Determination of an Efficient Regeneration Protocol for Bangladeshi Peanut Varieties (*Arachis hypogaea* L.)

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Dedicated

To

My beloved parents

DECLARATION

I hereby declare that the research work embodying the results reported in this thesis entitled "**Determination of an Efficient Regeneration Protocol for Bangladeshi Peanut Varieties (*Arachis hypogaea* L.)"** submitted by the undersigned has been carried out under supervision of Dr. Aparna Islam, Associate Professor, 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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Abstract

Peanut is an economically important oilseed crop with high nutrition value. This plant is imposing several yield constrains like biotic and abiotic stresses. However, fungal diseases are mostly seen. Like other legumes, it has physiological barriers in traditional hybridization in such case "tissue culture regeneration " offers a potential alternative for improving production in quantity and quality of this plant. Several attempts with various explants have already revealed in past few years but an efficient protocol with case by case study is yet to be revealed for popular Bangladeshi peanut varieties. In this study, the role and interaction of the most promising explant sources; effect of different growth hormones in different combination and concentration have been investigated for six farmer popular Bangladeshi cultivar to find out an efficient transformation amenable regeneration protocol of *Arachis hypogaea* . In this experiment, BAP singly gave indirect regeneration and BAP combined with kinetin gave direct regeneration from leaflet explant, 5 mg/l BAP with and without 0.5 mg/l Kn showed multiple shoots in lower length in case of BARI Chinabadam-7 and BARI Chinabadam-8. However, 2.00 mg/l BAP with and without 0.5 mg/l Kn showed multiple shoots in lower length in case of Dhaka Chinabadam-1, BINA Chinabadam-2, BINA Chinabadam-4, BINA Chinabadam-6 varieties. The another promising explant half decapitate embryo, gave rapid multiple longer shoots in 3.00 mg/l BAP and 1.00 mg/l Kinetin combined media for most of the cases. Among all the varieties BINA Chinabadam-6 was resulted to be best among all the varieties. At the stage of rooting, three root induction hormone were tried - IBA, IAA, NAA and the concentration was about 0.1 and 0.2 mg/l; among them 0.2 mg/l IBA shows better root formation and highest survival rate at hardening stage. Seasonal impact on rooting was also studied where January to June is the suitable period where September to November, no rhizogenesis was occurred. BINA Chinabadam-4 and BINA Chinabadam-6 gave the higher number of pod formation then the other.

Content

Abbreviations

The following abbreviations have been used throughout the text.

BARI	**Bangladesh Agricultural Research Institute**
BAP	**6-Benzylaminopurine**
BINA	**Bangladesh Institute of Nuclear Agriculture**
cm	**Centimeter**
gm	**Gram**
FAO	**Food and Agriculture Organization**
Fig	**Figure**
HCL	**Hydrochloric Acid**
IAA	**Indole-3 Acetic Acid**
IBA	**Indole-3 Butyric Acid**
Kg	**Kilogram**
Kn	**6- furfurylaminopurine**
ml	**Milliliter**
MS	**Murashige and Skoog (1962) medium**
NAA	**Napthalene acetic Acid**
NAOH	**Sodium Hydroxide**
NA_2-EDTA	**Sodium salt of Ferric Ethylene Diamine tera Acetate-log [H+]**

Chapter: 1

Introduction

Introduction

Peanut, *Arachis hypogaea* L. belongs to the largest plant family Fabaceae, which is commonly known as peanut or groundnut. Some other names are also present like, earthnuts, goober peas, monkey nuts, pygmy nuts or pignuts.

1.1 Description of peanut

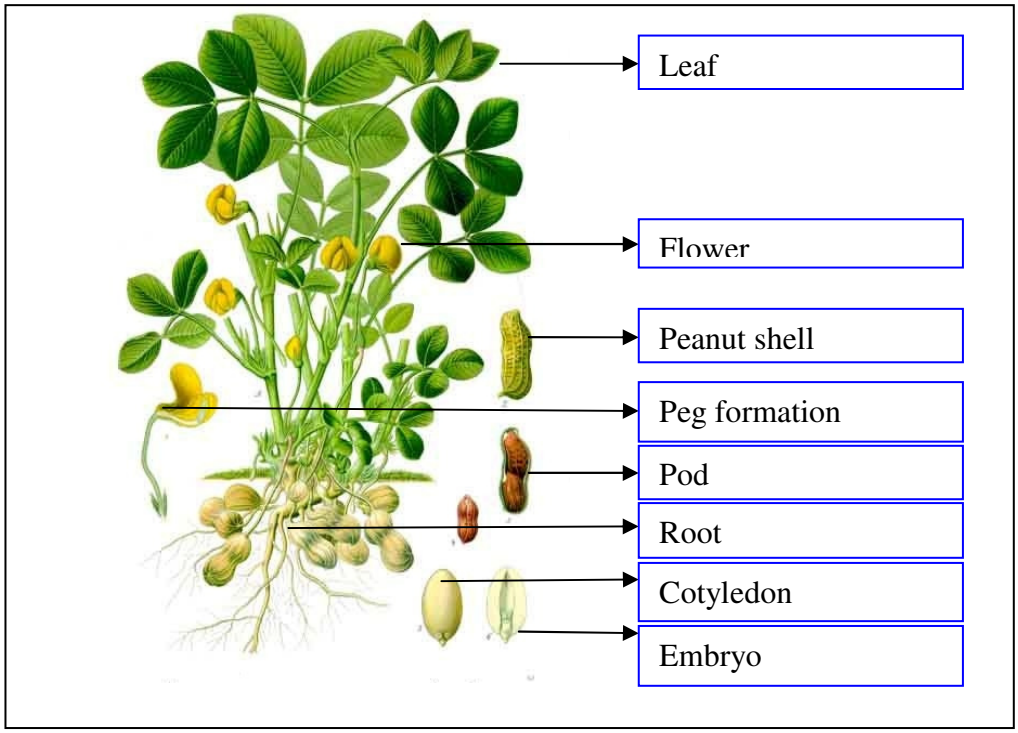

Fig. 1.1: A typical peanut plant

Peanut is a C3 plant but like C4 plants, it shows elevated rate of photosynthesis in high sunlight. It grows around 30 to 50 cm tall and its leaves are opposite, pinnate with four leaflets (Fig. 1.1). Flowers are similar to typical pea flower (Family-Papilionaceae or pea) in shape with reddish veining. After pollination, the flower stalk elongates, causing it to bend until the ovary touches the ground. It then pushes the ovary underground so that the mature fruit develops into a pod. These characteristics are the reasons for the origin of the name groundnut.

Cultivation of peanut

Groundnuts grow best in well-drained deep land with light-textured, loose, friable sandy-loam or sandy-clay-loam acidic soils (pH 5.9-7.0). Good soil condition is required for rapid and uniform germination, good root penetration and growth, steady pod development etc.

For peanut cultivation, 0.5–1.0 cm of annual rainfall is needed. This helps the pegs to penetrate the soil easily. Soil moisture is the most common limiting factor for better yield in groundnut production. Farmers should only plant when there is adequate moisture in the ground to ensure good germination.

Planting earlier in the season helps to improve yield and seed quality and reduce the incidence of rosette disease both in case of monsoon and winter crop. Maintaining adequate moisture is important for three major stages; during planting to encourage germination, after planting from 50-100 days as the pegs enter into the soil and during the pod filling until the pods mature. The other important factors for cultivation are spacing, weeding, soil fertilization, disease and pest control should be considered for better yield.

The oldest specimen of peanut was found by a team of archeologists dated around 7600 years back. Scientists have hypothesized that, peanut was probably first domesticated and cultivated in the valleys of Paraguay ("World Geography of the Peanut". University of Georgia, 2004-01-02. Retrieved 2009-08-18). The plant was later extended worldwide by European traders. Therefore, peanut plant is thought to be originated in South America and spread by European explorers to reach Asia, Africa, and North America. The current top three producers of peanuts are China, India, and USA (http://www.peanut-institute.org/peanut-facts/history-of-peanuts.asp, date: 24 May 2014).

According to the recent report of American peanut council (2013), world peanut production totals approximately 29 million metric tons per year. China is the largest producer followed by India and USA. On the other hand, USA is the leading exporter with average annual exports of 0.2 and 0.25 million metric tons while worldwide peanut export is approximately 1.25 million metric tons. Argentina and China are the other significant exporters, while countries, such as, India, Vietnam, and several African countries periodically enter the world market depending upon their crop quality and world market demand. The largest export markets within Europe are

the Netherlands, the UK, Germany and Spain. In Bangladesh, the peanut production is 33000 MT/year as of 2008 (FAO stat, 2008).

According to FAO statistical report 2008, in Bangladesh, 31182 hectares land is used for peanut cultivation, where 16658 hectogram production is achieved per hectare. Total production is 51944 tons (with shell), 4054 tons of seeds per year. Our country has great potential for peanut cultivation. About 80% area of our country consist of sediments deposit from nearby rivers and costal area covers almost 20%, which is 29000 km^2 and 30% cultivable lands are here. But 53% of this area is affected by salinity. Vegetable oils are imported in our country in large amounts. Therefore, if the vast coastal area can be utilized for peanut production, we will be able to increase peanut oil production to an extent that oil import may become redundant.

Nutritional value of peanut and use as food

The kernel of peanut contains 48-52% oil, 25-30% protein, and 8-14% soluble sugar, vitamin B and vitamin E providing over 30 essential nutrients. The oil contains about 30% essential fatty acid for human namely, linoleic acid. Unlike mustard oil, erucic acid is absent in peanut oil. Peanut oil is enriched with all amino acids except leucine and methionine. The monounsaturated fats of peanut make itself important in the heart healthy diet. In addition, an adult human being requires 55 gm protein in a day and peanut alone can meet 5-6 gm (10%) of that requirement. Peanut is a good source of niacin, foliate, fiber, magnesium, manganese, phosphorus, flavonoids, and isoflavones (Janila *et al*. 2013). Each 100 gm of peanut contain 600 kcal, 50gm fat, 800mg sodium and 10mg fiber and no cholesterol. For this reason, World Health Organization recommends 2 servings of 100 gm of processed nuts as a survival base for African children per day (http://news.bbc.co.uk/2/hi/europe/8610427.stm, Date: November 6, 2012). This valuable seed is also used to make processed food such as tasty dry roasted peanut, peanut butter, oil etc.

Some major food usages of peanut in different parts of the world:

1. Raw dry nuts in South and Central Asia, Africa
2. Fresh boiled and salted nut in Southeast Asia, Africa
3. Fried and mixed with sugar syrup in Asia, particularly in India, Pakistan and Bangladesh
4. Fried and coated with chickpea flour in South Asia and Mediterranean regions

5. Fermented and fried nuts in Southeast Asia, particularly Indonesia, Philippines and Thailand
6. Roasted and salted nuts in Asia, Africa, North and Central America and South America
7. Peanut butter in Europe, North and Central. America and South America
8. Candies and confections in North and Central America, Some European, Asian and African Countries

On the other hand, peanuts is also used in other areas, such as, dermal medicine for external use on the skin for arthritis and joint pain, dry skin, eczema, scalp crusting and scaling without hair loss or any other side effect. (http://www.stylecraze.com/articles/best-benefits-of-peanut-oil-for-skin-hair-and-health, http://naturalology.wordpress.com/2013/11/05/peanut-oil-benefits-for-hair).

Non-culinary use of peanut

In textile industry the peanut shell can be used to produce fiber. Peanut shells can also be used domestically as fuel. The oil of peanut is used as ointments. Peanut oil is used in skin care products and baby care products, nitroglycerin, plastics, dyes and paints. In fact, peanut oil has been found to lower LDL cholesterol without reducing beneficial HDL cholesterol. Peanut cultivation can protect soil erosions and can make the land more fertile and are useful for crop rotations. Commonly village women are involved in the cultivation of peanut in kitchen garden while farmers grow them in large scale in the field. Therefore, in one hand, peanut helps the farmers economically while meeting the nutritional value of the family and on the other hand it helps our country to reduce the import of costly edible oil**.** Being a legume, it can fix atmospheric nitrogen in the soil, which benefits subsequent crops; as it replenishes the ground with nitrogen thus maintaining soil fertility. All parts of the plant are useful. The stalks are fed to cattle in the form of green, dried and silage. Groundnut shell, the haulms and hay of peanut are good fodder.

Constrains of peanut cultivation and production

Diseases and pests

Immediate attention should to be paid on different serious diseases and pest problems of peanut. This plant is a very propitious one for disease infestation. Fungal diseases are the main constraints behind yield loss. The commonly seen diseases are late leaf spot (LLS) caused by *Phaeoisariopsis personata*, early leaf spot caused by *Cercospora arachidicola* and rust caused by *Puccinia arachidis* (Janila *et al*. 2013, Backman and Crawford 1984, Khaleque *et al*.1985). Iqbal and his colleagues (2012) have reported that wilt caused by *Fusarium oxysporum* also hamper the yield considerably. Other than these, a fungi named *Sclerotium rolfsii* causes stem and pod rot to peanut plant.

Major fungal diseases for peanut plants are leaf spot and rust. Foliar disease such as early leaf spot (caused by *Cercospora arachidicola*) usually appears on 2-3 weeks old seedlings. Small chlorotic spots appear on leaflets about 10 days after infection. The spots develop to maturity and sporulate like sub circular lesions. Late leaf spot (caused by *Phaeoisariopsis personata*) usually occurs on 3-month old crops and severity is generally low to moderate. Lesions caused in case of late leaf spot are nearly circular, and darker in color than those of early leaf spot. Rust (caused by *Puccinaia arachidis*) generally occurs sporadically and at low severity, but can sometimes cause significant yield losses. Long-distance spreading of the disease can occur through airborne spores, spores contaminating the surfaces of pods/seeds, or infected crop debris.

Aflatoxin contamination is a major problem of food safety. Yellow mold in peanuts is caused by *Aspergillus flavus*. This infect either before or after harvesting during field drying and also in storage. *A. flavus* can invade groundnut tissues, producing toxic compounds known as aflatoxins. Contaminated peanut produced can be poisonous to people and livestock, and cannot be exported. This toxin also leads to low germination percentage and poor seedling establishment. Pre-harvesting contamination is influenced by factors such as, soil moisture and temperature, and is likely to become severe under drought conditions. Post harvesting aflatoxin contamination occurs if the groundnuts become moist and / or damaged, and can occur at the time of harvesting or later.

Groundnut is generally free from serious insect pests, but some insects can cause economic damages. The pests which cause economic loss are aphids, thrips (frankliniella schultzi), jassids (*Empoasca dolichi*), white grubs, termites (mainly *Microtermes* sp), and *Hilda patruelis*. False wireworms and millipedes seem to occur less frequently. In general soil insects appear to cause more damage than foliage feeders or sucking pests. However, aphids are particularly harmful because they transmit groundnut rosette disease.

