties of peanuts:

i. Runner variety: Runners have become the dominant type due to the introduction in the early 1970's of a new runner variety, the Florunner, which was responsible for a

spectacular increase in peanut yields. Runners have rapidly gained wide acceptance because of the attractive, uniform kernel size.

ii. Spanish variety: The plant is erect and compact with 2 to 3 peanuts in each fruit. They have smaller kernels covered with reddish brown skin. The oil content is higher than the other types which gives it an advantage when crushing for oil.

iii. Valencia variety: Valencias usually have three or more small kernels to a pod and are covered in a bright-red skin. They are very sweet peanuts and are usually roasted and sold in-the-shell.

iv. Virginia variety: The plant is trailing with 2 peanuts in each pod. Virginias have the largest kernels that are sold as gourmet snack peanuts.

Peanut is either directly consumed as food or crushed for oil production. After oil extraction, the remaining meal is used as animal feed. In some places, peanut is used as a dermal medicine for external use to treat arthritis, joint pain, dry skin, etc.

1.2 Cultivation of peanut

Peanuts prefer to grow in light, sandy loam soil with a pH of 5.9-7. Adequate levels of phosphorous, potassium, calcium, magnesium and micronutrients are needed for good yield. However, due to its ability to fix nitrogen, they do not require nitrogen containing fertilizers. Peanuts thrive in warm temperatures but they are also frost tolerant and can grow in areas with a lower temperature. Their optimum growing temperature is between 20-27 degrees Celsius. Flower formation is hampered above 33 degrees Celsius and growth stops completely below 13 degrees Celsius (https://living.thebump.com/good-climate-growing-peanuts-5973.html Date: 2018-11-14).

Soil moisture is a very important factor in improving peanut yield. Water logged soil affects rhizomes. For a good yield, 0.5-1.0 cm of annual rainfall is needed. Peanut is a C3 plant but its rate of photosynthesis is elevated in high sunlight, much like a C4 plant. When planted in shade, the leaves are larger and the plant has fewer reproductive organs but the yield is usually unaffected (https://living.thebump.com/good-climate-growing-peanuts-5973.html Date: 2018-11-14).

For harvesting, the entire plant, including the roots, is removed from the soil. The peanut pods are removed and dried properly before storing. Storage conditions have to be sufficiently dry to prevent infection from *Aspergillus*.

1.3 Peanut cultivation in Bangladesh

Peanut is a very important crop in Bangladesh. In 2008, Bangladesh imported 27 metric tons of peanut (Deb & Pramanik, 2015). The soil and climate of the country are also favourable for growing the oilseed. In late 2000s, average peanut area and production in Bangladesh was 30.16 thousand hectares and 45.20 thousand metric tons, respectively (Deb & Pramanik, 2015). Faridpur, Noakhali and Panchagarh are the top districts with maximum peanut yield, while Chittagong and Rajshahi accounted for less than 1% of the yield. According to the Bangladesh Bureau of Statistics, Panchagarh was the highest producer of peanut in 2015 with 11809 million tons. Over time the average production of peanut in Bangladesh has increased. However, the cultivable land for peanut has decreased due to increased salinity in the soil (Deb & Pramanik, 2015). If the vast coastal areas around Chittagong and other districts can be used for peanut cultivation, the production level of the important oil crop can be increased so that oil import becomes redundant. Combating land salinization problem is necessary for ensuring food security in Bangladesh.

1.4 Limitation in peanut cultivation

Peanut is vulnerable to a variety of biotic and abiotic stresses. The genetic diversity of peanut is low as compared to other important crops as they are diploid and self-pollinating in nature. This narrow genetic base and low genetic diversity results in the susceptibility to various pests and diseases.

1.4.1 Biotic stresses

Fungal diseases are one of the main constraints of cultivating peanut. The peanut plant is vulnerable to late leaf spot and early leaf spot diseases caused by *Phaeoisariopsis personata* and *Cercospora arachidicola,* respectively (Janila *et al*. 2013; Backman and Crawford 1984; Khaleque *et al*.1985). Other fungal diseases include rust, wilt and stem and pod rot. Viral

diseases include peanut bud necrosis disease and peanut stem necrosis disease. The peanut cultivars found in Bangladesh, mainly the Spanish type, are especially susceptible to *Cercospora sp*. Peanut is not very susceptible to insects and pests in the growing stage but some pests and insects cause major economic loss in storage.

1.4.2 Abiotic stresses

Abiotic stress is also a very serious constraint on peanut cultivation. Abiotic stresses include drought, salinity, water logging, high temperature, etc. These constraints are a serious threat in mainly developing countries, leading to the loss of crop yield up to 50 percent (Boyer *et al*. 1982; Bray *et al*. 2000). A large percentage of agricultural land in Asia is highly affected by salinity. Bangladesh is one of the affected countries as two million hectares of coastal areas have a high salt concentration (Chowdhury, 2014). A high concentration of salt in the soil leads to a lower crop yield, marginal leaflet burn and severe pod rots in peanut. Water infiltration into soil is also decreased in soils with excessive salinity (http://agropedia.iitk.ac.in/content/abiotic-stress-groundnut Date: 2018-11-14).

1.5 Improving peanut cultivation

The use of pesticides, insecticides, etc. is frowned upon due to its environmental impact. Thus, genetic resistance is a sustainable and promising alternative for preventing diseases and improving crop yield. Conventional breeding techniques are not always a viable option for peanut because the necessary resistance genes are not available to the breeders and also because *Arachis hypogaea* is self-pollinating in nature. The available cross with *Arachis hypogaea*, ploidy differences, evaluation and the germplasm have been reviewed by many researchers (Gregory and Gregory 1979; Moss and Stalker 1987; Singh *et al.* 1980; Halward *et al.* 1992).

Introducing novel and beneficial genes into peanut is possible via genetic manipulation. Peanut tissue culture is the crucial prerequisite to genetic engineering. To develop protocols for an efficient regeneration, it is important to investigate the roles and interactions of different genotypes, explant sources and hormonal effects. Micropropagation can be achieved while working with different parts of the plant, such as, apical meristem, nodal bud, shoot bud, axillary bud or by producing somatic embryos through the process of somatic embryogenesis. Regardless what type of explant was used, the differentiation pathway also relies on the growth

regulators used in the regeneration media. Compounds with the plant hormone cytokinins, such as, BAP, Kn and TDZ promote shoot initiation and development while auxins 2, 4-D and picloram induce somatic embryogenesis.

In Bangladesh, peanut tissue culture techniques have been studied since 1990s. The efficacy of individual explants or genotypes are yet difficult to assess. Such research will help in identifying the best responsive explant and genotype for future use in genetic transformation studies.

1.6 Objectives

Under the current scenario in Bangladesh to meet the demand of peanut cultivation, the present study was conducted to achieve the following objectives:

1. A short review on *in vitro* regeneration protocol of peanuts
2. Determination of the best explant in the two farmer popular varieties of peanut
3. Determination of the best shooting medium for the two peanut varieties
4. Acclimatization of the regenerated plantlets

Chapter – 2: Materials and Methods

CHAPTER – 2

MATERIALS AND METHODS

This study was carried out at the Plant Biotechnology Laboratory of BRAC University. The materials and methods used in this study are described below.

2.1 Materials

2.1.1 Plant material

In this study two varieties of peanut (*Arachis hypogaea* L.) were used. They are:

i. BINA Chinabadam 2
ii. BINA Chinabadam 4

The seeds were collected from Bangladesh Nuclear Institute of Agriculture (BINA), Mymensingh. The seeds were preserved at 4°C temperature in Plant Biotechnology Laboratory, BRAC University, Mohakhali, Dhaka, Bangladesh.