Implication of diseases

These diseases are greatly hampering the net production of peanut. Indian peanut is affected by bud necrosis and stem necrosis viral diseases and peanut stripe potyvirus is another common disease in East and South East Asia. On the other hand, in the tropical and sub-tropical regions, peanut production has to face difficulties mainly due to several abiotic stresses (Sun *et al*. 2013; Rengasamy *et al.* 2008, Jie Zou *et al.* 2009).

Effects of abiotic factors

Abiotic stresses are also rather important hindrances to crop productivity specially for the semiarid tropics (SAT) that include parts of 55 developing countries. These regions are populated by about 1.4 billion people, where grain legumes are mainly cultivated. Abiotic stresses, such as, drought, salinity, water logging, high temperature, chilling, etc. are the primary cause behind loss of crops and yield up to as much as 50% (Boyer *et al*. 1982, Bray *et al*. 2000) There are some reports on responses to abiotic stresses of peanut plants (Wang *et al*. 2003; Rudrabhatla *et al*. 2002; Bhatnagar *et al*. 2007).

In Bangladesh, peanut cultivation has to face 21 diseases (Talukder *et al*. 1974; Ahmed *et al*. 1985). Among them, early and late spot disease are the most damaging; late spot diseases causes 30-48% yield loss and early and late spot causes up to 70% for Dhaka Chinabadam-1 varieties (Hossain *et al.* 2005). Some of the Bangladeshi varieties have the highest salt stress tolerance (level which is less than 5 dS /m). However, 2 million hectares of coastal areas are affected by high range of salt concentration, which is 12-18 dS/m/salt. So along with biotic stresses, the abiotic stress like salinity stress also challenge the betterment of peanut production in Bangladesh.

Improvement of peanut

Chemical control is neither a permanent solution nor the best approach environmentally and economically. A promising alternative, therefore, is the incorporation of genetic resistance. The problem with *Arachis hypogaea* is that, the necessary resistance genes are not always available to the breeders. Several wild *Arachis* species are important sources of novel genes that can be used for improvement of the cultivated peanut. Attempts of conventional hybridization between cultivated peanut and *A. paraguariensis* have failed, as they are cliestogamous (Rao *et al.* 2003). The available cross with *Arachis hypogaea*, ploidy differences, evaluation and the germplasm have been reviewed (Gregory and Gregory 1979; Moss and Stalker 1987; Singh *et al.* 1980; Halward *et al.* 1992). In addition to that, Stalker and his colleagues (Stalker *et al.* 1980) also discussed how tissue culture technique has helped in some cases.

Biotechnology techniques, like, genetic transformation offers potential routes for overcoming such barrier where tissue culture is prerequisite (Gardner 1993). The legumes hardly respond to various *in-vitro* techniques, as they are recalcitrant grain (Bajaj and Gossal 1981, Mroginski and Kartha 1984). In spite of this fact, several legumes, like, *Pisum sativum* L., *Cicer arintum* L., *Phaseolous vulgaris* L., *Vigna unguiculata* L., *Vigna radiate* L. *Wilczek, Gycine max* L., and *Arachis hypogaea* L. have been investigated to regenerate plantlets (Kartha *et al*. 1981, Rubluo *et al*. 1984, Allavana and Rossetti 1986, Barwale *et al*. 1986, Wright *et al.* 1987 and 1986, Bose 1991, Khanam 1994, Khanam *et al*. 1995, Ahmed *et al*. 1997, Little *et al*. 2000). In case of peanut, tissue culture was first studied by using embryo and embryonic segment (Harvey and Schulz 1943; Nuchowiz *et al*.1955). Afterward 0.3mm ovule culture was subjected to the formation of viable plant (Martin et al 1970). Ziv and Zamski (1975) showed that cultured gynophores tips could exhibit three major kinds of responses; callus formation at the cut end, gynophores elongation with its geotropic curvation and swelling of the ovary followed by pod formation. All the responses were found to be dependent upon physical factors, like, light, orientation of the explants with respect to their connection to the media, gravitational force and concentration or dosages of hormones. After few years, the histological study with chlorophyll development from mesophyll tissue culture was investigated and optimum vitamins, iron salt, magnesium sulphate were determined. One year later, aseptic embryo culture for making virus free plant and developing phytosanitation was proposed (Kumer *et al.* 1974). Based on this,

different stages of microsporogenesis from anther culture of *A. hypogaea* were examined (Saxena *et al.* 1999). In another report, fresh callus formation and early pollen embryogenesis with multicellular pollen grains were obtained via anther culture of *A. hypogaea* and *A. glabrata* (Bajaj *et al.* 1981). Both the reports were concerned with the ploidy of different varieties.
In the last few decades, several explants have been evaluated to find the regeneration protocols. Following that, *in vitro* regeneration of peanut plants has been achieved from different tissues through both organogenesis (McKently *et al.* 1991 and 1989; Cheng *et al.* 1992; Pestana *et al.* 1999; Eapen and George 1993) and embryogenesis (Saxena *et al.* 1992; Baker and Wetzstein 1992; Gill *et al.* 1992,1999; Hajra *et al.* 1989; Haque *et al.* 1991; Sarkar *et al.* 2000; Ozias-Akins *et al.* 1992); genotypic impact on embryogenesis was revealed by Chengalrayan *et al.* 1998. Among the explants, leaflets are the most widely used explants in peanut tissue culture. Several other types of explants, such as, cotyledonary nodes (Srinivasan *et al.* 2010), epicotyls and hypocotyls (Marion *et al.* 2008), auxiliary meristems (Singh and Hazra 2009) and cotyledons (Bhatnagar *et al.* 2010, Tiwari and Tuli 2008) have also been used in peanut regeneration systems. Hassan and his colleagues found decapitated embryo as an effective explant for regeneration of another recalcitrant plant, lentil (2007).

Although great efforts have been made to enhance the frequency of regeneration in peanuts, it is still difficult to obtain a sufficient number of explants in a short period of time. It can even take as long as 4 to 6 months for explants to regenerate (Bhatnagar *et al.* 2010).

In Bangladesh, scientists are trying to improve peanut production via gamma irradiation and *in vitro* tissue culture method. Not only that, sometimes they experimented wide range of plant extract for the foliar spray (Hossain *et al.* 2013). Genetic analysis for Bangladeshi agronomic traits has been done (Alam *et al.* 2013). In the neighboring country India, Rohini and Rao (2000) established an *in planta* method for peanut because of its recalcitrant nature. However, during the same time frame, a successful protocol for *in vitro* direct and indirect organogenesis along with *Agrobacterium*-mediated transformation was established (Sarker *et al.* 1997; Islam *et al.* 1999; Sarker *et al.* 2000).

Tissue culture is a pre-requisite for transformation. To develop protocols for an efficient regeneration, it is important to investigate the roles and interactions of different genotypes, explant sources, and hormonal effects. In Bangladesh, several attempts have been made since late 1990s with many farmer popular peanut varieties but these reports severely lack relevance. For example, several explants from all the possible valuable plants were never evaluated in one research attempt. As a result, efficacy of individual explants or genotypes cannot be assessed from the available reports. If such research is conducted, the research data will help in identifying the best responsive explants and genotype for future use in transformation in Bangladesh. Therefore, it is extremely important to evaluate the explants' efficiency in addition to easy regeneration system to develop an efficient reproducible tissue culture protocol for transformation experiments.

Under this background, in the present investigation, tissue culture of six farmer popular peanut varieties has been done to compare and determine:

1. The best explant in the farmer popular varieties of peanut
2. The best hormonal combination with less complexity of the regeneration system to achieve highest plantlets
3. Reproducibility of the tissue culture protocol, also
4. To study seasonal response on i*n-vitro* regeneration.

Chapter: 2

Materials

Materials

2.1 Plant materials

In the present investigation, six farmer popular locally grown peanut (*Arachis hypogeae* L.) varieties were used. These are,

i. BARI Chinbadam-7
ii. BARI Chinbadam-8
iii. Dhaka Chinbadam-1
iv. BINA Chinbadam-2
v. BINA Chinbadam-4
vi. BINA Chinbadam-6 **(Fig. 2.1)**

The seeds were collected from two institutions for this experiment. All the BARI varieties along with Dhaka-1 were collected from Bangladesh Agricultural Research Institute (BARI) Joydevpur; Gazipur. Seeds of BINA varieties were collected from BINA (Bangladesh Institute of Nuclear Agriculture), Mymensing. Some of the cultivars are Rabi crop (it is the spring harvest, also known as the "winter crop" in Indian subcontinent) and some are Kharif crop (sown in the monsoon season on the Asian subcontinent and harvested in autumn. These are also called the summer or monsoon crop in Indian subcontinent). Seeds were preserved at 4°C temperature in Plant Biotechnology Laboratory, BRAC University, Mohakhali, Dhaka, Bangladesh.

Fig. 2.1: Six varieties of locally grown peanut (*Arachis hypogeae* L.) collected from BARI and BINA. **A.** BARI Chinabadam-7, **B.** BARI Chinabadam-8, **C.** Dhaka Chinabadam-1, **D.** BINA Chinabadam-2, **E.** BINA Chinabadam-4, **F.** BINA Chinabadam-6.

2.1.1 BARI Chinabadam-7

Seeds are Virginia type; non-dormant; shell of the nuts is whitish brown; smooth and soft; life cycle is 145-155 days in Rabi season and 130-140 days in Kharif season; average yield is 2.8-3.0 ton/ha in Rabi season and 1.8-2.0 ton/ha in Kharif season; comparatively less leaf spot and rust diseases are found in leaves.

2.1.2 BARI Chinabadam-8

The height of the plant is 35-42 cm.; leaf deep green; seeds grow in clusters; veins are not conspicuous, 2 seeds per pod; seeds are large in size, Virginia type; non-dormant; shell of the nuts is smooth and soft; testa color reddish brown; life cycle is 140-150 days on Rabi season and 125-140 days in Kharif season; 100 seed-weight is 55-60 gm; shelling percentage is 65-70%; highly susceptible to *Cercospora* sp.; average yield is 2.5 tons per hectare.

2.1.3 Dhaka Chinabadam-1

Spanish type; widely grown recommended cultivar; height is (30-40 cm); leaves are pale green in color; flowers are found in both the main stem and branches; 1 to 2 seeds per pod; seeds are light brown in color; plants are thermo-sensitive and drought resistant to some extent; matures in 140-150 days in winter and 120-140 days in summer; size of pods and seeds are comparatively smaller; testa color reddish; 100-seed weight is 30-35gm; shelling percentage is 70% kernel 30% husk; highly susceptible to *Cercospora sp.*; average yield 1.85-2.05 ton/ha in 'Rabi' and 1.60-1.80 ton/ha in 'Kharif'.

2.1.4 BINA Chinabadam-2

Developed by gamma irradiation to Dhaka Chinabadam-1. Plants are dwarf (av. 28cm); leaves are small, darker green and ovate shape; pods and seeds are 24% and 28% bolder than the mother variety Dhaka-1; pods are shiny, without constriction; this is a mutant variety; . Maximum pod yield potential is 3.19 tons/ha in winter and 2.29 tons/ha in winter season; maturity period ranges between 150 and 160 days in winter and 125 and 135 days in summer; seeds contain 50% oil and 28% protein; moderately resistant to collar rot, *Cercospora sp.* leaf spot and rust diseases.

2.1.5 BINA Chinabadam-4

BINA Chinabadam-4 variety was developed by mutation through gamma irradiation of Dhaka Chinabadam-1. Plants are intermediate dwarf; leaves mostly upright, lanceolate, dark green in color; pods and seeds are 27% and 40% bolder than the mother plant; pods are shiny, without constriction and strong venation; maximum pod yield potential is 2.6 tons/ha in winter and 1.04 tons/ha in winter season; maturity period ranges between 150 and 160 days in winter and 125 and 135 days in summer; seeds contain 52% oil and 29% protein; moderately resistant to collar rot, *Cercospora* leaf spot and rust diseases.

2.1.6 BINA Chinabadam-6:

Plants are dwarf (av. 28cm); leaves are small, darker green and oval shape; pods and seeds are 24% and 18% bolder than the mother variety Dhaka Chinabadam-1; this variety is also a mutated product achieved through Gamma irradiation. Pods are shiny, without constriction; maximum pod yield potential is 3.2 tons/ha in winter and 1.7 tons/ha in summer; maturity period ranges

between 150 and 160 days in winter and 140 and 150 days in summer; seeds contain 48.51% oil and 28.68 % protein; moderately resistant to collar rot and rust diseases.

Chapter: 3

Methods

Methods

3.1 Seed sterilization

Seeds of all varieties were surface sterilized using 70% ethanol and 0.1% mercuric chloride treatment. The treatment time was optimized in this study.

3.2 Seed germination and explant collection

Surface sterilized seeds were placed on autoclaved cotton soaked (with distilled water) medium for immature leaflet explant collection. For collection of decapitated half embryo explant treated seeds were kept soaked in double distilled water over night or for two consecutive nights.

3.2.2 Media for multiple shoot regeneration

For the induction and development of multiple shoots MS medium (Murashige and Skoog 1962) supplemented with various concentrations and combinations of BAP and Kn were used.

3.2.3 Root induction

For induction of roots from the *in vitro* grown shoots, half MS (half the strength of macro and micronutrients of MS) medium supplemented with NAA was used.

3.3Media preparation

In the present study, Murashige and Skoog (MS) medium (1962) in full or half strength were used for *in-vitro* regenerations.

3.3.1 Preparation of stock solutions

The various constituents of the medium were prepared as different stock solutions as the first step for the preparation of the medium. As different constituents were required in different

concentrations, separate stock solutions for macro- and micro-nutrients, vitamins, plant growth regulators etc. were prepared. (**Table 3.2**)

3.3.1 Stock solutions (Macro-nutrients) for MS medium

The stock solution of macro-nutrient was made in such a way that its strength was 10 times more than the full strength of the medium. The stock solution was numbered I and made in 500 ml distilled water. For this purpose, 5 times the weight of different salts required for 500 ml of stock were weighed accurately and were sequentially dissolved one after another in a 500 ml volumetric flask with 350 ml of distilled water. The final volume of the solution was made up to 500 ml by further addition of distilled water. The solution was autoclaved and poured into a clean plastic container. After proper labeling, the solution was stored in a refrigerator at 4°C temperature for several weeks.

3.3.2 Stock solution (Micro-nutrients) for MS medium

To prepare this constituent of the medium two separate stock solutions were prepared.

(i) Stock solution II (all the micronutrients except iron)

This part of the stock solution was made with all the micronutrients except Iron chelate ($FeSO_4.7H_20$) and Na-EDTA. This was made 100 times the full strength of components and volume up to 500 ml of distilled water as described for the stock solution II. This solution was autoclaved and stored at 4°C.

(ii) Stock solution III (Iron Chelate solution)

Solution III was made 100 times the final strength of Iron Chelate ($FeSO4.7H_20$) and $Na_2EDTA.2H_2O$ and volume up to 500 ml of distilled water as described for the stock solution III. Heat was applied to dissolve the salts. The solution was

autoclaved and stored at 4°C for several weeks in an amber bottle or clear bottle covered by aluminum foil to prevent penetration of sunlight into the solution as the solution is sun sensitive.

3.3.3 Stock solution IV (Organic constituents) for MS medium

It was also made 100 times the final strength of medium up to 500 ml of distilled water. This solution was autoclaved and stored at 4°C for future uses.

3.3.4 Stock solution for growth regulators

The effect of following plant growth regulators were examined in present study:

I. Auxins

Indole -3- acetic- acid (IAA)
Indolebutyric acid (IBA)
Napthalene acetic-acid (NAA)

II. Cytokines

6- Benzyl amino purine (BAP)
6- Furfuyl amino purine (Kinetin/Kn)

The growth regulators along with their solvents and molecular weight are listed in **Table 3.1.**

Table 3.1: growth regulator, their solvent and molecular weight

Growth regulator	Solvent	Molecular weight
IAA	1 N NaOH	175.2
IBA	1 N NaOH	203.24
NAA	1 N NaOH	186.21
BAP	1 N NaOH	225.3
Kn	1 N NaOH	215.2

Preparation of all the growth regulators is more or less similar**.** To prepare any of the above mentioned hormonal stock solutions, 20 mg of hormone powder was placed on a clean beaker, where required amount of appropriate solvent was added to dissolve the hormone powder. The dissolved mixture was then made up to 200 ml in volume with the addition of distilled water. This will give the stock solution a concentration of 10x. This solution was sterilized and preserved in a refrigerator at 4°C for several weeks.

3.4 Preparation of one liter of MS medium

MS medium was prepared from these stock solutions. The amounts of using prepared stock solutions are given bellow in **Table 3.2.**

Table 3.2: Stock solution requirement for the preparation of one liter MS medium

Components	**Amount**
Macro-nutrients (10X)	100 ml
Micro-nutrients (100X)	10 ml
Na-Fe- EDTA (100X)	10 ml
Organic (100X)	10 ml
Myo-inositol	0.10 gm
Sucrose	30 gm

The following steps were carried out successively:

I. For the preparation of desired medium (MS) 250 ml distilled water was taken in a one-liter volumetric flask.

II. 100 ml of stock solution Stock solution I (Macro-nutrients), 10 ml of Stock solution II (Micro-nutrients), Stock solution III (iron chelate) and Stock solution IV was added sequentially and mixed thoroughly.

III. 100 mg of Myo-inositol (Sigma, USA) was added to this solution and were dissolved completely.

IV. Then 30 gm of sucrose was dissolved as carbon source.

V. To obtain different required concentrations of various hormone(s) the stock solutions were added singly or in case of multiple supplementations added sequentially to this solution and were mixed thoroughly. Since each of the hormonal stock solution contained 10 mg in 100 ml of solution, the addition of 10 ml of any hormonal stock solution to make 1 liter of medium resulted in 1.0 mg/l concentration of that hormonal supplement. Different concentrations of hormonal supplements were prepared by adding required amount of the stock solution of the medium following this procedure.

VI. The whole mixture was then made up of 1 liter with further addition of distilled water.

VII. The pH of the medium was adjust to 5.8 using a digital pH meter (pHep,HI-98107, Hanna Instruments) with the help of 1N NaOH or 1N HCl, whichever was required.

VIII. To solidify either 6.0 g (at 0.6%) of plant-agar (Duchefa, Biochemie) or 3.0 g (at 0.3%) of phytagel (Sigma, USA) was added to the desired medium. To dissolve the solidifying agent (agar, phytagel etc.) the whole mixture was heated in a microwave oven (Model: MH6548SR, LG, China).

IX. Finally the melted medium was dispensed in culture vessels like conical flask or test tube and sealed properly using cotton plug and aluminum foil. The vessels were marked with the help of a permanent marker to indicate specific hormonal supplement.

3.5 Sterilization

The culture vessels were autoclaved (Model: CL-32L,APL Co.Ltd, Japan) at 15 lbs/sq. inch pressure at 121°C temperature for 20 minutes. The medium was allowed to cool after sterilization.

3.6 Preparation of seed germination media

To obtain sufficient leaflet explants, seeds were germinated aseptically. In this case germination of the seeds was carried out in autoclaved flasks containing sterile non-absorbent cotton soaked with sterile distilled water.

3.7 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 at least 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 by autoclave machine. During the entire period of inoculation the instruments were kept immersed in absolute alcohol containing 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.

3.8 Culture techniques

Techniques implemented in this present study are presented below

(i) Axenic Culture

(ii) Explant designing and explant culture

(iii) Subculture

(iv) Root induction

(v) Transplantation

(vi) Maintenance of transplanted plants

3.8.1 Axenic culture

As a first step, seeds were washed under running tap water for 3-5 minutes. At that time, floating seeds were discarded, as they had abnormality. Later the seeds were stirred gently with 70% ethanol for 1 minutes followed by thorough washing with double distilled water inside laminar air flow cabinet.

Finally, the seeds were treated with 0.1% $HgCl_2$ solution for 18 minutes for surface sterilization. During this period, the sterilant was continuously agitated. For complete removal of the harsh chemical, the seeds were thoroughly washed with sterilized distilled water after decanting the sterilant following treatment. The surface sterilized seeds were then inoculated in to conical flask, containing cotton soaked with sterilized distilled water. Then the flasks were placed in dark chamber to create proper environment for germination.

3.8.2 Seedling development

Seeds in germination medium were kept in the dark chamber to imitate the darker environment under soil. In that condition, seeds germinate and gave explant. Generally, explants of different ages were collected and used in various experiments.

3.8.3 Explant designing and explant culture

Germinated seeds of various varieties were the source of the two different explants, leaflet and half decapitated embryo. Leaflet explants were collected from 7, 9 and 11 days old germinated seeds. On the other hand, immature embryos were collected from overnight and 2 days long emerged seeds. These explants were cultured in MS media with various concentration and combination of hormonal supplements to achieve direct and indirect regeneration.

3.8.4 Subculture

All the *in-vitro* cultures were transferred into fresh media at an interval of 3 weeks. Morphogenic changes were under regular observation for proper data maintenance.

3.8.5 Root induction

To obtain sufficient root, well developed shoots were placed individually into different auxin containing media and effect of those auxin on root induction were experimented.

3.8.6 Transplantation

The healthy shoots with sufficient roots were taken out carefully from the media. Attached media at the root system was washed under running tap water carefully so that the root system remains intact. The plants were then transplanted to small size pots containing a sterilized mixture of ground soil, sand and cow dung in the ratio of 1:2:1. Pots were covered with transparent perforated polythene bags. The inner side of the bag was moistened with water to prevent desiccation of the newly transplanted plantlet. To reduce sudden environmental shock, the pots were kept in growth room condition for 2 weeks. However, after one week, the polythene covers were removed. In next two weeks the plantlets were exposed to natural environment for 2-7 hours and again placed in growth room condition. Generally, after three week from transformation, when plants looks established enough with natural environment, they were transferred to larger pots and shifted to net house. All the plants were then kept under routine observation through proper agronomic practice. Their growth and development and was monitored in this stage.

Chapter: 4

Result

Results

In the present study, attempts were taken to establish a genotype independent regeneration protocol for six locally grown farmer popular peanut varieties, namely, BARI Chinabadam-7, BARI Chinabadam-8, Dhaka Chinabadam-1, BINA Chinabadam-2, BINA Chinabadam-4, and BINA Chinabadam-6. During this investigation, experiments were performed using two explants, viz., immature leaflet and half of decapitated embryo. Suitability of these explants for regeneration and optimization of different hormonal supplements were accomplished through repeated experiments. Finally, regenerated plantlets acclimatized in sandy soil following proper hardening and placed in net house.

4.1 Aseptic seed germination

Germination rate and days requirement of all peanut varieties were observed. BINA varieties showed better germination capacity than that of BARI varieties. Among all the varieties, BARI Chinabadam-7 showed less germination and longer time to germinate where others need 6 days. On the other hand, BINA Chinabadam -6 took only 5 days (Table 4.1).

Two totipotent plant parts immature leaflet and decapitated half embryo were collected from these to do further experiments. Decapitated half embryo explants were collected from 1-2 night soaked seed where germination has just initiated.

Table 4.1: Germination response of six farmer popular peanut cultivars following sterilization

Varieties	No. of seeds germinated *in-vitro*	Percentage of seeds germination	Average time required for germination (days)
BARI Chinabadam-7	24	80.00 (6.24)	7.8 (0.53)
BARI Chinabadam-8	26	86.67 (5.91)	7.6 (0.47)
Dhaka Chinabadam-1	26	86.67 (3.20)	6.8 (0.77)
BINA Chinabadam-2	28	93.33 (4.66)	6.6 (0.38)
BINA Chinabadam-4	29	96.67 (5.89)	6.0 (0.69)
BINA Chinabadam-6	29	96.67 (4.81)	5.5 (0.51)

All experiments were performed three times with 30 seeds in every experiment. SD value is contained within parenthesis.

4.2 Analysis of overall responses of two different explants of peanut

4.2.1 Effect of age of leaflet explants on regeneration response

Leaflet explants of three different ages were examined to find out the optimum age for regeneration. For all the varieties 9 days old leaflets showed the highest rate of response with less time requirement for regeneration initiation (**Table 4.2**).

Table 4.2: Determination of the suitable age of leaflet explants for regeneration

Varieties	Age of leaflet explants (days)	No. of explants responded	% of responsive explants	Days required for callus initiation
BARI Chinabadam-7	7	23	76.66 (2.32)	13.32 (1.28)
	9	25	83.33 (1.97)	11.54 (1.30)
	11	23	76.66 (2.07)	10.21 (1.66)
BARI Chinabadam-8	7	23	76.33 (2.41)	14.47 (1.21)
	9	26	86.66 (1.77)	12.36 (1.55)
	11	22	73.33 (2.15)	11.45 (1.63)
Dhaka Chinabadam-1	7	28	93.33 (3.02)	14.62 (1.18)
	9	26	86.66 (1.85)	13.82 (1.09)
	11	20	66.66 (2.49)	10.35 (1.24)

Varieties	Age of leaflet explants (days)	No. of explants responded	% of responsive explants	Days required for callus initiation
BINA Chinabadam-2	7	26	86.66 (3.11)	16.64 (1.83)
	9	28	93.33 (0.89)	12.92 (1.03)
	11	22	73.33 (2.37)	11.33 (1.44)
BINA Chinabadam-4	7	25	83.33 (1.89)	16.36 (1.08)
	9	28	93.33 (1.09)	13.81 (1.26)
	11	24	80.00 (1.86)	11.16 (1.39)
BINA Chinabadam-6	7	27	90.00 (2.05)	15.62 (1.51)
	9	27	90.00 (2.39)	14.37 (0.94)
	11	21	70.00 (2.71)	11.95 (1.11)

All experiments were carried out with different aged leaflet explant. SD value is contained within parenthesis.

4.2.2 Regeneration response of leaflet explant under various hormonal supplementations

During this experiment both direct and indirect regeneration were observed in all the six peanut varieties in response to different hormonal concentrations and combinations.

Except Dhaka Chinabadam-1, the other two varieties from BARI, viz, BARI Chinabadam-7 and BARI Chinabadam-8, showed higher percentage of indirect shoot regeneration on MS medium supplemented with 5 mg/l BAP. Direct shoot regeneration was observed in 5 mg/l BAP and 0.5 mg/l Kn combination. On the other hand, for indirect regeneration 2mg/l BAP alone and in combination with 0.5 mg/l Kn resulted the optimized hormonal supplements in MS for the other four varieties, namely, Dhaka Chinabadam-1, BINA Chinabadam-2, BINA Chinabadam-4, and BINA Chinabadam-6 (**Table 4.3**). Though regeneration from 7 mg/l BAP containing media gave high rate of callus induction but unexpectedly the entire callus became necrosed within 1 month.

After analyzing the data through ANOVA, the F-value (df = 5, 25; p = 0.1676004, p = 0.106652 for varieties and hormonal usage accordingly) indicates that, there is no significant difference among the cultivars and hormonal impact in case of regeneration response with leaflet explants (**Annex 2**). However, according to F-value (df = 5, 25p = 6.36008 and p = 8.6014 for shoot per explant and shoot regeneration response, respectively) showed that, regeneration from leaflet explant presented significant difference among different hormonal supplementation in all the observations (**Annex 3 and 4**). But as the p value is 0.293092 and 0.92521, respectively for those same factors, there is no significant difference among the cultivars.