2.1.1.1 BINA Chinabadam 2

The plants of BINA Chinabadam 2 maintain an average height of 28 cm with small leaves that are dark green and ovate in shape. This mutant variety has shiny pods with a maximum yield of 2.29 tons and 3.19 tons in winter and summer, respectively. The maturity period of this variety ranges from 150-160 days in winter and 125-135 days in summer. The seed contains 50% oil and 28% protein. It is moderately resistant to collar rot, *Cercospora sp*. leaf spot and rust diseases.

2.1.1.2. BINA Chinabadam 4

The plants of BINA Chinabadam 4 are intermediate dwarf with upright, lanceolate and dark green leaves. This mutant variety has shiny pods with a maximum yield of 2.47 tons and 2.6 tons in winter and summer, respectively. The maturity period of this variety ranges from 140-150 days in summer and 100-120 days in winter. The seed contains 48.6% oil and 27.5%

protein. This variety is also moderately resistant to collar rot, *Cercospora sp.* leaf spot and rust diseases.

2.1.2. Sterilizing agents

Seeds of both varieties were surface sterilized with 70% ethanol and 0.1% Mercury Chloride.

2.1.3. Germination media

In order to obtain the needed explants, seeds were germinated aseptically. The germination process was carried out in autoclaved flasks containing sterile non-absorbent cotton soaked with sterile distilled water.

2.1.4. Stock solutions for plant regeneration media

In this investigation, Murashige and Skoog (MS) medium (1962) in full or half strength were used for *in-vitro* regeneration. The various constituents of the media were prepared as different stock solutions. The different components for preparation of 1 litre stock solutions in MS media are given in Table 2.1.

2.1.5. Stock solutions for growth regulators

The effect of the following plant growth regulators: 6- Benzyl amino purine (BAP), 6- Furfuryl amino purine (Kinetin/Kn) and Indolebutyric acid (IBA), were evaluated in this study.

Table 2.1: Different components for preparation of 1 litre stock solution in MS media

Composition of Macro 10X		**Composition of Micro 100X**	
Components	**Amount in milligrams/litre**	**Components**	**Amount in milligrams/litre**
KNO_3	1900	KI	0.83
NH_4NO_3	1650	H_3BO_3	6.2
$MgSO_4.2H_2O$	370	$MnSO_4.4H_2O$	22.3
$CaCl_2.2H_2O$	440	$ZnSO_4.7H_2O$	8.6
KH_2PO_4	170	$Na_2MoO_4.2H_2O$	0.25
		$CuSO_4.5H_2O$	0.025
		$CoCl_2.6H_2O$	0.025
Composition of Organic 100X		**Composition of Iron EDTA 100X**	
Components	**Amount in milligrams/litre**	**Components**	**Amount in milligrams/litre**
Nicotinic Acid	0.5	$FeSO_4.7H_2O$	27.8
Pyridoxin HCl	0.5	$Na_2EDTA.2H_2O$	37.3
Thiamine HCl	0.1		
Glycine	2.0		

2.2 Methodology

2.2.1. Database search for review

The search engine Google Search, PubMed and NCBI were used for the review. Relevant journals were obtained from ResearchGate, PeanutScience, Springer, Bangladesh Journals Online, Bangladesh Association for Plant Tissue Culture and Biotechnology, etc. The main keywords were *in vitro* regeneration, tissue culture, peanut, micropropagation, etc.

2.2.2. Preparation of MS medium

MS medium was prepared from the stock solutions listed in Table 2.1. The amounts of using prepared stock solutions for making 1 litre of MS medium are given in Table 2.2:

Table 2.2: Components and their quantities required to prepare 1L basal MS medium

Components	Amount for 1 L full strength MS medium	Amount for 1 L half strength MS medium
Macronutrients (10x)	100 ml	50 ml
Micronutrients (100x)	10 ml	5 ml
Organic (100x)	10 ml	5 ml
Fe-EDTA (100x)	10 ml	5 ml
Sucrose	30.0 gm	30.0 gm
Myo-inositol	0.1 gm	0.1 gm
Agar	8.0 gm	8.0 gm

All the components were carefully measured and taken into a conical flask. If hormone supplements were needed, those solutions were added too. Then, the final volume was made to 1000 ml by adding distilled water. The pH of the media was adjusted to 5.8 by either adding NaOH or HCl. Then, agar was added in 0.8% (w/v) ratio and the media was heated in a microwave oven. Finally, the media was split into several conical flasks or test tubes and were sealed properly with two layers of aluminium foil papers and sterilized by autoclaving at 15psi pressure at 121°C for 20 minutes (Model: CL – 32L, ALP Co., Ltd). The media was cooled and stored at 25±2°C.

2.2.3. Preparation of MS media supplemented with growth regulators

The growth regulators were added to basal MS media in the appropriate quantities before autoclaving the media. In this study, four different hormonal combinations were used for shoot regeneration:

i. MS + 3 mg/l BAP + 1 mg/l Kn
ii. MS + 3 mg/l BAP + 0.5 mg/l Kn
iii. MS + 3 mg/l BAP
iv. MS + 5 mg/l BAP

For root induction, half strength MS supplemented with 0.2 mg/l IBA was used. The vessels were marked with a permanent marker to indicate the hormonal combination used.

The stock solutions of the growth regulators were prepared by measuring 20 mg of hormone on the clean beaker and adding few drops of the appropriate solvent to the powder. The dissolved mixture was then made up to 200 ml in volume by adding double distilled water. This solution was preserved at 4° C for several weeks.

2.2.4. Precaution for aseptic culture

All inoculation and aseptic manipulations were carried out in a laminar air flow cabinet (Streamline, Model: SCV-4AI, Singapore). The cabinet was switched 'ON' along with the UV light for 30 minutes before use. The inner condition of that clean bench was sterilized by UV rays and thoroughly washed by 70% ethanol to overcome the surface contaminants. All the necessary instruments, like, scalpels, forceps, and Petri dishes were sterilized prior to use by the autoclave machine.

During the entire period of inoculation the instruments were kept immersed in absolute alcohol contained in a flask inside the cabinet. At the time of inoculation these instruments were carefully sterilized by flaming method inside the cabinet. Both the hands were rinsed with 70% alcohol. All measures were taken to obtain maximum contamination free condition during the work. Any contaminants and older non-regenerative plant parts were autoclaved before disposal as per bio-safety demands.

2.2.5. Culture techniques

Techniques utilized in this present study are given below:

i. Seed sterilization
ii. Seed germination
iii. Explant designing and culture
iv. Callus induction and shoot regeneration
v. Subculture
vi. Root induction
vii. Acclimatization of *in vitro* grown plantlets

2.2.5.1. Seed sterilization

Seeds were sterilized inside the laminar air flow cabinet. At first the seeds were washed in sterile distilled water three times. After that the seeds were rinsed with 70% ethanol for 1 minute followed by a thorough washing in sterile distilled water. Finally, the seeds were surface sterilized with 0.1% HgCl for 15 minutes. During those 15 minutes, the sterilant was continuously agitated. In order to remove the harsh chemical, the seeds were washed in sterile distilled water thrice.

2.2.5.2. Seed germination

The sterilized seeds were then inoculated in conical flasks containing cotton soaked with sterilized distilled water. Each flask had 10 seeds. The flasks were placed in a dark chamber to ensure proper environment for germination. The seeds were allowed to germinate for 1 or 2 days, depending on the explant type.