Table 4.3: Effect of cytokinins on shoot regeneration from leaflet explants

Varieties	Hormone		Responsive explants (%)	Shoot regeneration response (%)	Shoot bud/ explant	Shoots/ explant	Shoot formation (after 3 months)
	BAP (mg/l)	Kn (mg/l)					
BARI Chinabadam-7	2.0	-	85.71 (11.45)	35.51 (2.29)	10	8.2 (0.09)	+
	2.0	0.5	85.71 (12.88)	32.67 (1.95)	09	7.21 (0.12)	+
	5.0	-	92.86 (10.33)	46.42 (1.87)	15	9.42 (0.06)	+
	5.0	0.5	85.71 (09.61)	48.14 (1.92)	17	11.78 (0.02)	+
	7.0	-	78.57 (11.89)	0 (0)	Died	0 (0)	0 (0)
	7.0	0.5	100 (06.51)	0 (0)	Died	0 (0)	0 (0)
BARI Chinabadam-8	2.0	-	85.71 (16.69)	32.85 (02.11)	15	8.46 (0.05)	+
	2.0	0.5	71.43 (10.34)	40.20 (02.38)	16	11.03 (0.02)	+
	5.0	-	100 (05.81)	52.98 (02.07)	18	15.61 (0.06)	+
	5.0	0.5	100 (06.77)	58.32 (02.71)	18	16.32 (0.14)	+
	7.0	-	71.43 (08.27)	0 (0)	Died	0 (0)	0 (0)
	7.0	0.5	71.43 (10.49)	0 (0)	Died	0 (0)	0 (0)

Varieties	Hormone		Responsive explants (%)	Shoot regeneration response (%)	Shoot bud/ explant	Shoots/ explant	Shoot formation (after 3 months)
	BAP (mg/l)	Kn (mg/l)					
Dhaka Chinabadam-1	2.0	-	71.43 (11.53)	46.14 (02.67)	8	6.31 (0.11)	+
	2.0	0.5	92.86 (10.37)	53.57 (02.09)	9	7.28 (0.07)	+
	5.0	-	85.71 (10.02)	42.07 (02.76)	8	6.21 (0.10)	+
	5.0	0.5	85.71 (10.52)	40.81 (02.89)	10	8.23 (0.08)	+
	7.0	-	85.71 (10.79)	0 (0)	Died	0 (0)	0 (0)
	7.0	0.5	85.71 (10.05)	0 (0)	Died	0 (0)	0 (0)
BINA Chinabadam-2	2.0	-	85.71 (11.26)	48.57 (01.77)	17	11.20 (0.31)	+
	2.0	0.5	85.71 (12.11)	47.42 (01.73)	16	15.33 (0.37)	+
	5.0	-	78.57 (10.04)	37.14 (01.25)	8	7.85 (0.05)	+
	5.0	0.5	71.43 (09.51)	37.14 (02.59)	9	7.49 (0.07)	+
	7.0	-	71.43 (08.98)	0 (0)	Died	0 (0)	0 (0)
	7.0	0.5	78.57 (10.03)	0 (0)	Died	0 (0)	0 (0)

Varieties	Hormone		Responsive explants (%)	Shoot regeneration response (%)	Shoot bud/ explant	Shoots/ explant	Shoot formation (after 3 months)
	BAP (mg/l)	Kn (mg/l)					
BINA Chinabadam-4	2.0	-	92.86 (06.77)	51.42 (02.47)	20	13.05 (0.12)	+
	2.0	0.5	85.71 (07.66)	48.25 (01.83)	17	11.67 (0.51)	+
	5.0	-	71.43 (10.59)	30.82 (01.49)	8	6.22 (0.04)	+
	5.0	0.5	71.43 (12.03)	42.85 (01.50)	8	5.41 (0.07)	+
	7.0	-	57.14 (14.55)	0 (0)	Died	0 (0)	0 (0)
	7.0	0.5	57.14 (10.62)	0 (0)	Died	0 (0)	0 (0)
BINA Chinabadam-6	2.0	-	92.86 (05.81)	47.14 (02.42)	15	13.2 (0.21)	+
	2.0	0.5	100 (04.22)	51.61 (01.66)	13	10.5 (0.08)	+
	5.0	-	71.43 (09.93)	35.37 (01.91)	7	5.8 (0.06)	+
	5.0	0.5	71.43 (13.99)	41.28 (02.17)	8	6.34 (0.08)	+
	7.0	-	57.14 (11.08)	0 (0)	Died	0 (0)	0 (0)
	7.0	0.5	57.14 (11.57)	0 (0)	Died	0 (0)	0 (0)

+ = shoot regeneration present; 0 = Died, no shoot regeneration

SD value is contained within parenthesis.

4.2.3 Analysis of overall response of decapitated half embryo under various hormonal supplementations

The decapitated half embryo explants were observed to give good response in 3.0 mg/l BAP in combination with 1.0 mg/l Kn. In this medium on an average 2-3 shoots were observed per explant within 25 days **(Table 4.4)**. Further shoot regeneration was observed on those explants in following subcultures.

In this study, MS medium supplemented with 3.0 mg/l BAP and 1.0 mg/l Kn was found optimum for better shoot regeneration. According to F- value (df = 5, 25; P = 4.2547 and P =2.5709) (**Annex 6 and 7**) and there is significant difference among the hormonal supplements in terms of Number of shoot per explant, Length and Time requirement for regeneration from decapitated half embryo but (df = 5, 25; P = 0.4802, P= 0.29565) no significant difference among cultivars (**annex 5, annex 6 and annex 7**). This indicates that regeneration is highly dependent on hormonal supplementation.

Table 4.4: Shoot regeneration response from decapitated half embryo explant of BARI Chinabadam varieties

Hormone		BARI Chinabadam -7			BARI Chinabadam -8			Dhaka Chinabadam -1		
BAP (mg/l)	**Kn (mg/l)**	**Number of shoots/ Explant**	**Length of shoots in 30 days (cm)**	**Time required for >3cm length (days)**	**Number of shoots explant**	**Length of shoots in 30 days (cm)**	**Time required for >3cm length (days)**	**Number of shoots Explant**	**Length of shoots in 30 days (cm)**	**Time required for >3cm length (days)**
2.0	0.5	2.24 (0.23)	1.59 (0.04)	46.75 (0.65)	2.03 (0.12)	2.32 (0.22)	42.80 (0.78)	2 (0.27)	2.50 (0.53)	40.20 (3.13)
2.0	1.0	1.75 (0.19)	1.50 (0.06)	50.25 (0.72)	2.32 (0.11)	2.63 (0.19)	39.11 (0.90)	2.62 (0.35)	1.50 (0.47)	49.35 (3.16)
3.0	0.5	2.29 (0.24)	1.62 (0.03)	48.52 (0.88)	2.47 (0.19)	2.87 (0.24)	39.92 (1.04)	2.65 (0.29)	1.40 (0.28)	52.0 (3.53)
3.0	1.0	3.36 (0.12)	1.35 (0.06)	50.88 (0.75)	2.53 (0.18)	2.65 (0.18)	41.02 (1.25)	2.74 (0.31)	1.32 (0.41)	50.82 (3.34)
5.0	0.5	2.26 (0.25)	1.42 (0.01)	50.74 (0.33)	2.27 (0.10)	2.39 (0.19)	43.75 (0.89)	2 (0.32)	1.4 (0.33)	51.78 (3.80)
5.0	1.0	2.23 (0.09)	1.46 (0.07)	50.57 (0.80)	2.18 (0.16)	2.32 (0.23)	43.19 (0.84)	2 (0.18)	1.26 (0.32)	55.21 (3.42)

SD value is contained within parenthesis.

Table 4.5: Shoot regeneration response from decapitated half embryo explant of BINA Chinabadam varieties

Hormone		BINA Chinabadam-2			BINA Chinabadam-4			BINA Chinabadam-6		
BAP (mg/l)	**Kn (mg/l)**	**Number of shoots per explant**	**Length of shoots in 30 days (cm)**	**Time required for >3cm length (days)**	**Number of shoots per explant**	**Length of shoots in 30 days (cm)**	**Days required for >3cm length (days)**	**Number of shoots per explant**	**Length of shoots in 30 days (cm)**	**Days required for >3cm length (days)**
2.0	0.5	1.63 (0.42)	1.91 (0.13)	40.9 (1.58)	1.78 (0.16)	2.43 (0.06)	40.33 (0.78)	2.20 (0.14)	2.25 (0.15)	40.8 (1.68)
2 .0	1.0	1.68 (0.38)	1.94 (0.10)	47.8 (2.03)	2.11 (0.28)	2.47 (0.10)	39.44 (1.08)	2.33 (0.06)	1.98 (0.21)	40.17 (1.56)
3 .0	0.5	3 (0.32)	1.93 (0.06)	42.6 (1.88)	3.33 (0.18)	2.56 (0.03)	39.89 (1.11)	3.39 (0.05)	2.4 (0.19)	39.43 (1.23)
3.0	1.0	3 (0.36)	2.11 (0.09)	41.13 (1.79)	3.63 (0.23)	2.31 (0.05)	41.25 (0.56)	3.50 (0.08)	2.43 (0.13)	40.12 (1.16)
5 .0	0.5	2.29 (0.54)	1.94 (0.16)	40.71 (1.98)	2.30 (0.21)	2.29 (0.10)	42.4 (1.24)	2.53 (0.12)	2.61 (0.21)	39.38 (1.64)
5 .0	1.0	2 (0.63)	1.80 (0.15)	40.0 (2.23)	2.25 (0.26)	2.30 (0.09)	42.13 (1.17)	2.25 (0.14)	2.29 (0.22)	42.5 (1.71)

SD value is contained within parenthesis.

4.3 Variation of regeneration response of decapitated half embryo from different peanut varieties

4.3.1 Effect of different concentrations and combinations of BAP and Kn towards regeneration in BARI Chinabadam-7 variety

In case of BARI Chinabadam-7, regeneration response in MS medium supplemented with two hormones, BAP and Kn, was observed. Application of only BAP in MS media resulted in indirect regeneration, whereas direct regeneration was achieved when small amount of Kn was added along with BAP.

In case of immature leaflet explants, shoot regeneration percentage was 32.67 to 48.14. this percentage is too low but number of shoots per explants was as high as 7.21 to 11.78. Nevertheless, the length of those shoots was not enough for root induction (**Table 4.6**) (**Fig. 4.1**).

On the other hand, in case of decapitated half embryo explants, rapid response was observed. Mean number of shoots per explant was 3.36. Newly regenerated shoots were healthy and became as long as 3 cm within 46-51 days (**Table 4.7**) (**Fig. 4.1**).

Table 4.6: Regeneration response of leaflet explants towards different concentrations and combinations of BAP and Kn in BARI Chinabadam-7 variety

Hormone		Responsive explants (%)	Type of response	Shoot regenerating explants (%)	Shoots per explants
BAP (mg/l)	Kn (mg/l)				
2.0	-	85.71 (11.45)	Indirect	35.51 (2.29)	8.2 (0.09)
2.0	0.5	85.71 (12.88)	Direct	32.67 (1.95)	7.21 (0.12)
5.0	-	92.86 (10.33)	Indirect	46.42 (1.87)	9.42 (0.06)
5.0	0.5	85.71 (09.61)	Direct	48.14 (1.92)	11.78 (0.02)
7.0	-	78.57 (11.89)	Indirect	0 (0)	0 (0)
7.0	0.5	100 (06.51)	Direct	0 (0)	0 (0)

SD value is contained within parenthesis.

Table 4.7: Effect of different concentrations and combinations of BAP and Kn on regeneration of BARI Chinabadam-7 from decapitated half embryo explant

MS + Hormone		Number of shoots /Explant	Length of shoots in 30 days (cm)	Days required for length >3cm (days)
BAP (mg/l)	**Kn (mg/l)**			
2.0	0.5	2.24 (0.23)	1.59 (0.04)	46.75 (0.65)
2.0	1.0	1.75 (0.19)	1.50 (0.06)	50.25 (0.72)
3.0	0.5	2.29 (0.24)	1.62 (0.03)	48.52 (0.88)
3.0	1.0	3.36 (0.12)	1.35 (0.06)	50.88 (0.75)
5.0	0.5	2.26 (0.25)	1.42 (0.01)	50.74 (0.33)
5.0	1.0	2.23 (0.09)	1.46 (0.07)	50.57 (0.80)

SD value is contained within parenthesis.

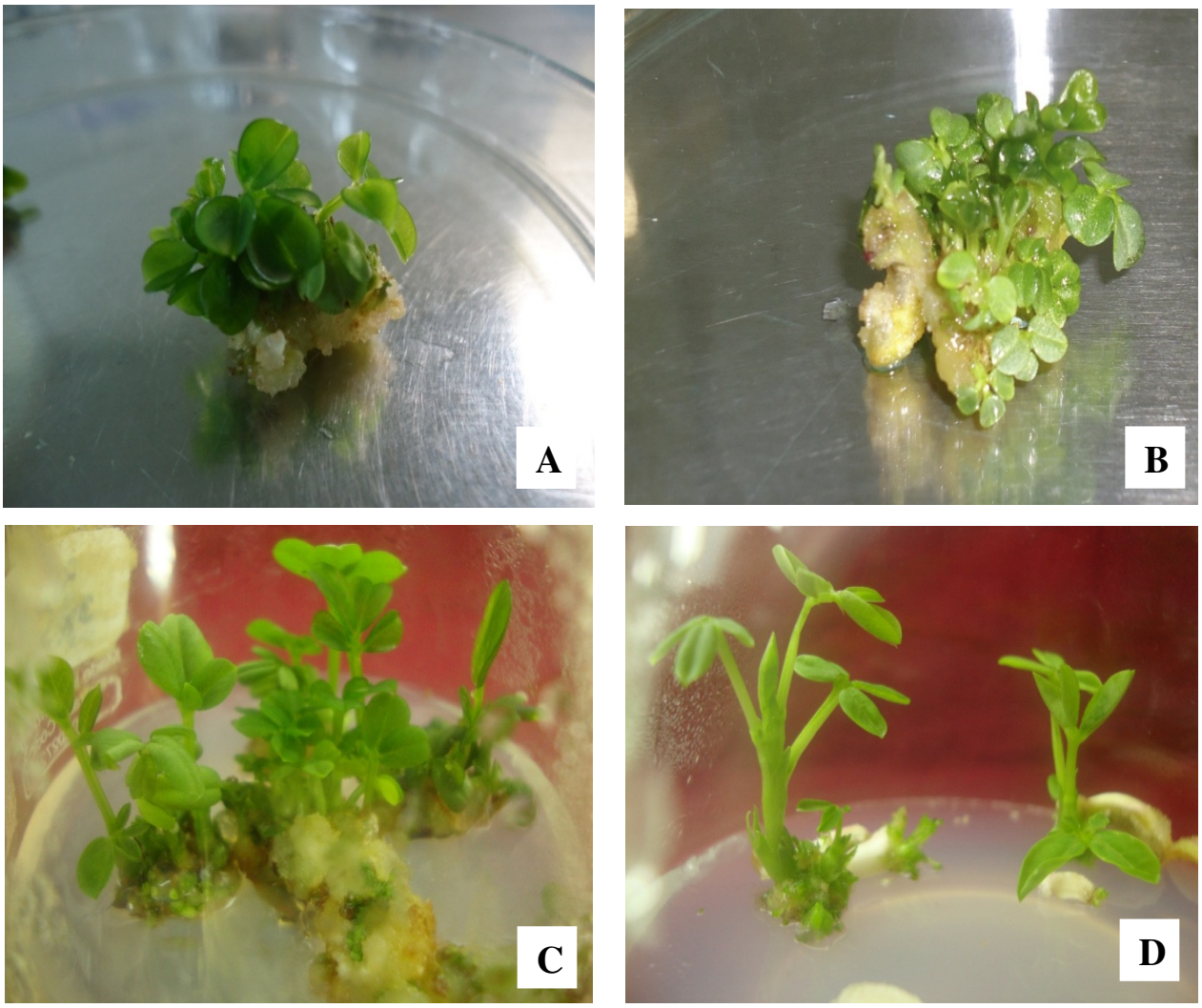

Fig. 4.1: A. Indirect regeneration from leaflet explants of BARI Chinabadam-7 in MS medium with 5 mg/l BAP and **B.** Indirect regeneration from leaflet explants of BARI Chinabadam-7 in MS medium with 5 mg/l BAP along with 0.5 mg/l Kn, **C-D.** Regeneration from decapitated half embryo explants of BARI Chinabadam-7 in MS medium with 3 mg/l BAP along with 0.5 mg/l and 3 mg/l BAP along with 1 mg/l Kn respectively.

(Photographs were taken 3 months after inoculation)

4.3.2 Effect of different concentrations and combinations of BAP and Kn towards regeneration in BARI Chinabadam-8 variety

To achieve regeneration in case of BARI Chinabadam-8, MS medium was supplemented with BAP with or without addition of Kn. When BAP alone was applied, there was indirect regeneration but direct regeneration was observed with BAP and small amount of Kn supplementation in MS medium.