2.2.5.3. Explant designing and culture

The germinating seeds of the two varieties, BINA Chinabadam 2 and BINA Chinabadam 4, were used as the source for the explants. Immature embryos were used as explants from 1 and 2 days long emerged seeds. The explants were collected from seeds where germination had just initiated. Four different types of explants were studied in this investigation:

i. Whole decapitated embryo from 1 day old seedling
ii. Half decapitated embryo from 1 day old seedling
iii. Whole decapitated embryo from 2 days old seedling
iv. Half decapitated embryo from 2 days old seedling

In order to prepare the explants, the seed coat was removed and the embryo was carefully detached, using sterile scalpels, from the cotyledons. The plumule and radicle were then discarded and the half decapitated embryos were sliced longitudinally.

These explants were cultured in MS media with different concentrations of hormonal supplements to achieve shoot regeneration. Five conical flasks of the same hormonal treatment were randomly assigned to individual explant types for culture. Each conical flask had 5 explants. All experiments were performed thrice with 30 seeds in every experiment.

2.2.5.4. Callus induction and shoot regeneration

Daily observations were made to keep track of callus and shoot initiation from the explants. Morphogenic changes, such as, callus induction and shoot regeneration, were regularly observed and recorded. Brownish callus was discarded during subculture and when the shoots grew up to a height of approximately 3 cm, they were transferred to the rooting media for rhizogenesis.

2.2.5.5. Subculture

All the *in vitro* cultures were transferred to fresh media of the same hormonal concentration at an interval of 3 weeks.

2.2.5.6. Root induction

To obtain sufficient roots, well developed shoots were placed individually in test tubes containing half strength MS media supplemented with 0.2 mg/l IBA.

2.2.5.7. Acclimatization of *in vitro* plantlets

When the shoots had developed a proper and sufficient rooting system, they were transferred to the soil for acclimatization in nature. The plantlets were taken out of the test tubes and carefully washed under running water to remove any traces of media. Once the roots were clean, they were transferred to plastic cups containing autoclaved soil. The pots were then covered with clear plastic bags and water was sprayed inside the bag to maintain the moisture content.

After 3 weeks, the plastic bags were daily removed and replaced with the duration of removal gradually increasing until they were no longer needed. The pots were watered daily.

Chapter – 3: Results

CHAPTER 3

RESULTS

3.1. A brief review of the *in vitro* regeneration of peanut (*Arachis hypogaea* L.)

The successful exploitation of *in vitro* techniques in plant biotechnology depends on the establishment of efficient regeneration systems. Even though there is a vast amount of literature available for peanut tissue culture, there still exists difficulties in obtaining satisfactory response from explants. Due to the variability of genotypes, the plant growth regulator requirements also vary for callus induction and successful plant regeneration.

This review will focus on research concerned with the tissue culture and *in vitro* regeneration of peanut (*Arachis hypogaea* L.). Attempts to establish a reproducible tissue culture protocol go as early as the 18th century (Harvey and Schulz, 1943). Since then, several protocols have been established for both organogenesis and embryogenesis of peanut.

3.1.1. Explant type

Although the cultivated peanut is still known to be relatively recalcitrant to tissue culture, successful protocols of micropropagation, organogenesis and somatic embryogenesis have been reported from different explant types. Various explants have been used to initiate a regenerable tissue culture in peanut, such as, cotyledonary nodes (Okello *et al*., 2015; McKently, 1990; Iqbal *et al*., 2011; Matand *et al*., 2013; Al Joboury, 2011; Verma *et al*., 2009; Venkatachalam *et al*., 1996; Alam and Khaleque, 2010; Farhat *et al*., 2011; Radhakrishnan *et al*., 2000; Li *et al*., 1994; Ozias-akins, 1989), immature leaflets (Cheng *et al*., 1992; Zhao *et al*., 2012; Venkatachalam *et al*., 1998; Tiwari and Tuli, 2009; Li *et al*., 1994; Chowdhury, 2014), mature leaflets (Gill and Saxena, 1992; Baker and Wetzstein, 1992; Chengalrayan *et al*., 1998; Chengalrayan and Gallo-Meagher, 2004; Pittman *et al*., 1983), epicotyl (Cheng *et al*., 1992; Venkatachalam *et al*., 1996, 1998; Shan *et al*., 2009; Alam and Khaleque, 2010) and hypocotyl sections (Venkatachalam *et al*., 1996, 1998; Li *et al*., 1994; Alam and Khaleque, 2010), embryo axes (McKently *et al*., 1990; McKently, 1991; Radhakrishnan *et al*., 2000; Li *et al*., 1994; Singh and Hazra, 2009; Shan *et al*., 2009; Venkatachalam *et al*., 1996), shoot tips

(Heatley and Smith, 1996; Eapen *et al*., 1998; Ozudogru *et al*., 2005; Ozudogru and Lambardi, 2012), root tissue (Matand *et al*., 2013) and mature whole seeds (Radhakrishnan *et al*., 2000; Li *et al*., 1994).

In all the literature reviewed, Li *et al*. (1994) investigated the maximum number of different types of explants and reported that the half seed without the plumule or the radicle is the best explant as it produced the most number of shoots per explant. This article further reported that the removal of either the plumule or the radicle results in a greater number of shoots compared to an intact whole seed. Other investigations that focused on a number of different explants reported that the frequency of shoot formation was highest from cotyledonary sections (McKently *et al*., 1990; Matand *et al*., 2013; Venkatachalam *et al*., 1996). The proximal end of a diced cotyledon and leaflet is more likely to produce a higher number of shoots than the distal region (Matand *et al*., 2013; Mroginski *et al*., 1980). However, Radhakrishnan *et al*. (2000) found that the embryo axes is a better explant than the cotyledonary sections.

Matand *et al*. (2013) reported the first successful use of root tissue to induce direct adventitious plant formation. Upon investigation of the epicotyl and hypocotyl sections, it was found that the epicotyl section is better than the hypocotyl section for shoot formation but the hypocotyl section is better than the epicotyl for root formation (Venkatachalam *et al*., 1998; Alam and Khaleque, 2010). Seed explants, such as, embryo axes (McKently *et al*., 1990; McKently, 1991) and shoot tip (Heatley and Smith, 1996; Ozudogru *et al*., 2005) were utilized for organogenesis successfully. From investigating the somatic embryogenesis of embryo axes explant, Singh and Hazra (2009) demonstrated that by changing the orientation of the explant, the determined organogenic cells can be made embryogenic. Immature leaflets were found to be the most efficient explants for callogenesis among the explants studied by Venkatachalam *et al*. (1996). Cheng *et al*. (1992) reported that the leaflet segments and petiole sections are less responsive for shoot formation.

Regeneration capacity depends on the size, type and age of an explant as well as its degree of differentiation and how it is implanted in the medium. The induction rate of shoots have been reported to decrease significantly with the use of aged seedlings (Shan *et al*., 2009).

In peanuts, the cotyledonary and leaf sections have been the explants of choice thus far, mainly due to their high potential in producing adventitious shoots (Gill and Saxena, 1992). To induce somatic embryogenesis, mature leaflet has been the most frequently used explant (Baker and Wetzstein, 1992; Chengalrayan *et al*., 1998; Chengalrayan and Gallo-Meagher, 2004).

3.1.2. Plant growth regulators

Plant tissue culture is generally dependent for its success on the inclusion of plant growth regulators. Auxins, cytokinins and auxin-cytokinin interactions are usually considered to be the most important for regulating growth and development in plant tissue and organ cultures (Gaspar *et al*., 1996). The reported ratios of plant growth regulators resulting in the differentiation and proliferation differ markedly among the many studies, indicating that the results are strongly influenced by both explant type and genotype.