Shoot regeneration was about 32.85% to 58.32% for immature leaflet explant. The number of shoots per explant was remarkably high (between 8.46% and 16.32%). However, the length of those shoots was not enough for root induction (**Table 4.8**) (**Fig. 4.2**).

On the other hand, in decapitated half embryo explants, rapid response was observed with healthy long shoots (**Table 4.9**) (**Fig. 4.2**).

Table 4.8: Regeneration response of leaflet explants towards different concentrations and combinations of BAP and Kn in BARI Chinabadam-8 variety

Hormone		Responsive explants (%)	Type of regeneration	Shoot regenerating explants (%)	Shoots/ explant
BAP (mg/l)	Kn (mg/l)				
2.0	-	85.71 (14.04)	Indirect	32.85 (25.45)	8.46 (7.247)
2.0	0.5	71.43 (14.04)	Direct	40.20 (25.45)	11.03 (7.247)
5.0	-	100 (14.04)	Indirect	52.98 (25.45)	15.61 (7.247)
5.0	0.5	100 (14.04)	Direct	58.32 (25.45)	16.32 (7.247)
7.0	-	71.43 (14.04)	Indirect	0 (0)	0 (0)
7.0	0.5	71.43 (14.04)	Direct	0 (0)	0 (0)

SD value is contained within parenthesis.

Table 4.9: Effect of different concentrations and combinations of BAP and Kn on regeneration of BARI Chinabadam-8 from decapitated half embryo explant

Hormone		Length of shoots in 30 days (cm)	Days required for length >3cm (days)	Number of shoots /explant
BAP (mg/l)	**Kn (mg/l)**			
2.0	0.50	2.03 (0.12)	2.32 (0.22)	42.80 (0.78)
2.0	1.00	2.32 (0.11)	2.63 (0.19)	39.11 (0.90)
3.0	0.50	2.47 (0.19)	2.87 (0.24)	39.92 (1.04)
3.0	1.00	2.53 (0.18)	2.65 (0.18)	41.02 (1.25)
5.0	0.50	2.27 (0.10)	2.39 (0.19)	43.75 (0.89)
5.0	1.00	2.18 (0.16)	2.32 (0.23)	43.19 (0.84)

SD value is contained within parenthesis.

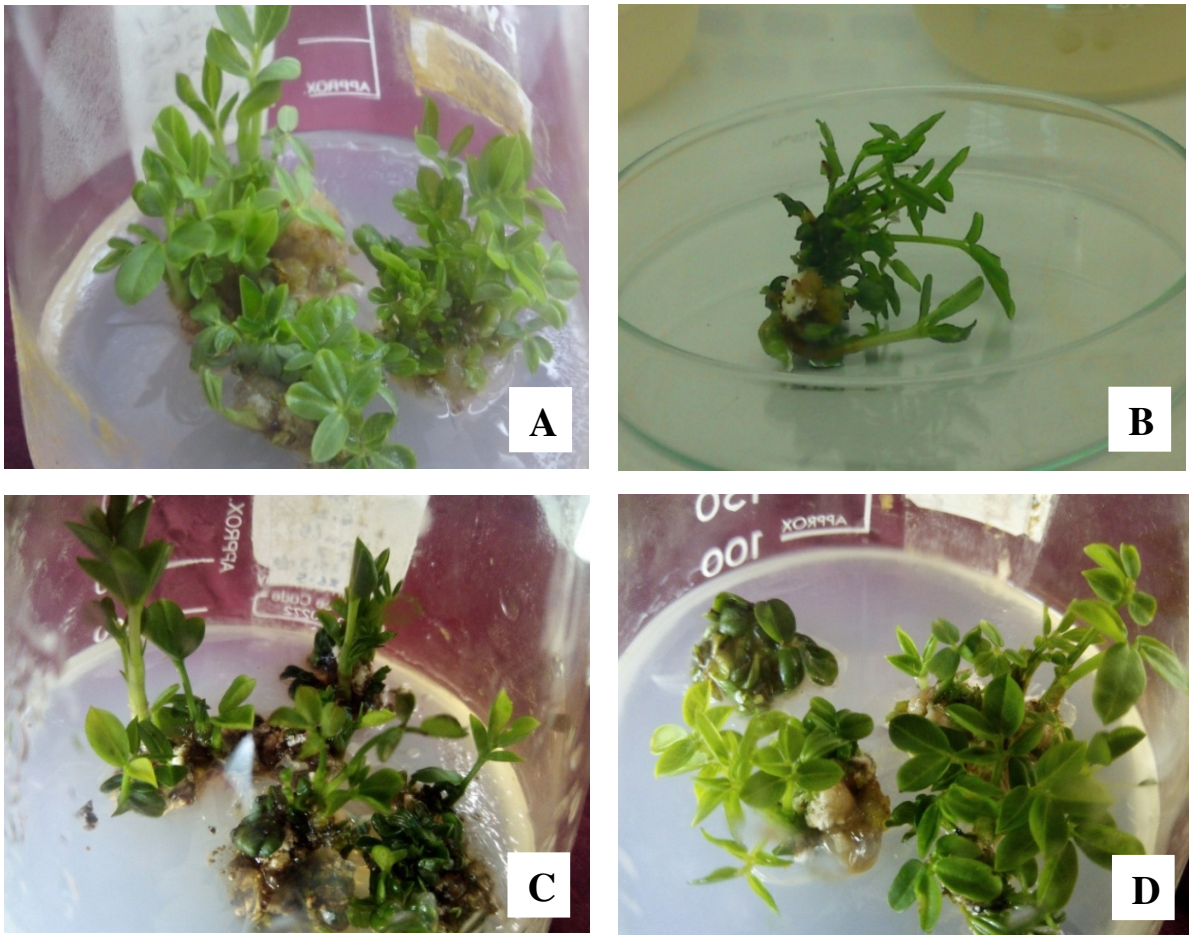

Fig. 4.2: A. Indirect regeneration from leaflet explants of BARI Chinabadam-7 in MS medium with 5 mg/l BAP and **B.** Indirect regeneration from leaflet explants of BARI Chinabadam-7 in MS medium with 5 mg/l BAP along with 0.5 mg/l Kn, **C-D.** Regeneration from decapitated half embryo explants of BARI Chinabadam-7 in MS medium with 3 mg/l BAP along with 0.5 mg/l and 3 mg/l BAP along with 1 mg/l Kn respectively.

(Photographs were taken 3 months after inoculation)

4.3.3 Effect of different concentrations and combinations of BAP and Kn towards regeneration in Dhaka Chinabadam-1 variety from leaflet explants

The hormonal treatments applied in other varieties were also followed. In case of Dhaka Chinabadam-1, MS media containing only BAP resulted in indirect regeneration and low amount of Kn addition along with BAP caused direct regeneration.

Percentage of leaflet explants that responded towards shoot regeneration was between 40.81 and 53.57. This percentage was low but the number of shoots per explant was remarkably high, ranging from 6.21 to 8.23. However, the length of those shoots was not high enough for root induction (**Table 4.10**, **Fig. 4.3**).

On the other hand, in case of decapitated half embryo explants response was rapid. Mean number of shoots per explant was 2.74. Newly regenerated shoots were healthy and became as long as 3 cm within 40-56 days (**Table 4.11**, **Fig. 4.3**).

Table 4.10: Regeneration response of leaflet explants towards different concentrations and combinations of BAP and Kn in Dhaka Chinabadam-1 variety

Hormone		Responsive explants (%)	Type of regeneration	Shoot regenerating explants (%)	Shoots / explant
BAP (mg/l)	Kn (mg/l)				
2.0	-	71.43 (7.02)	Indirect	46.14 (23.99)	6.31 (3.69)
2.0	0.5	92.86 (7.02)	Direct	53.57 (23.99)	7.28 (3.69)
5.0	-	85.71 (7.02)	Indirect	42.07 (23.99)	6.21 (3.69)
5.0	0.5	85.71 (7.02)	Direct	40.81 (23.99)	8.23 (3.69)
7.0	-	85.71 (7.02)	Indirect	0 (0)	0 (0)
7.0	0.5	85.71 (7.02)	Direct	0 (0)	0 (0)

SD value is contained within parenthesis.

Table 4.11: Effect of different concentrations and combinations of BAP and Kn on regeneration of Dhaka Chinabadam-1 from decapitated half embryo explants

Hormone		Length of shoots in 30 days (cm)	Days required for length >3cm (days)	Number of shoots/ explant
BAP (mg/l)	**Kn (mg/l)**			
2.0	0.5	2 (0.27)	2.50 (0.53)	40.20 (3.13)
2.0	1.0	2.62 (0.35)	1.50 (0.47)	49.35 (3.16)
3.0	0.5	2.65 (0.29)	1.40 (0.28)	52.0 (3.53)
3.0	1.0	2.74 (0.31)	1.32 (0.41)	50.82 (3.34)
5.0	0.5	2 (0.32)	1.4 (0.33)	51.78 (3.80)
5.0	1.0	2 (0.18)	1.26 (0.32)	55.21 (3.42)

SD value is contained within parenthesis.

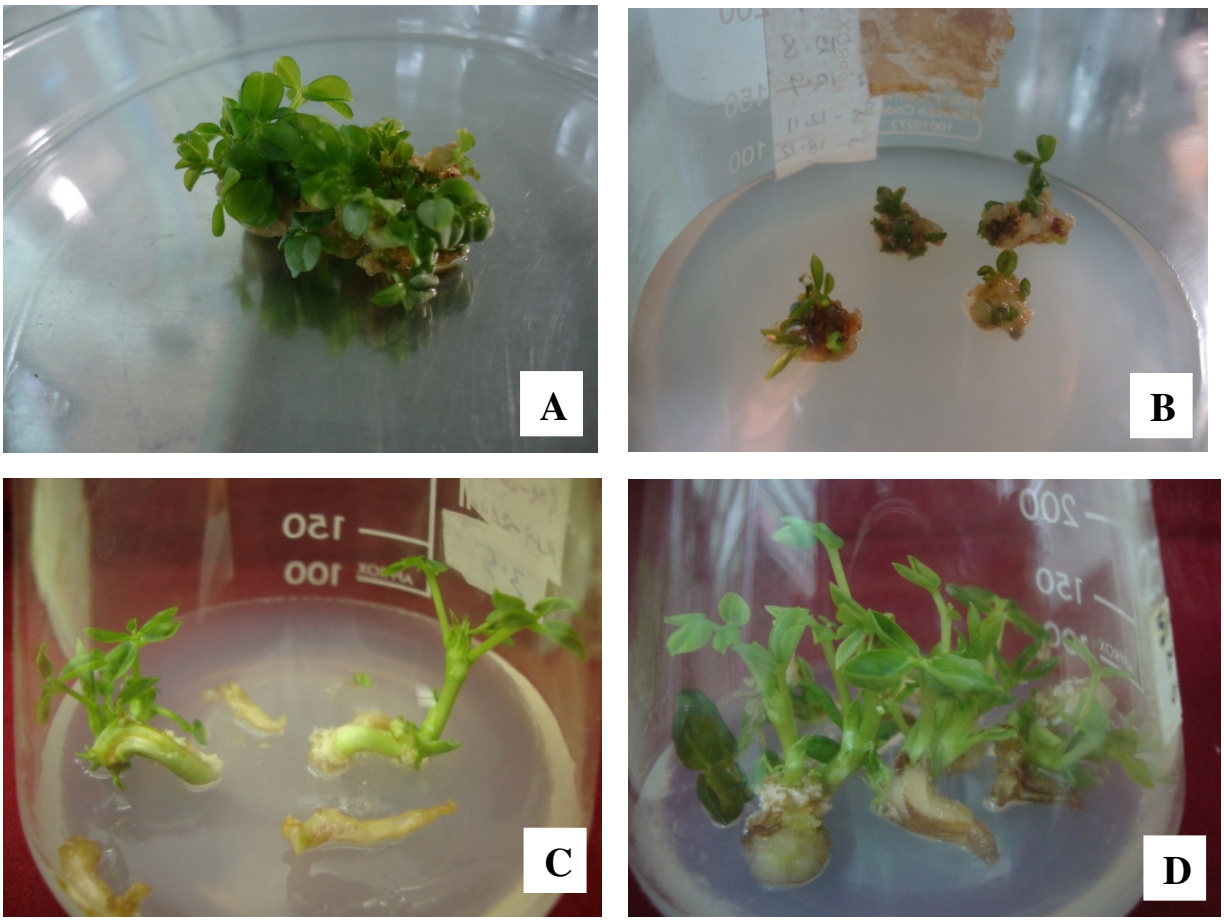

Fig. 4.3: A. Indirect regeneration from leaflet explants of BARI Chinabadam-7 in MS medium with 2 mg/l BAP and **B.** Indirect regeneration from leaflet explants of BARI Chinabadam-7 in MS medium with 2 mg/l BAP along with 0.5 mg/l Kn, **C-D.** Regeneration from decapitated half embryo explants of BARI Chinabadam-7 in MS medium with 3 mg/l BAP along with 0.5 mg/l and 3 mg/l BAP along with 1 mg/l Kn respectively.

(Photographs were taken 3 months after inoculation)

4.3.4 Effect of different concentrations and combinations of BAP and Kn towards regeneration in BINA Chinabadam-2 variety

Response towards hormone supplementation was similar in BINA Chinabadam-2. Indirect regeneration was observed when BAP was applied singly in MS media but regeneration was direct in case of low amount of Kn along with BAP.

Immature leaflet explant showed shoot regeneration between 37.14 and 48.57 percentage. This percentage is not highly significant but number of shoots per explant ranged from 7.49 to 15.33 which are considerably high. Nevertheless, the length of those shoots was not high enough for root induction (**Table 4.12**, **Fig. 4.4**).

In case of decapitated half embryo explants, response was rapid with mean number of shoots per explant recorded to be approx 3. Newly regenerated shoots were healthy and as long as 3 cm within 40-47 days (**Table 4.13**, **Fig. 4.4**).

Table 4.12: Regeneration response of leaflet explants towards different concentrations and combinations of BAP and Kn in BINA Chinabadam-2 variety

Hormone		Responsive explants (%)	Callus type	Shoot regenerating explants (%)	Shoots/ explant
BAP (mg/l)	Kn (mg/l)				
2.0	-	85.71 (11.26)	Indirect	48.57 (01.77)	11.20 (0.31)
2.0	0.5	85.71 (12.11)	Direct	47.42 (01.73)	15.33 (0.37)
5.0	-	78.57 (10.04)	Indirect	37.14 (01.25)	7.85 (0.05)
5.0	0.5	71.43 (09.51)	Direct	37.14 (02.59)	7.49 (0.07)
7.0	-	71.43 (08.98)	Indirect	0 (0)	0 (0)
7.0	0.5	78.57 (10.03)	Direct	0 (0)	0 (0)

SD value is contained within parenthesis.

Table 4.13: Effect of different concentrations and combinations of BAP and Kn on regeneration of BINA Chinabadam-2 from decapitated half embryo explant

Hormone		Number of shoots explant	Length of Shoots in 30 days (cm)	Days required for shoot length >3cm (days)
BAP (mg/l)	**Kn (mg/l)**			
2.0	0.5	1.63 (0.42)	1.91 (0.13)	40.9 (1.58)
2 .0	1.0	1.68 (0.38)	1.94 (0.10)	47.8 (2.03)
3 .0	0.5	3 (0.32)	1.93 (0.06)	42.6 (1.88)
3.0	1.0	3 (0.36)	2.11 (0.09)	41.13 (1.79)
5 .0	0.5	2.29 (0.54)	1.94 (0.16)	40.71 (1.98)
5 .0	1.0	2 (0.63)	1.80 (0.15)	40.0 (2.23)

SD value is contained within parenthesis.

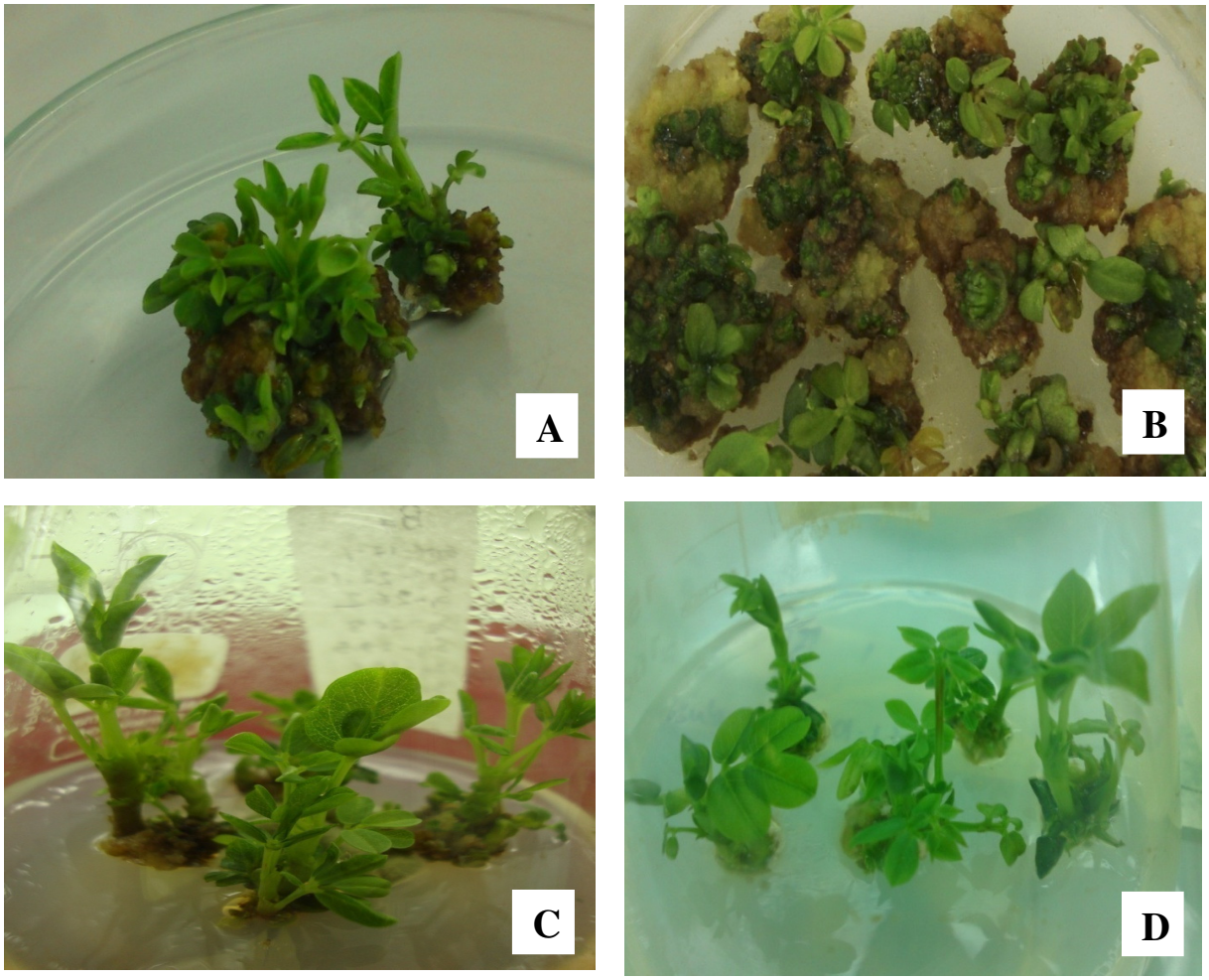

Fig. 4.4: A. Indirect regeneration from leaflet explants of BARI Chinabadam-7 in MS medium with 2 mg/l BAP and **B.** Indirect regeneration from leaflet explants of BARI Chinabadam-7 in MS medium with 2 mg/l BAP along with 0.5 mg/l Kn, **C-D.** Regeneration from half decapitated embryo explants of BARI Chinabadam-7 in MS medium with 3 mg/l BAP along with 0.5 mg/l and 3 mg/l BAP along with 1 mg/l Kn respectively.

(Photographs were taken 3 months after inoculation)

4.3.5 Effect of different concentrations and combinations of BAP and Kn towards regeneration in BINA Chinabadam-4 variety

When similar hormonal treatment was given in BINA Chinabadam-4 similar response was observed. Indirect regeneration was observed with only BAP supplementation but regeneration was direct when low amount of Kn alone with BAP was added in MS media. Here from 30.82 to 51.42 percent of immature leaflet explant regenerated. Though this percentage is not high but the number of shoots per explant was as high as 13.05 cm. But the length of those shoots was not high enough for root induction (**Table 4.14**, **Fig. 4.5**).

On the other hand, in case of decapitated half embryo explants, quick response was observed. Mean number of shoots per explant was 2.63 and newly regenerated shoots were healthy. They found to obtain 3 cm of length within 40-42 days (**Table 4.15**, **Fig. 4.5**).

Table 4.14: Regeneration response of leaflet explants towards different concentrations and combinations of BAP and Kn in BINA Chinabadam-4 variety

Hormone		Responsive explants (%)	Type of regeneration	Shoot regenerating explants (%)	Shoots/ explant
BAP (mg/l)	Kn (mg/l)				
2.0	-	92.86 (06.77)	Indirect	51.42 (02.47)	13.05 (0.12)
2.0	0.5	85.71 (07.66)	Direct	48.25 (01.83)	11.67 (0.51)
5.0	-	71.43 (10.59)	Indirect	30.82 (01.49)	6.22 (0.04)
5.0	0.5	71.43 (12.03)	Direct	42.85 (01.50)	5.41 (0.07)
7.0	-	57.14 (14.55)	Indirect	0 (0)	0 (0)
7.0	0.5	57.14 (10.62)	Direct	0 (0)	0 (0)

SD value is contained within parenthesis.

Table 4.15: Effect of different concentrations and combinations of BAP and Kn on regeneration of BINA Chinabadam-4 from decapitated half embryo explant

Hormone		Number of shoots/ explant	Length of shoots in 30 days (cm)	Days required for length >3cm (days)
BAP (mg/l)	**Kn (mg/l)**			
2.0	0.5	1.78 (0.16)	2.43 (0.06)	40.33 (0.78)
2 .0	1.0	2.11 (0.28)	2.47 (0.10)	39.44 (1.08)
3 .0	0.5	3.33 (0.18)	2.56 (0.03)	39.89 (1.11)
3.0	1.0	3.63 (0.23)	2.31 (0.05)	41.25 (0.56)
5 .0	0.5	2.30 (0.21)	2.29 (0.10)	42.4 (1.24)
5 .0	2.25	2.25 (0.26)	2.30 (0.09)	42.13 (1.17)

SD value is contained within parenthesis.

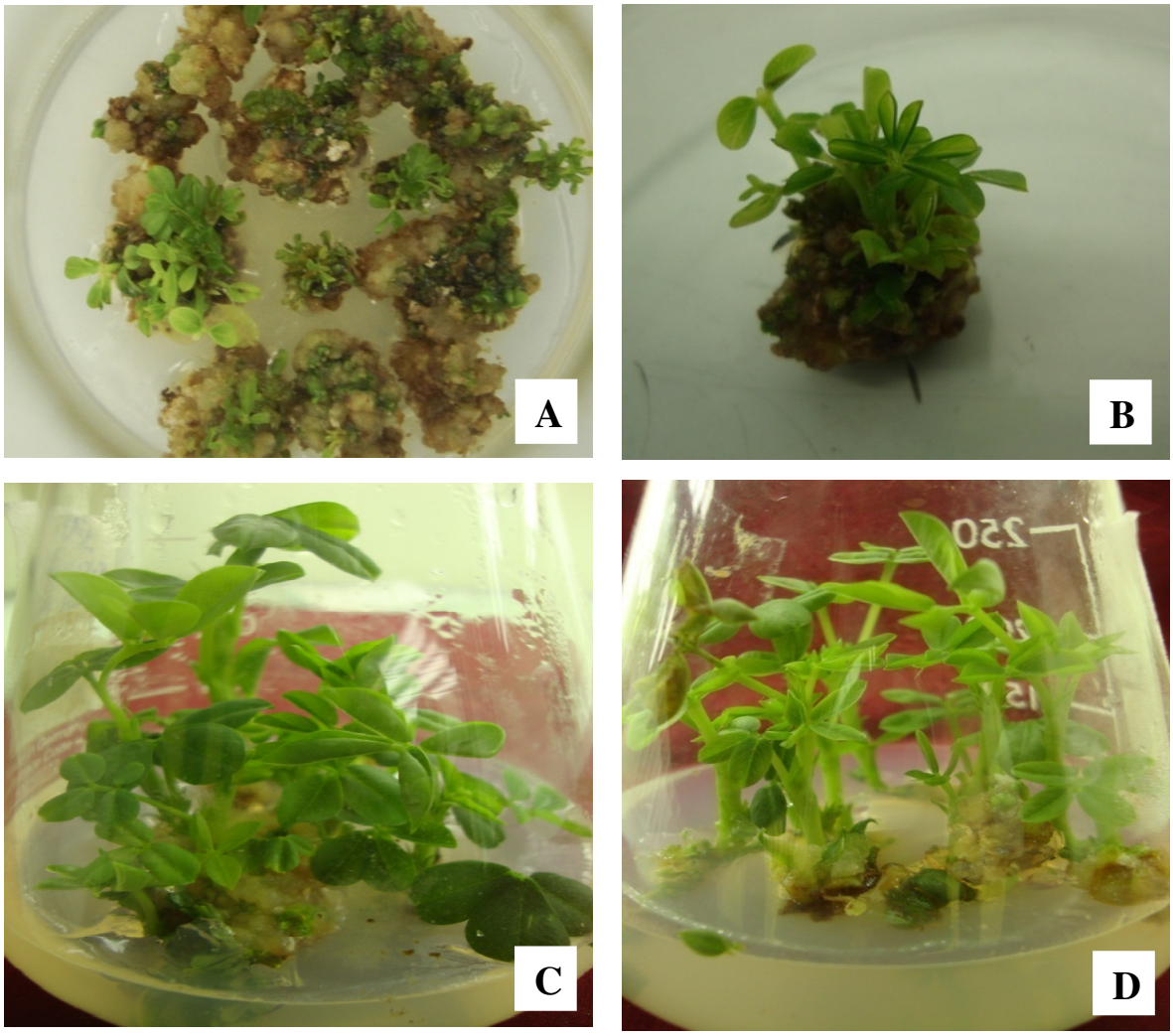

Fig. 4.5: A. Indirect regeneration from leaflet explants of BARI Chinabadam-7 in MS medium with 2 mg/l BAP and **B.** Indirect regeneration from leaflet explants of BARI Chinabadam-7 in MS medium with 2 mg/l BAP along with 0.5 mg/l Kn, **C-D.** Regeneration from decapitated half embryo explants of BARI Chinabadam-7 in MS medium with 3 mg/l BAP along with 0.5 mg/l and 3 mg/l BAP along with 1 mg/l Kn respectively.

(Photographs were taken 3 months after inoculation)

4.3.6 Effect of different concentrations and combinations of BAP and Kn towards regeneration in BINA Chinabadam-6 variety

Indirect regeneration was observed when only BAP was applied in MS media but direct regeneration was achieved when BAP and low amount of Kn was added from in BINA Chinabadam-6 variety.

Here 35.37 to 51.61 percent of leaflet explants showed shoot regeneration. This percentage is low but the number of shoots per explant was as high 13.20. However, here also the length of those shoots was not high enough for root induction (**Table 4.16**, **Fig. 4.6**).

On the other hand, in case of decapitated half embryo explants, response was faster than that of the previous explant. Mean number of shoots per explant was 2.63 and newly regenerated shoots were healthy. They became as long as 3 cm within 39-42 days (**Table 4.17**, **Fig. 4.6**).

Table 4.16: Regeneration response of leaflet explants towards different concentrations and combinations of BAP and Kn in BINA Chinabadam-6 variety

Hormone		Responsive explants (%)	Type of regeneration	Shoot regenerating explants (%)	Shoots/ explant
BAP (mg/l)	Kn (mg/l)				
2.0	-	92.86	Indirect	47.14	13.20
2.0	0.5	100	Direct	51.61	10.50
5.0	-	71.43	Indirect	35.37	5.80
5.0	0.5	71.43	Direct	41.28	6.34
7.0	-	57.14	Indirect	0	0
7.0	0.5	57.14	Direct	0	0

SD value is contained within parenthesis.

Table 4.17: Effect of different concentrations and combinations of BAP and Kn on regeneration of BINA Chinabadam-6 from decapitate half embryo explant

Hormone		Number of shoots/ explant	Length of shoots in 30 days (cm)	Days required for length >3cm (days)
BAP (mg/l)	**Kn (mg/l)**			
2.0	0.5	2.20 (0.14)	2.25 (0.15)	40.8 (1.68)
2 .0	1.0	2.33 (0.06)	1.98 (0.21)	40.17 (1.56)
3 .0	0.5	3.39 (0.05)	2.4 (0.19)	39.43 (1.23)
3.0	1.0	3.50 (0.08)	2.43 (0.13)	40.12 (1.16)
5 .0	0.5	2.53 (0.12)	2.61 (0.21)	39.38 (1.64)
5 .0	1.0	2.25 (0.14)	2.29 (0.22)	42.5 (1.71)

SD value is contained within parenthesis.