Auxins exert a strong influence over processes, such as, cell growth expansion, cell wall addification, initiation of cell division and organization of meristems giving rise to either callus or defined organs, generally roots. Cytokinins are useful in cell culture for stimulation of all division, release of lateral bud dormancy and induction of adventitious bud formation. Cell division is regulated by the combined action of auxins and cytokinin, each of which influences different phases of the cell cycle (Gaspar *et al*., 1996). Thus, the levels of these phytohormones in cultures need to be carefully balanced and controlled.

In tissue culture of peanut, various levels and combinations of hormones have been utilized to induce callogenesis, shoot and root regeneration as well as embryogenesis.

The most commonly used hormone for callus induction is 2, 4- D (Okello *et al*., 2015; Iqbal *et al*., 2011; Alam and Khaleque, 2010). At concentrations ranging from 2-5 mg/l, 2, 4- D is capable of producing creamy, white, friable callus (Iqbal *et al*., 2011). NAA has also been used for callus induction (Alam and Khaleque, 2010; Farhat *et al*., 2011; Hazra *et al*., 1989) and successful callogenesis was achieved at 1 mg/l NAA (Farhat *et al*., 2011). However, callus growth is slower in the presence of NAA compared to 2, 4- D (Alam and Khaleque, 2010). When used in combination with a cytokinin, such as, Kn, the callus formation can be improved. Venkatachalam *et al*. (1996) reported that high levels of auxin and low levels of cytokinin is the most effective for producing callus. Increasing concentrations of cytokinins, such as, BAP, results in a decrease in callus formation (Farhat *et al*., 2011; Gill and Saxena, 1992).

Among the factors that influence growth, the type of cytokinin, such as, BAP or TDZ, is critical for initiation of shoot primordia from cultured explants (Shan *et al*., 2009). In all the literature reviewed, the most commonly used hormone for shoot development was BAP alone (Okello *et al*., 2015; Gill and Saxena, 1992; McKently *et al*., 1990; Iqbal *et al*., 2011; Matand *et al*., 2013; Zhao *et al*., 2012; Verma *et al*., 2009; Alam and Khaleque, 2010; Radhakrishnan *et al*., 2000; Heatley and Smith, 1996; Chowdhury, 2014) or in combination with another hormone (Al-

Joboury, 2012; Venkatachalam *et al*., 1996; Chowdhury, 2014; Okello *et al*., 2015; Cheng *et al*., 1992; Venkatachalam *et al*., 1998; Farhat *et al*., 2011; Tiwari and Tuli, 2009; Chengalrayan and Gallo-Meagher, 2004; Ozudogru and Lambardi, 2012; Pittman *et al*., 1983; Mroginski *et al*., 1981; Iqbal *et al*., 2011; Eapen *et al*., 1998; Shan *et al*., 2009; Ozudogru *et al*., 2005).

A combination of auxin and cytokinin was found to be suitable for several cultivars of peanut. A satisfactory level of shoot bud regeneration was reported from using BAP along with IAA (Okello *et al*., 2015; Iqbal *et al*., 2011; Venkatachalam *et al*., 1998). Use of BAP along with NAA produced the highest number of shoots per explant in many studies (Okello *et al*., 2015; Cheng *et al*., 1992; Venkatachalam *et al*., 1998; Farhat *et al*., 2011; Tiwari and Tuli, 2009; Chengalrayan and Gallo-Meagher, 2004; Ozudogru and Lambardi, 2012; Pittman *et al*., 1983; Mroginski *et al*., 1981; Eapen *et al*., 1998; Shan *et al*., 2009). This combination of hormones is capable of producing greater bud primordia and greater number of shoots (Cheng *et al*., 1992). A satisfactory level of shoot induction has also been observed in using BAP in concert with Kn (Al-Joboury, 2012; Venkatachalam *et al*., 1996; Chowdhury, 2014). A higher level of BAP than Kn was used in all three studies.

Using BAP singly results in multiple shoot production (McKently *et al*., 1990). McKently *et al*. (1990) reported that average number of shoots initially increased with increasing concentrations of BAP but decreased after the concentration reached 40 mg/l. Even though the shoot number has been found to increase with higher levels of BAP, it has also been reported that a high concentration of this cytokinin results in a lower shoot length (Verma *et al*., 2009; Eapen *et al*., 1998).

Other cytokinins, such as, TDZ and Kn were also utilized to study their effect in shoot development (Matand *et al*., 2013; Zhao *et al*., 2012; Li *et al*., 1994; Shan *et al*., 2009). Matand *et al*. (2013) reported a greater number of shoot primordia in media containing higher levels of TDZ. However, in general, BAP has been considered as a better hormone for shoot induction and development than Kn (Venkatachalam *et al*., 1996) and TDZ (Zhao *et al*., 2012). Use of auxins alone for shoot regeneration has not been reported as successful so far (Alam and Khaleque, 2010; Okello *et al*., 2015; Iqbal *et al*., 2011).

In micropropagation, there is plenty of evidence showing that control of the gaseous phytohormone (ethylene) biosynthesis and control of the gaseous environment within the culture vessels can improve adventitious bud induction (Ozudogru *et al*., 2005). Among the various compounds that have been widely utilized in these studies to promote or inhibit

ethylene biosysthesis or to counteract its action, silver nitrate ($AgNO_3$) is one that has often proved to influence *in vitro* shoot development and multiplication of different herbaceous species including *Brassica campestis* (Chi and Pua, 1989), *Capsicum annuum* (Hyde and Philips, 1996) and *Vanilla planifolia* (Giridhar *et al*., 2001). Similarly, silver nitrate has a positive effect on shoot tip proliferation and shoot elongation of *in vitro* grown peanut (Ozudogru and Lambardi, 2012). Combining silver nitrate with phytohormones also results in low callus proliferation (Ozudogru *et al*., 2005).

In a few studies, adventitious root formation was seen in hormone free medium (Ozudogru *et al*., 2005; Radhakrishnan *et al*., 2000; Mroginski *et al*., 1981) but rooting has been more successful with the use of an auxin (Heatley and Smith, 1996). Thus, in most studies, a low level of NAA was added to the culture medium to produce roots (Cheng *et al*., 1992; McKently *et al*., 1990; Iqbal *et al*., 2011; Verma *et al*., 2009; Heatley and Smith, 1996; Li *et al*., 1994; Chowdhury, 2014; Mroginski *et al*., 1981; Rossi Jaume & Cucco, 2000; Eapen *et al*., 1998; Shan *et al*., 2009). Increasing concentration of NAA stimulates root formation and increasing concentration of BAP impedes root formation (Mroginski *et al*., 1981.On the other hand, Shan *et al*. (2009) reported that root formation is best on lower concentrations of NAA. A higher concentration produces a short and thick root system that lacks lateral roots.

3.1.3. Morphogenic pathway

Plant regeneration from cultures occurs via organ differentiation or somatic embryogenesis. Regeneration of plants from callus cultures often show genetic variability; direct somatic embryogenesis from organs can be more efficiently used for application of biotechnology in the improvement of crops (Hazra *et al*., 1989).

2, 4- D has been widely used for inducing somatic embryos in tissue culture of peanut (Iqbal *et al*., 2011; Zhao *et al*., 2012; McKently, 1991; Hazra *et al*., 1989; Chengalrayan *et al*., 1998; Chengalrayan and Gallo-Meagher, 2004; Ozias-Akins, 1989; Singh and Hazra, 2009; Rossi Jaume & Cucco, 2000). Singh and Hazra (2009) reported that by monitoring the exposure of the explants in 2, 4- D, it is possible to obtain both types of morphogenic activity in two parts of the same explant. Rossi Jaume & Cucco (2000) investigated the effect of various plant growth regulators on producing somatic embryos, including BAP, Picloram, DMA and 2, 4- D and concluded that only 2, 4- D was capable of producing somatic embryos.

Ozias-akins (1989) also investigated the role of various hormones on embryogenesis and found that instead of 2, 4- D, NAA induced the formation of normal embryos which were more easily converted into plants. This literature also reported that the response of explants can be improved by using Picloram. The same result was reflected by Iqbal *et al*. (2011) who found that using 19 mg/l Picloram is the most efficient for somatic embryogenesis. Successful embryogenesis was achieved using other hormones as well, including 2, 4, 5- T (McKently, 1991), TDZ (Gill and Saxena, 1992) and a combination of Kn and 2,4 D (Baker and Wetzstein 1992), In general, auxins seem to play a more efficient role in somatic embryogenesis than cytokinins.

3.2. An investigation of the *in vitro* regeneration of peanut

In light of the previous studies, *in vitro* regeneration was studied for two farmer popular peanut varieties, namely, BINA Chinabadam 2 and BINA Chinabadam 4. During this investigation, experiments were performed using two explants: decapitated whole embryo and decapitated half embryo. Shooting and rooting capability of the explants was observed using various hormonal concentrations and combinations. Shooting was accomplished via cytokinins BAP and Kn and rooting was accomplished via auxin IBA. The experiments were repeated to establish reproducibility of the results. Finally, the regenerated plantlets were acclimatized in sandy soil for proper hardening.

3.2.1. Aseptic seed germination

The germination rate and the days required for the seeds to germinate was observed and recorded for the two varieties. BINA Chinabadam 2 had a lower germination rate and it also took more time to germinate compared to BINA Chinabadam 4. 97% of the seeds of BINA Chinabadam 4 germinated in 2 days whereas only 66.7% of the seeds of BINA Chinabadam 2 in 5 days. The germination response of the two varieties is presented in Table 3.1.

Table 3.1: Germination response of two varieties of peanut following sterilization

Variety	No. of seeds germinated *in vitro*	Percentage of germinated seeds	Time taken for germination (days)
BINA Chinabadam 2	29	97	2
BINA Chinabadam 4	20	66.7	5

3.2.2. Regeneration response of two peanut varieties

Whole and half decapitated embryos of two different ages, 1 and 2 days old, were examined to find their shoot regeneration response. All data presented in the following tables were recorded on the 18th day post inoculation.

During this investigation, both direct and indirect regeneration was observed in both the peanut varieties in response to various concentrations and combinations of cytokinins. All the explants of both varieties showed 100 percent response rate. Shoot formation was also seen in all the various hormonal supplements.

3.2.2.1. Effect of different concentrations of BAP and Kn on the shoot formation of BINA Chinabadam 2

In case of BINA Chinabadam 2, regeneration response in MS medium supplemented with BAP with or without Kn was observed.

For decapitated whole embryo, all the explants responded in the various hormonal supplements but the shoot regeneration rate decreased for all the hormonal combinations used. The percentage of shoot regeneration varied from 65 to 80 in case of 1 day old decapitated whole embryo and 65 to 90 in case of 2 days old decapitated whole embryo. The average number of shoots recorded in each culture was the highest when 3 mg/l BAP and 0.5 mg/l Kn was used together for the 1 day old decapitated whole embryo explant and when 3 mg/l BAP and 1 mg/l Kn was used for 2 days old whole decapitated embryo explants. (Table 3.2) (Figure 3.1)

In case of decapitated half embryo, the explant responsiveness was decreased to 95 percent when 5 mg/l BAP was used singly. The percentage of shoot regeneration varied from 60 to 80 in case of 1 day old decapitated half embryo and 47 to 70 in case of 2 days old decapitated half embryo. The average number of shoots recorded in each culture was highest for 1 day old decapitated half embryo when 3 mg/l BAP and 1 mg/l Kn was used and when 3 mg/l BAP was used alone for 2 days old decapitated half embryo. (Table 3.3) (Figure 3.1)

For all explant types of BINA Chinabadam 2, hormonal combination of 3 mg/l BAP and 1 mg/l Kn was seen to give the highest rate of shoot regeneration from explants and the lowest rates were seen in cultures containing 5 mg/l BAP alone. The average number of shoots was also higher when the cytokinins were used in combination.

Table 3.2: Effect of different concentrations and combinations of BAP and Kn on regeneration BINA Chinabadam 2 from decapitated whole embryo

Age of Explant	Plant Hormones (mg/l)	Responsive Explants (%) ± SD	Shoot regenerating explants (%) ± SD	Mean shoots per flask ± SD
Day 1	BAP 3.0 + Kn 1.0	100 ± 0	80 ± 0	3.33 ± 0.58
Day 1	BAP 3.0 + Kn 0.5	100 ± 0	76 ± 0.32	3.50 ± 1.29
Day 1	BAP 3.0 + Kn 0	100 ± 0	70 ± 0.26	2.50 ± 1.29
Day 1	BAP 5.0 + Kn 0	100 ± 0	65 ± 0.19	3.50 ± 2.12
Day 2	BAP 3.0 + Kn 1.0	100 ± 0	90 ± 0.14	4.00 ± 1.41
Day 2	BAP 3.0 + Kn 0.5	100 ± 0	80 ± 0.31	2.75 ± 0.23
Day 2	BAP 3.0 + Kn 0	100 ± 0	80 ± 0.20	3.67 ± 2.05
Day 2	BAP 5.0 + Kn 0	100 ± 0	65 ± 0.10	3.25 ± 0.50

Table 3.3: Effect of different concentrations and combinations of BAP and Kn on regeneration BINA Chinabadam 2 from decapitated half embryo explants

Age of Explant	Plant Hormones (mg/l)	Responsive Explants (%) ± SD	Shoot regenerating explants (%) ± SD	Mean shoots per flask ± SD
Day 1	BAP 3.0 + Kn 1.0	100 ± 0	80 ± 0	4.50 ± 0.71
Day 1	BAP 3.0 + Kn 0.5	100 ± 0.1	80 ± 0.26	2.33 ± 1.49
Day 1	BAP 3.0 + Kn 0	100 ± 0	60 ± 0.14	4.00 ± 1.41
Day 1	BAP 5.0 + Kn 0	95 ± 0.1	60 ± 0.16	3.25 ± 1.71
Day 2	BAP 3.0 + Kn 1.0	100 ± 0	70 ± 0.27	2.33 ± 0.52
Day 2	BAP 3.0 + Kn 0.5	100 ± 0	53 ± 0.12	1.0 ± 0
Day 2	BAP 3.0 + Kn 0	100 ± 0	70 ± 0.43	3.00 ± 1.73
Day 2	BAP 5.0 + Kn 0	93 ± 0.11	47 ± 0.31	2.00 ± 0