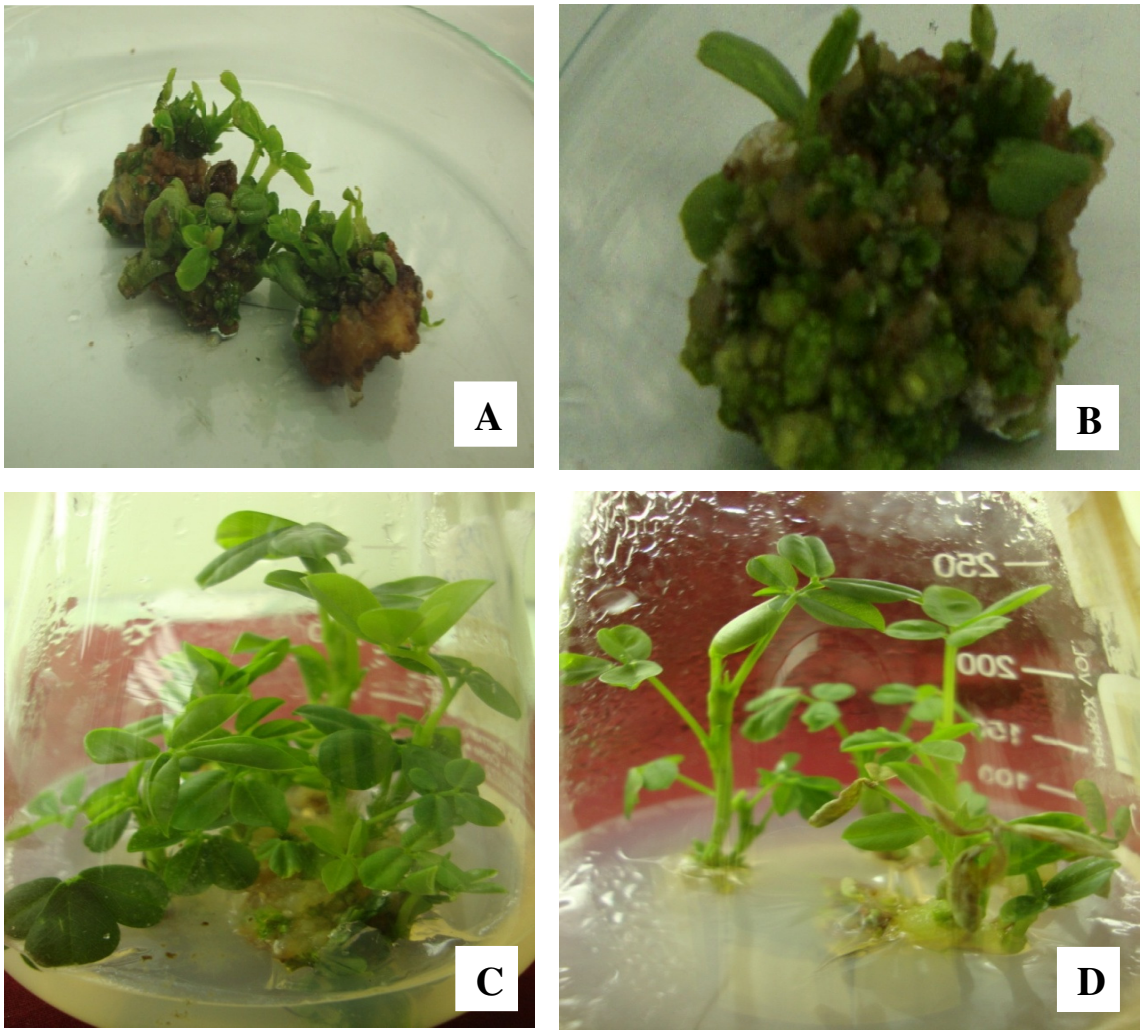

Fig. 4.6: A. Indirect regeneration from leaflet explants of BARI Chinabadam-7 in MS medium with 2 mg/l BAP and **B.** Indirect regeneration from leaflet explants of BARI Chinabadam-7 in MS medium with 2 mg/l BAP along with 0.5 mg/l Kn, **C-D.** Regeneration from decapitated half embryo explants of BARI Chinabadam-7 in MS medium with 3 mg/l BAP along with 0.5 mg/l and 3 mg/l BAP along with 1 mg/l Kn respectively.

(Photographs were taken 3 months after inoculation)

4.4 Induction of roots from regenerated shoots

Root formation is a vital step to produce plantlet. Spontaneous *in vitro* root induction did not occur during this study. Therefore, 2.5-4.2 cm long shoots were excised, and then cultured on half strength of MS media, supplemented with various concentrations of three auxines. The result of root induction in all the six varieties is presenting in **Table 4.18**.

Here the effectiveness of the auxines, namely, IAA, IBA, and NAA was investigated. The best response for root induction was obtained on half strength MS medium supplemented with 0.2 mg/l IBA and resulted well developed root were giving highest survival rate at acclimatization stage (**Table 4.18**, **Fig. 4.7**).

No significant difference was found among the cultivars during rhizogenesis but highly significant difference was found among different auxin supplementation usage (**Table 4.18)**. An interesting observation in this stage, in using the same hormone supplementation highly significant variation on rooting response was seen different season of the year. During March-May highest percentage of root induction was found but no rooting response was observed during December-November. Not a single root formation was observed in winter. This seasonal effect on rhizogenesis was present in all the peanut varieties (**Table 4.19**).

Table 4.18: Comparison of effect of different auxines on rooting in all the peanut varieties

Varieties	No. of shoots inoculated for rooting	Factors	IAA (mg/l)		NAA (mg/l)		IBA (mg/l)	
			0.1	0.2	0.1	0.2	0.1	0.2
BARI Chinabadam-7	10	Response (%)	20	30	20	40	30	60
		Roots per shoot	2	2.5	2.5	4.5	4	6.5
		Length of roots (cm)	2.5	3	2.5	3.5	4	6
		Survival rate (%)	0	33.33	0	50	33.33	50
BARI Chinabadam-8	10	Response (%)	20	30	30	40	40	60
		Roots per shoot	2	2.5	3	3.5	3	4
		Length of roots (cm)	2.5	3.5	2.5	3.5	4	7
		Survival rate (%)	0	33.33	0	50	50	83.33
Dhaka Chinabadam-1	10	Response (%)	10	20	20	30	30	70
		Roots per shoot	2	2.5	2.5	2.5	3.5	4.5
		Length of roots (cm)	2.5	3.5	2.5	4	4	6
		Survival rate (%)	0	50	0	66.66	33.33	85.70
BINA Chinabadam-2	10	Response (%)	20	20	20	40	40	70
		Roots per shoot	2.5	3.5	2.5	3.5	3	4
		Length of roots (cm)	2.5	3.5	2.5	3.5	4	5.5
		Survival rate (%)	0	50	0	50	50	71.42
BINA	12	Response (%)	8.33	16.66	16.66	25	25	83.33

Varieties	No. of shoots inoculated for rooting	Factors	IAA (mg/l)		NAA (mg/l)		IBA (mg/l)	
			0.1	**0.2**	**0.1**	**0.2**	**0.1**	**0.2**
Chinabadam-4		Roots per shoot	2	2.5	4	3	2.5	5
		Length of roots (cm)	2.5	2.5	2.5	3.5	4	8
		Survival rate (%)	0	50	0	33.33	66.66	70
BINA Chinabadam-6	15	Response (%)	13.33	20	6.66	20	13.33	80
		Roots per shoot	2.5	3	2.5	3.5	3	4
		Length of roots (cm)	2.5	2.5	2.5	4	3.5	8
		Survival rate (%)	0	0	0	50	50	75

Table 4.19: Seasonal impact on root regeneration

Season	Percentage of the developed roots	Mean no. of root per shoot
March - May	97.5	6.5
June - August	30	3.5
September- November	0	0
December - February	63.55	4.5

Fig. 4.7: Rhizogenesis and root development capacity of different Auxines. **A-F.** root formation during the effect of 0.1 mg/l IAA, 0.2 mg/l IAA, 0.1 mg/l IBA, 0.2 mg/l IBA, 0.1 mg/l NAA, 0.2 mg/l NAA.

(All the photographs were taken after 40 days of inoculation.)

Graph 4.1: Seasonal impact on root regeneration from regenerated shoots

4.5 Establishment and reproducibility examination of the plantlets

After sufficient development of roots, the plantlets of all six peanut varieties were successfully transplanted into small plastic pots. The survived plantlets were transferred to larger clay pots for their further growth and development **(Fig. 4.8)**.

After 1-3 months of transplantation, plantlets were matured and set flowers in 4-5 months **(Fig. 4.9)**, they started pod formation After planting, within 6 months matured pods was collected from those plants **(Fig. 4.10)**.

The seeds of different varieties were again allowed to germinate in the same germination medium and viability of these seed was 60-90% **(Table 4.20, Fig. 4.11)**

Fig. 4.8: A. Tissue cultured plantlets of different varieties.

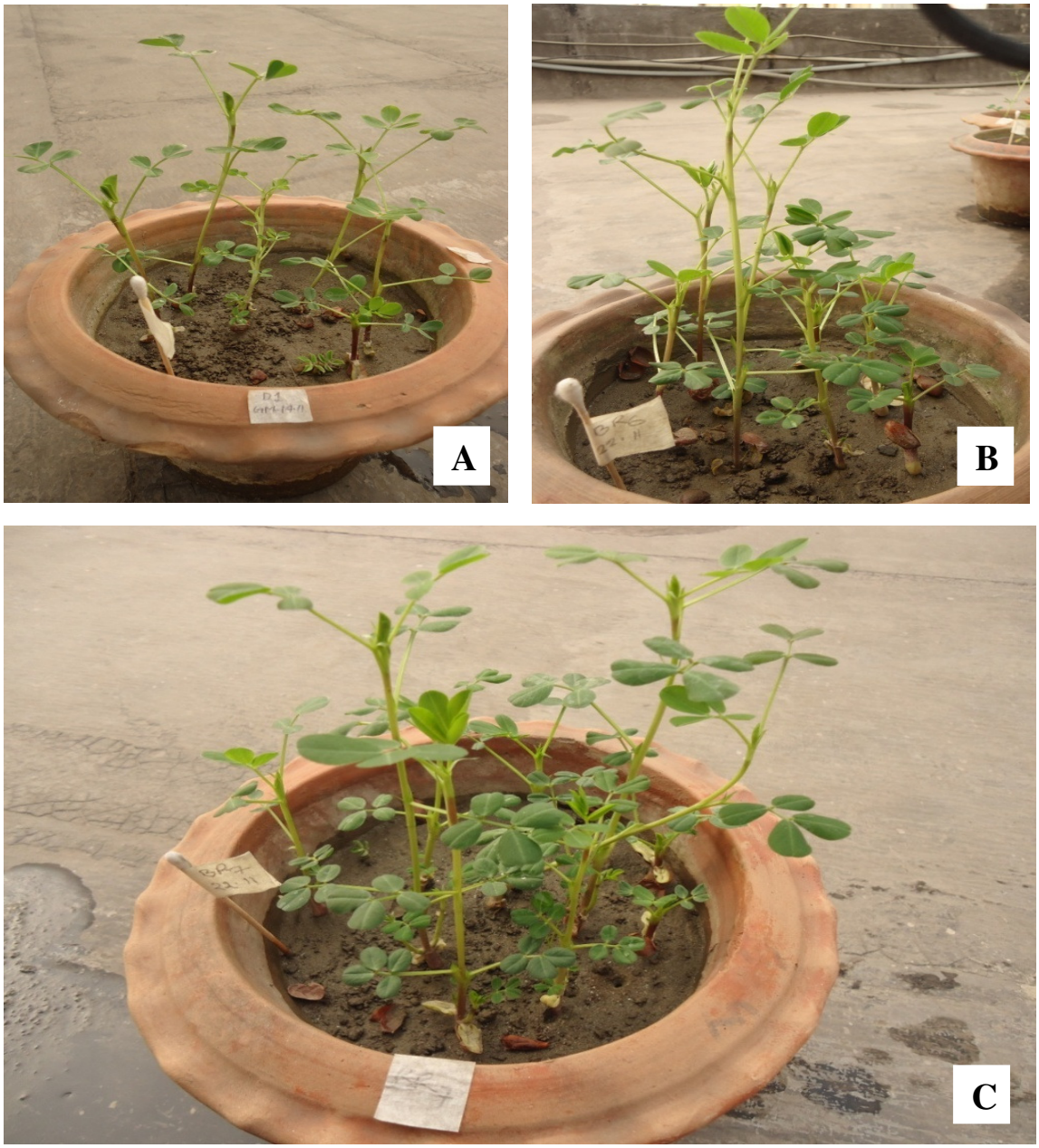

Fig. 4.9: R_1 generation seedlings after seed viability test. R_1 generation seedlings of **A.** Dhaka Chinabadam -1, **B.** BINA Chinabadam -6, **C.** BARI Chinabadam -7.
(Photograph was taken after 15 days of seed sowing)

Fig. 4.10: Flowering of different chinabadam varieties. A-F, Flowering of BINA Chinabadam -6, BINA Chinabadam -4, BINA Chinabadam -2, BARI Chinabadam -7, BARI Chinabadam -8, Dhaka Chinabadam -1 respectively.