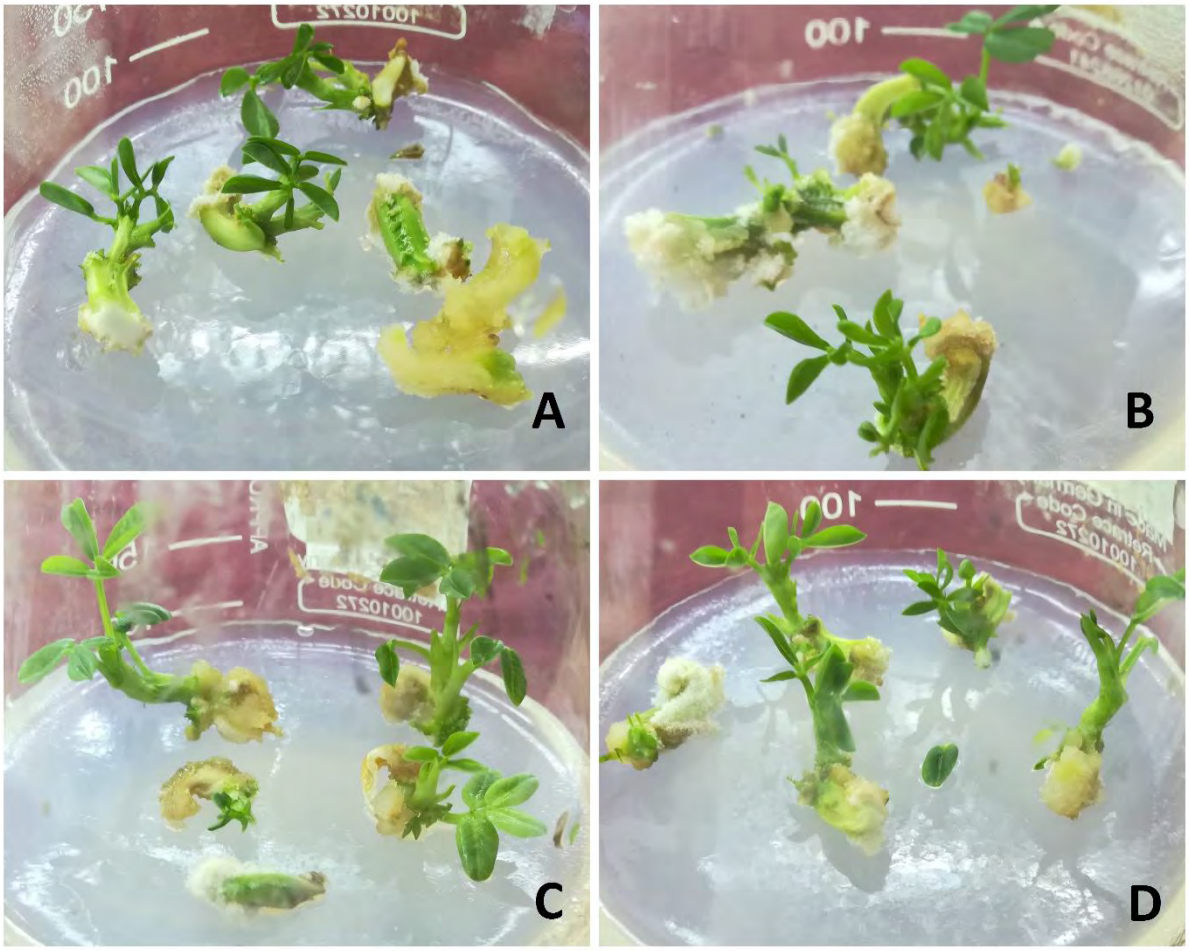

Fig 3.1: Regeneration of BINA Chinabadam 2 in MS medium with 3 mg/l BAP and 1 mg/l Kn on day 18 since inoculation of A. 1 day old whole decapitated embryo B. 2 days old whole decapitated embryo C. 1 day old half decapitated embryo D. 2 days old half decapitated embryo.

3.2.2.2. Effect of different concentrations of BAP and Kn on the shoot formation of BINA Chinabadam 4

The hormonal supplements applied to BINA Chinabadam 2 was followed for BINA Chinabadam 4 as well.

For decapitated whole embryo, all the explants showed a 100 percent regeneration capacity in all the different hormonal combinations and concentrations used. However, all the responsive explants did not form shoots. The percentage of shoot regenerating explants varied from 45 to 80 for 1 day old decapitated whole embryo and 35 to 80 for 2 days old decapitated whole embryo. The average number of shoots recorded in each culture was highest for 1 day old

decapitated whole embryo when 3 mg/l BAP and 0.5 mg/l Kn was used and when 3 mg/l BAP and 1 mg/l Kn was used for 2 days old decapitated whole embryo. (Table 3.4) (Figure 3.2)

In case of decapitated half embryo, the percentage of responsive explants was lower when BAP was used without Kn. The percentage of shoot regenerating explants varied from 50 to 85 for 1 day old decapitated half embryo and from 55 to 75 for 2 days old decapitated half embryo. The average number of shoots recorded in each culture was highest for both 1 and 2 days old decapitated whole embryo when 3 mg/l BAP and 0.5 mg/l Kn was used together. (Table 3.5) (Figure 3.2)

Overall for BINA Chinabadam 4, the hormonal combination of 3 mg/l BAP and 0.5 mg/l Kn was seen to give the best response for all explant types both in case of shoot regeneration rate from explants and average number of shoots.

Table 3.4: Effect of different concentrations and combinations of BAP and Kn on regeneration BINA Chinabadam 4 from decapitated whole embryo

Age of Explant	Plant Hormones (mg/l)	Responsive Explants (%) ± SD	Shoot regenerating explants (%) ± SD	Mean shoots per flask ± SD
Day 1	BAP 3.0 + Kn 1.0	100 ± 0	45 ± 0.12	2.05 ± 0.80
Day 1	BAP 3.0 + Kn 0.5	100 ± 0	80 ± 0	4.05 ± 1.04
Day 1	BAP 3.0 + Kn 0	100 ± 0	70 ± 0.14	1.75 ± 0.98
Day 1	BAP 5.0 + Kn 0	100 ± 0	67 ± 0.42	2.75 ± 0.54
Day 2	BAP 3.0 + Kn 1.0	100 ± 0	80 ± 0.29	5.10 ± 0.95
Day 2	BAP 3.0 + Kn 0.5	100 ± 0	75 ± 0.12	3.55 ± 1.92
Day 2	BAP 3.0 + Kn 0	100 ± 0	60 ± 0.24	1.75 ± 0.49
Day 2	BAP 5.0 + Kn 0	100 ± 0	35 ± 0.53	2.25 ± 0.67

Table 3.5: Effect of different concentrations and combinations of BAP and Kn on regeneration BINA Chinabadam 4 from decapitated half embryo explants

Age of Explant	Plant Hormones (mg/l)	Responsive Explants (%) ± SD	Shoot regenerating explants (%) ± SD	Mean number of shoots ± SD
Day 1	BAP 3.0 + Kn 1.0	100 ± 0	50 ± 0.30	1.95 ± 0.76
Day 1	BAP 3.0 + Kn 0.5	100 ± 0	85 ± 0	3.75 ± 1.60
Day 1	BAP 3.0 + Kn 0	95 ± 0.1	65 ± 0.2	2.00 ± 1.35
Day 1	BAP 5.0 + Kn 0	95 ± 0.1	70 ± 0.11	2.75 ± 0.26
Day 2	BAP 3.0 + Kn 1.0	100 ± 0	55 ± 0.84	3.00 ± 0.54
Day 2	BAP 3.0 + Kn 0.5	100 ± 0	75 ± 0.46	4.00 ± 1.41
Day 2	BAP 3.0 + Kn 0	100 ± 0	60 ± 0.16	2.00 ± 1.02
Day 2	BAP 5.0 + Kn 0	95 ± 0.1	60 ± 0.32	1.75 ± 1.23

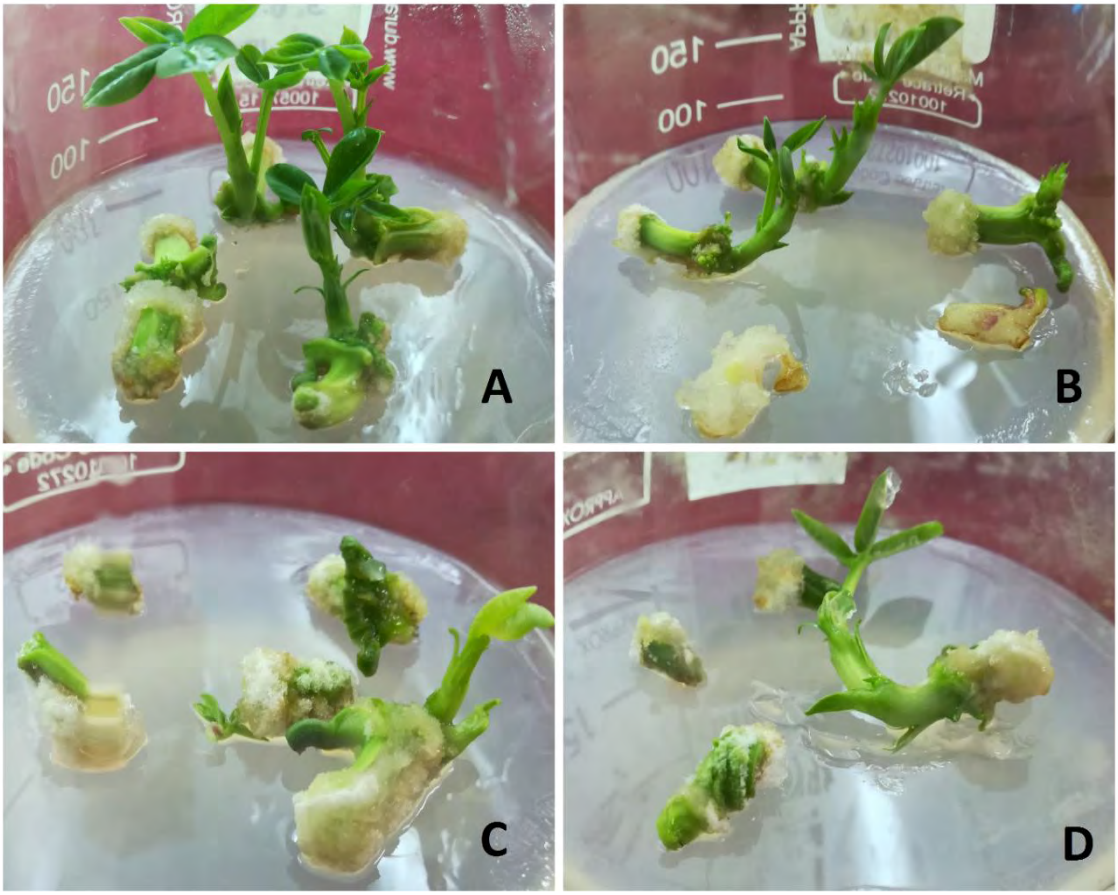

Fig 3.2: Regeneration of BINA Chinabadam 4 in MS medium with 3 mg/l BAP and 0.5 mg/l Kn on day 18 since inoculation of A. 1 day old whole decapitated embryo B. 2 days old whole decapitated embryo C. 1 day old half decapitated embryo D. 2 days old half decapitated embryo.

3.2.2.3. Effect of explant type on the shoot length of the regenerated plantlets

The average shoot lengths of the plantlets of all explant types grown in hormonal combination of 3 mg/l BAP and 1 mg/l Kn are shown in Table 3.6. Overall, it was seen that 2 days old explants produce longer shoots than 1 day old explants. Decapitated whole embryo that was allowed to germinate for 2 days produced the longest shoots for both varieties. The shoot length of 2 days old decapitated whole embryo of BINA Chinabadam 2 was 3.38 cm and 3.79 cm in case of BINA Chinabadam 4.

Table 3.6: Mean shoot length of BINA Chinabadam 2 and BINA Chinabadam 4 cultured in 3 mg/l BAP and 1 mg/l Kn

Variety	Explant type	Age of explant (Days)	Mean shoot length (cm) ± SD
BINA Chinabadam 2	Decapitated whole embryo	1	2.73 ± 0.31
		2	3.38 ± 0.37
	Decapitated half embryo	1	3.28 ± 0.25
		2	3.18 ± 0.17
BINA Chinabadam 4	Decapitated whole embryo	1	3.35 ± 0.28
		2	3.79 ± 3.33
	Decapitated half embryo	1	3.33 ± 0.11
		2	3.55 ± 0.45

3.2.3. Induction of roots from regenerated shoots

Formation of a healthy rooting system is a crucial step in plantlet formation. Spontaneous *in vitro* root induction was seen in the callus tissue of two explants of BINA Chinabadam 4 prior to shoot differentiation. Therefore, after the shoots had grown at least 3 centimetres long, they were excised at the base and cultured on half strength MS media supplemented with 0.2 mg/l IBA.

No significant difference was observed during rhizogenesis among the two varieties. Both varieties, BINA Chinabadam 2 and BINA Chinabadam 4, successfully grew roots in 0.2 mg/l IBA. The type of explant or the hormonal concentration used during shooting also seemed to have no effect on rhizogenesis.

Roots emerged from the cut end of the shoots in all cultures within 15-20 days. The roots were white, long and slender (Figure 3.3). Multiple roots were formed in most cultures and some cultures also had branched roots. Some cultures formed white, friable callus at the base of the excision before forming roots. It took approximately 30 days for the plantlets to form a healthy rooting system that was suitable for transplantation in soil.

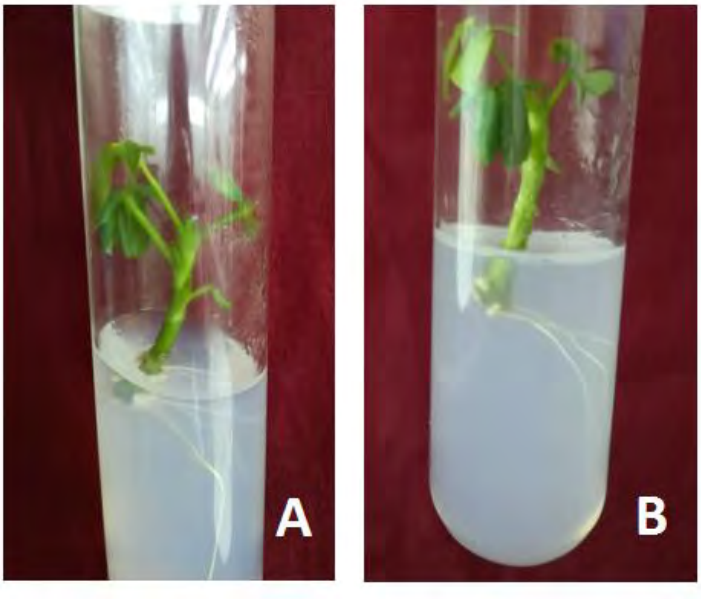

Fig 3.3: Rhizogenesis and root development of A. BINA Chinabadam 2 B. BINA Chinabadam 4.

3.2.4. Transplantation and acclimatization in nature

After establishment of a healthy and sufficient rooting system, the plantlets were transferred to the soil for acclimatization in nature. Most of the plantlets survived the acclimatization phase. Since transplantation, it took two months for the plantlets to mature into healthy and well developed plants and after two more months, the plants set flowers (Figure 3.4).