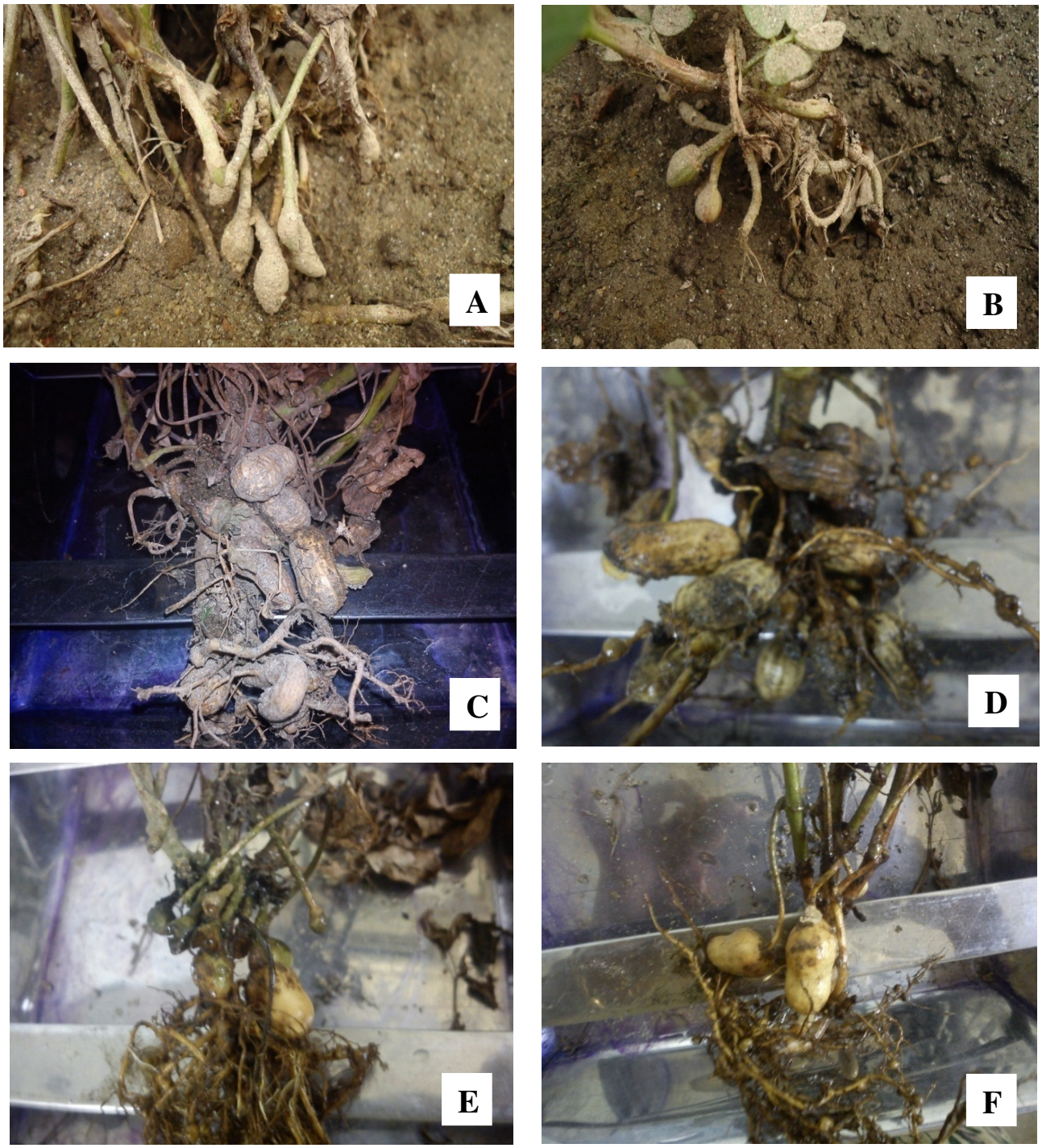

Fig. 4.11: Pod formation of different chinabadam varieties. A-F, Pod formation of BINA Chinabadam -6, BINA Chinabadam -4, BINA Chinabadam -2, BARI Chinabadam -7, BARI Chinabadam -8, Dhaka Chinabadam -1 respectively.

Table 4.20: Performance of tissue cultured plantlet of different varieties during acclimatization

Varieties	No. of acclimatized plantlets	No. of survived plantlets in the net house (%)	Pod producing plants (%)	No. of pods per plant	Seed viability (%)
BARI Chinabadam-7	10	60.00	83.33	1.5 (0.75)	60
BARI Chinabadam-8	10	70.00	85.71	3 (1.03)	70
Dhaka Chinabadam-1	10	60.00	83.33	2.5 (0.82)	70
BINA Chinabadam-2	15	86.67	92.31	2.5 (0.84)	80
BINA Chinabadam-4	15	93.33	100.00	5.5 (2.63)	90
BINA Chinabadam-6	18	94.44	100.00	6.5 (2.50)	90

Therefore, reproducibility of the seeds from tissue cultured plants were achieved as well as, the reproducibility of the protocol was established.

Chapter: 5

Discussion

Discussion

Attempts on developing tissue culture protocol for peanut go as early as 18th century (Harvey and Schulz 1943, Nuchowiz *et al*. 1955). Since then, several attempts have been reported through both organogenesis and embryogenesis (Kently *et al.* 1991 and 1989, Cheng *et al.* 1992, Pestana *et al.* 1999, Eapen and George 1993, Saxena *et al.* 1992, Baker and Wetzstein 1992, Gill *et al.* 1992 and 1999, Hajra *et al.* 1989, Haque *et al.* 1991, Sarkar *et al.* 2000, Ozias-Akins *et al.* 1992, Marion *et al.* 2008, Singh and Hazra 2009, Bhatnagar *et al.* 2010, Tiwari and Tuli 2008).

There are also some reports also on transgenic peanut using these regeneration systems (Higgins and Dietzgen 2000, Sarker *et al.* 2003, Sharma *et al.* 2000, Anuradha *et al.* 2006, Tiwari and Tuli 2011, Sarkar and Islam 1999 and 2000). However, due to inconsistency of regeneration response and variation of totipotency among peanut varieties and its explants, peanut is still considered as a recalcitrant crop. This is also true for Bangladeshi varieties. Few regeneration reports are available on Bangladeshi varieties using various explants (Sarker and Islam 1997 and 1999, Sarker *et al.* 2000, Nahar *et al*. 2002, Akter *et al*. 2006, Mondol *et al*. 2009, Honey *et al*. 2010), but we are yet to identify any ultimate regeneration protocol that can be applied for efficient transformation.

Considering this background, the present study was aimed to develop an efficient and reproducible regeneration protocol for farmer popular Bangladeshi peanut (*Arachis hypogaea* L.) varieties. In this study, six varieties, namely, Dhaka Chinabadam – 1, BARI Chinabadam – 7, BARI Chinabadam – 8, BINA Chinabadam – 2, BINA Chinabadam – 4, and BINA Chinabadam – 6 were used. The study was performed in three phases. The first phase was carried out to compare two explants and find out the most efficient explant for regeneration, second phase was for the optimization of hormone uses for shoot and root induction, and the last phase was to investigate the reproducibility of the methodology adopted in the process.

In the past, several attempts had been made to develop a suitable plant regeneration protocol in peanut. The plant regeneration was reported through tissue culture *via* organogenesis using a wide range of explants, like, leaflet, mature leaf, cotyledon, epicotyls and hypocotyls (Morginski *et al.* 1981, Atreya *et al.* 1984, Pittman and Dunbar 1992, Mc Kently *et al.* 1991, Hoque *et al.* 1991, Cheng *et al.* 1992, Bhuiyan 1994, Sarker *et al.* 1997, Sarker and Islam 1999) and via somatic embryogenesis from immature zygotic embryos and leaflets (Hazra *et al.* 1989, Ozias-

Akins *et al.* 1989, Baker and Wetzstein 1992). Sarker and Islam (1999) had established an efficient regeneration protocol for Bangladeshi peanut varieties through callus as well as without the intervention of callus from leaflet explants. There are many reports available on these two explants, one is leaflet and another one is embryo. These are prominent plant parts for regeneration but case-by-case comparison of these explants need to be evaluated to find out a concise outcome.

In addition, a few more immature, young explants are also explored for regeneration of legumes. Embryo and decapitated half embryo with and without single cotyledon were reported for pea (Schroeder *et al*. 1993), chickpea and lentil (Jayanand *et al*. 2003, Tewari-Singh *et al*. 2004). So, at the first phase of this study, leaflet and decapitated half embryo explants were examined to find out the most responsive explants for *in vitro* culture.

In this present study, regeneration was obtained and compared from both leaflet and decapitated half embryo explant. Cotton soaked with distilled water has been used to germinate seeds for collecting immature leaflet. This media was found to be efficient which is in accord with Sarker and Islam (1999). In this present study, 8-15 days old germinated seeds were found to be effective for immature leaflet collection.

The dependent factors for callus and shoots induction are composition, concentration, and different combination of growth regulators, like, BAP and Kn in culture medium. In this present study MS medium supplemented with 2, 5 and 7 mg/l BAP with and without 0.5 mg/l Kn were used. BAP singly gave regeneration via callus but Kn containing media resulted in direct regeneration from leaflet explants in all the varieties. Here, 5mg/l BAP with 0.5mg/l Kn was found best for BARI Chinabadam-7 and BARI Chinabadam-8 to attain direct regeneration from leaflet explants. On the other hand, 2mg/l BAP with 0.5mg/l Kn was found best for Dhaka Chinabadam-1, BINA Chinabadam-2, BINA Chinabadam-4, and BINA Chinabadam-6. Therefore, optimum hormonal supplementation for shoot development was found to be variety dependent. This is contradictory to previous report by Sarker and Islam (1997). They reported, 5mg/l BAP with 0.5mg/l Kn was best of direct regeneration in Dhaka Chinabadam-1, DM-1, Acc no.-12, DG-2 varieties. Moreover, they did not study the varieties produced by BINA which have been evaluated here. The difference in response may be due to use of different genotypes.

Regeneration of sufficient number of elongated shoots was reported from same explant earlier (Sarker and Islam 1997) by using BAP and Kn as the growth hormones whereas, in the present study, shoots were found not elongated enough in the same condition from leaflet explant.

Earlier reports with Bangladeshi varieties show that, higher concentration of BAP gave the best (5 mg/l BAP) result there (Sarker and Islam 1997, Sarker and Islam 1999, Sarker and Islam 2000, Sarker *et al.* 2000). So, effect of more than 5 mg/l BAP was also examined in present study to find out whether or not there is actually any significance of higher BAP concentration. Though highest callus regeneration was obtained from such culture, they eventually died due to overdose of hormone afterward. Some were facing problem of length of shoots so, they started adding GA_3, IAA for shoot elongation (Nahar *et al*. 2002, Akter *et al*. 2006, Mondol *et al*. 2009, Honey *et al*. 2010)

Previously embryo was found as a prominent explant in case of the recalcitrant plants, like, peanut as presence of embryo axes shows better regeneration capability than cotyledon segments (Atreya *et al*. 1984). For several Bangladeshi varieties, viz, Dhaka Chinabadam-1, BARI Chinabadam-8, BINA Chinabadam-2 and BINA Chinabadam-3, decapitated half embryo explant was reported as viable explant (Honey *et al*. 2010). According to that context, the apical part and root cap was detached to get only the radical which was halved through longitudinal section to create three wound sites. The decapitated half embryo was cultured in presence of 2, 3 and 5 mg/l of BAP with 0.5 or 1 mg/l Kn. Here, 3mg/l BAP with 1 mg/l Kn supplemented medium was found suitable for rapid regeneration from this explant. Moreover, all six cultivars gave more or less identical level of shoot regeneration except for BARI Chinabadam-8 which showed better response than others. There are previous reports, supported on genotype dependent regeneration (Chowdhury *et al.* 1992, Mckently *et al.* 1989). Large numbers of shoots were regenerated and through subculture in same media leading to up to five times increase of shoot numbers. Additionally, from this explant, enough elongated multiple shoots in short span of time were obtained in contrast to leaflet explant.

Root was not spontaneously induced on the regenerated shoots. So after having sufficiently elongated shoots, root induction became essential for successful plantlet development. For these purposes, half strength MS medium supplemented with 0.1 and 0.2 mg/l of IAA, IBA or NAA were examined. Half strength MS medium consisting of 0.2 mg/l IAA was reported to be the best

for root induction previously by Sarker and Islam (2000) and Akhter (2006). However, 0.2 mg/l. NAA was reported as the best auxin by Sarker and Islam (1999) and Nahar (2002). In previous reports of Mandol (2009) and Honey (2010), auxin preference was dependent upon genotypes. Hormone IBA was reported to be the best in some BARI and BINA varieties (Honey *et al*. 2010). Similarly here, 0.2 mg/l IBA resulted to be the best hormonal supplement for root induction for both BARI and BINA Chinabadam varieties.

All of the six varieties were same in root formation but the BINA varieties responded better in root induction as it gave the highest number of developed roots *in vitro*.

One of the most interesting aspects of this study was that, seasonal impact was observed during *in vitro* root induction. Root induction occurred from December to August but not during the rest of the year. Moreover, the regeneration percentage also varies from seasons to seasons. On the other hand, from September to November no root was developed. Such observation has not been reported in any previous study.

After the acclimatization stages, the survived plantlets were introduced in net house. The plant physiology was routinely examined. In natural course of time plantlets set to form flowers and pods as well. After getting matured, fruits were developed from the pods. The pod of the tissue-cultured plants was investigated. In the previous studies, with some Bangladeshi popular peanut varieties, germination percentage was reported to be slightly low (Mandol *et al.* 2009), compared to the parent seeds. In the present experiment, germination percentages were similar compared to that of the parental line. Among all the varieties, the BINA varieties showed highest percentage of reproducibility of tissue cultured plants.

Chapter: 6

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Annex 1. Stock solution composition of MS medium.

Component	Amount

Macro nutrients (10x)	Mg/l
KNO3	1900
NH4NO3	1650
MgSO4.2H2O	370
CaCl2.2H2O	440
KH2PO4	170

Minor salts (100x)	Mg/l
KI	0.83
H3BO	3 6.2
MnSO4.4H2O	22.3
ZnSO4.7H2O	8.6
Na2MoO4.2H2O	0.25
CuSO4.5H2O	0.025
CoCl2.6H2O	0.025

Iron EDTA solution (100x)	Mg/l
FeSO4.7H2O	27.8
Na2EDTA.2H2O	37.3
Organics (100x)	Mg/l
Nicotinic acid	0.5
PyridoxinHCl	0.5
ThaiminHCl	0.1
Glycine	2.0

Annex 2. Two-way ANOVA analysis of shoots per explant from leaflet explant

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Rows	1066.99	5	213.398	1.716439	0.167604
Columns	1271.361	5	254.2721	2.045205	0.106652
Error	3108.15	25	124.326		
Total	5446.5	35			

Annex 3: Two-way ANOVA analysis of shoots per explant from leaflet explant

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Rows	50.23518	5	10.04704	1.306468	0.293092
Columns	752.7522	5	150.5504	19.57685	6.36E-08
Error	192.2557	25	7.690227		
Total	995.2431	35			

Annex 4. Two-way ANOVA analysis of shoot regeneration response from leaflet explant

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Rows	60.31278	5	12.06256	0.268729	0.925921
Columns	15420.78	5	3084.157	68.70881	8.6E-14
Error	1122.184	25	44.88736		
Total	16603.28	35			

Annex 5. Two-way ANOVA analysis of number of shoots per explant for the regeneration from half decapited embryo explants

ANOVA

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***
Rows	6.33872222	5	1.26774444	13.93588591	1.48644
Columns	0.89562222	5	0.17912444	1.969054436	0.11841
Error	2.27424444	25	0.09096977		
Total	9.50858888	35			

Annex 6. Two-way ANOVA analysis of length of shoots for the regeneration from half decapited embryo explants

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Rows	0.269113889	5	0.053822778	0.926975592	0.480179936
Columns	5.923013889	5	1.184602778	20.40210309	4.25466E-08
Error	1.451569444	25	0.058062778		
Total	7.643697222	35			

Annex 7. Two-way ANOVA analysis of time requirement for the regeneration from half decapited embryo explants

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Rows	45.06013333	5	9.012026667	1.299990287	0.295651363
Columns	585.8555667	5	117.1711133	16.90200383	2.57088E-07
Error	173.3095	25	6.93238		
Total	804.2252	35			