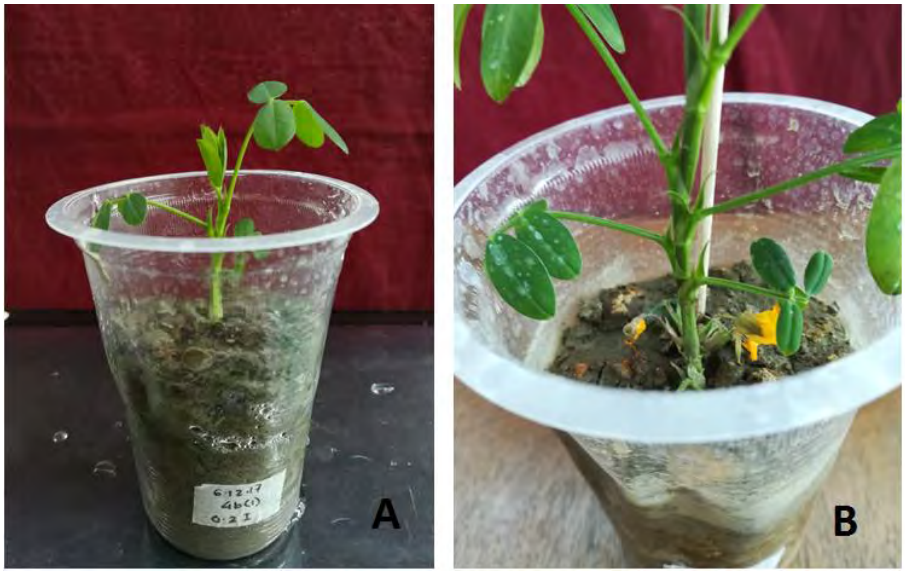

Fig 3.4: A. Tissue culture regenerated plantlet of BINA Chinabadam 4 B. Flowering of *in vitro* grown BINA Chinabadam 2.

Chapter – 4: Discussion

CHAPTER 4

DISCUSSION

Peanut is a legume of economic importance, whose improvement could greatly benefit from the integration of both classical and modern techniques. The economic importance of the crop is mainly due to its relatively high protein and oil content of its seeds (Ozias-akins, 1989). However, its narrow germplasm base, with unsatisfactory resistance to diseases and to several major pathogens, leads to reductions and great economic losses annually (Stalker, 1997). Hence, the development of genetic transformation systems, for example the introduction of resistance or stress tolerance genes, and the optimization of efficient *in vitro* regeneration procedures, would be of great benefit to peanut as it would increase yields and improve the quality of the seed.

Attempts to develop an efficient protocol for *in vitro* regeneration of peanut has been going on since the 18th century (Harvey and Schulz, 1943). Since then several attempts have been reported that investigate the organogenesis (Cheng *et al*., 1992; McKently *et al*., 1990; Matand *et al*., 2013; Al-Joboury, 2011; Verma *et al*., 2009; Venkatachalam *et al*., 1996, 1998; Alam and Khaleque, 2010; Farhat *et al*., 2011; Tiwari and Tuli, 2009; Radhakrishnan *et al*., 2000; Ozudogru and Lambardi, 2013; Li *et al*., 1994; Pittman *et al*., 1983; Chowdhury, 2014; Eapen *et al*., 1998; Shan *et al*., 2009) and embryogenesis (Rossi Jaume and Cucco, 2000; Ozias-akins, 1989; Chengalrayan *et al*., 1998; Chengalrayan and Gallo-Meagher, 2004; Hazra, 1989; Baker and Wetzstein, 1992; McKently, 1991; Iqbal *et al*., 2011; Zhao *et al*., 2012; Okello *et al*., 2015; Gill and Saxena, 1992) of peanut. There are also reports on transgenic peanuts using these regeneration systems (Higgins and Dietzgen, 2000; Sarker *et al*., 2003; Sharma *et al*., 2000; Anuradha *et al*., 2006; Tiwari and Tuli, 2011; Sarker and Islam, 1999, 2000).

Considering this background, this present study was carried out to review the literature available for peanut tissue culture so far and to develop an efficient and reproducible regeneration protocol for two farmer popular Bangladeshi peanut varieties. The tissue culture phase of the study involved finding out the best regeneration response of two explants in various combinations and concentrations of plant growth regulators.

In terms of maximum shooting response, the hormonal combination of 3 mg/l BAP along with 1 mg/l Kn gave the best response for BINA Chinabadam 2 and the hormonal combination of 3 mg/l BAP along with 0.5 mg/l Kn gave the best response for BINA Chinabadam 4. This

difference in response is on account of the genotypic variance of peanut. Similar results were reported in Chengalrayan *et al*. (1998) where 16 genotypes of *Arachis hypogaea* were investigated. The study reported that although the same protocol was effective for all the genotypes, the variation in frequency of response at each stage of development indicate that, with the exception of root meristem differentiation and subsequent radicle emergence, the whole process depends on the genetic constitution of the original plant. The effect of BAP together with Kn was also studied by Chowdhury (2014), Al-Joboury (2011) and Venkatachalam *et al*. (1996). In all three studies, a satisfactory level of shooting frequency was reported. Earlier reports with Bangladeshi peanut varieties showed that a higher concentration of BAP alone (5 mg/l) produced the best result (Sarker and Islam, 1997, 1999, 2000). Therefore, the effect of 3 mg/l and 5 mg/l BAP was also investigated in the current study. However, the frequency of shoot regenerating explants was lower in these cultures compared to cultures utilizing both BAP and Kn. This result was in accordance with Chowdhury (2014). Increasing the concentration of BAP also produced smaller shoots, compared to the combination of cytokinins (data not shown). This finding reflects that of Verma *et al*. (2009) and Eapen *et al*. (1998).

In previous investigations, the embryo was found to be a prominent explant since the presence of the embryo axes shows a better regeneration capability than cotyledon segments (Radhakrishnan *et al*., 2000; Chowdhury, 2014). Li *et al*. (1994) reported that the removal of either the plumule or radicle results in a greater number of shoots. According to such findings, the apical part and root cap was detached to obtain the decapitated whole embryo and it was halved longitudinally to obtain the decapitated half embryo. Overall, decapitated whole embryo produced a greater frequency of shoots than decapitated half embryo for both varieties.

Spontaneous root regeneration did not occur in the regenerated shoots. Thus, half strength MS consisting of 0.2 mg/l IBA was utilized to induce roots. This concentration of auxin was found to be optimal for root induction by Chowdhury (2014), where the same varieties were investigated.

After root induction, the regenerated plantlets were transferred to soil for acclimatization. The plant morphology was routinely examined. In natural course of time, the plantlets set to form flowers which were similar in size and colour to the control. However, it took longer for the regenerated plantlets to set flowers compared to the control. This may be on account of the fact

that plant cells in culture are in a stressful environment in comparison to the highly integrated and balanced environment of the whole plant (Eapen *et al*., 1998).

Plant regeneration in this study was achieved largely via direct organogenesis. The protocol for *in vitro* plant regeneration from decapitated embryo of peanut (*Arachis hypogaea* L.) described here can provide an experimental system for genetic transformation studied using *Agrobacterium*-mediated gene transfer. As this regeneration system does not involve an intermediary callus stage, the likelihood of regenerating genetically normal plants should be high (Cheng *et al*., 1992).

Chapter – 5: References

CHAPTER - 5

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