IN VITRO REGENERATION AND DEVELOPMENT OF STRESS TOLERANT PEANUT (*ARACHIS HYPOGAEA* L.) THROUGH *AGROBACTERIUM*-MEDIATED GENETIC TRANSFORMATION

Ph.D. THESIS

BY

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DECEMBER, 2017

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IN VITRO REGENERATION AND DEVELOPMENT OF STRESS TOLERANT PEANUT (*ARACHIS HYPOGAEA* L.) THROUGH *AGROBACTERIUM*-MEDIATED GENETIC TRANSFORMATION

A DISSERTATION
SUBMITTED TO THE UNIVERSITY OF DHAKA
IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
BOTANY

BY

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Dedicated
To
My Respected Parents
And
Beloved Family

CERTIFICATE

This is to certify that the research work embodying the results reported in this thesis entitled "In vitro regeneration and development of stress tolerant peanut (Arachis hypogaea L.) through Agrobacterium-mediated genetic transformation" submitted by Tanjina Akhtar Banu, has been carried out under our supervision in the Plant Breeding and Biotechnology Laboratory of the Department of Botany, University of Dhaka. It is further certified that the research work presented here is original and suitable for submission for the degree of Doctor of Philosophy in Botany, under the University of Dhaka.

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ACKNOWLEDGEMENT

My heartiest thanks to almighty ALLAH for providing me patience, strength, energy and courage to complete my research work within the limited time frame.

This is my greatest opportunity to acknowledge my sincere gratitude to my supervisors Professor Dr. M. Imdadul Hoque and Professor Dr. Rakha Hari Sarker of the Department of Botany, University of Dhaka, for their intellectual guidance, constructive criticism and untiring supervision during my entire study period. My deepest gratitude goes to them for their support and incessant encouragement. I am always grateful to them for giving me a chance to work under their generous supervision.

I express my gratitude to Professor Dr. M. M. Haque and Professor Dr. M. Nurul Islam, Department of Botany, University of Dhaka, for their valuable suggestions, support and encouragement during my research work.

Sincere and heartiest thanks goes to Rita Sarah Borna and Dr. Tahmina Islam, Assistant Professor, Department of Botany, University of Dhaka, for their inspiration, immense assistance to my work and kind help to complete the study.

The research work was undertaken under the United States Department of Agriculture (USDA) funded project entitled "Development of Stress Tolerant Peanut (*Arachis hypogaea* L.) Breeding Lines Using Modern Biotechnology" which is gratefully acknowledged.

I wish to express my deepest sense of gratitude to Md Faruque Ahmad, Chairman BCSIR for granting me leave to carry out my Ph.D. work. I am also grateful to Dr. Sarwar Jahan, Director, BCSIR laboratories Dhaka; Dr. Md Salim Khan (PSO), Section-in-charge, Plant Tissue Culture Section, BCSIR; Dr. Shahina Islam (CSO), BCSIR and all my colleagues at Plant Tissue Culture Section, BCSIR for their active support and co-operation during the study.

I am grateful to Professor Dr. Md. Abul Bashar, Chairman, Department of Botany as well as Professor Dr. Moniruzzaman Khondker and Professor Dr. Abdul Aziz, former Chairman, Department of Botany, University of Dhaka, for providing me necessary facilities of the Department to carry out my work.

I am also grateful to Professor Dr. Sheikh Shamimul Alam and Professor Dr. Mihir Lal Saha, Department of Botany, University of Dhaka, for their valuable advice and encouragement during this study. Sincere thanks goes to Dr. Sayeeda Sharmin Sultana, Assistant Professor, Department of Botany, University of Dhaka, Kishwar Jahan Shethi and Nadra Tabassum Lecturers, Department of Botany, University of Dhaka, for their inspiration.

All my appreciation goes to the members of Plant Breeding and Biotechnology Laboratory for their constant help and encouragement during the study. My special thanks to Iffat Ara Rahman, Research Associate; Dr. Subroto Kumar Das, Assistant Professor, Department of Botany, Barisal University; Dr. Md Ahashan Habib, SSO, BCSIR; Shefali Boonarjee, SSO, Tea Research Institute, Sylhet; Md. Hazrat Ali, Asst. Director, DSHE, Dhaka; Sujoy Kumar Bhajan, Research Associate; Ummey Honey, Assistant Commissioner, Peoples Republics of Bangladesh; Sanjida Rahman Mollika, Assistant Professor, Department of Botany, Jagannath University and all my seniors and juniors of Plant Breeding and Biotechnology Lab. whose co-operation had eased me long arduous laboratory hours. I also want to thank all the members of Cytogenetics laboratory, Department of Botany, University of Dhaka, for their generous cooperation.

Thanks to Md. Younus Miah, Md Shah Alam, Administration Officer, Rita Rani and Md. Salahuddin Lab. Attendant, Plant Breeding and Biotechnology Lab. for their kind help and assistance.

Last but not the least, I express my deepest and heartiest gratitude to my husband Muhammad Mehedi Hasan for his endless encouragement and providing an enabling environment as I pursued my Ph.D. degree. I express my deepest gratitude to my father, mother, mother -in-law, father in-law for their blessings and constant encouragement. I also thankful to my sister, brother, sister-in laws, brother-in-laws and cousins for their well wishes. I am also greatly indebted to my babies; Areesha and Umaiza. It is needless to say that without the support and endless encouragement of my family member it would not have been possible to complete my dream.

-The Author

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ABSTRACT

ABSTRACT

Peanut (*Arachis hypogaea* L.) is one of the most economically important oilseed crop of the world. Bangladesh has a great opportunity to cultivate peanut in the marginal lands where hardly any other economically important crops are growing. However, in Bangladesh peanut production is hampered due to the incidence of various fungal diseases. The modern techniques of biotechnology such as plant genetic transformation can be applied for its improvement towards the development of disease resistance.

The objective of the present investigation was to develop a fungal disease resistant peanut line through *Agrobacterium*-mediated genetic transformation. As a prerequisite of the transformation protocol, an efficient *in vitro* regeneration system was established for three locally grown peanut varieties, namely, Dhaka-1, BARI Chinabadam-8 and Binachinabadam-4. Four different types of explants, namely, immature leaflet, decapitated half embryo, de-embryonated cotyledon and single cotyledon attached decapitated embryo were used for regeneration. Highest percentage (71.8%) of multiple shoot regeneration was achieved when de-embryonated cotyledon explants initially cultured on 88.8 μM BAP, followed by two subsequent cultures on lower concentrations of BAP (66.6 μM BAP and 13 μM BAP) containing medium. It took about 45 days for the regeneration of multiple shoots.

Best response towards induction of roots for all the varieties was obtained when regenerated shoots were cultured on half strength of MS medium supplemented with either 2.5 μM IBA or 1.0 μM IAA. Fully developed *in vitro* regenerated plantlets were successfully established in soil for further growth and development.

For optimization of genetic transformation protocol, *Agrobacterium* strain LBA4404 containing binary vector plasmid pBI121GUS-NPTII harboring *GUS* (β-Glucoronidase) and *npt*II (neomycin phosphotransferase) genes (construct I) was used. Transient GUS histo-chemical assay revealed that among the three explants used maximum transient GUS expression (86.66%) was observed from de-embryonated cotyledon explants of variety Dhaka-1 having an optical density of 0.8 at 600 nm with 10 minutes incubation period. Transformed shoots were cultured on 200 - 250 mg/l kanamycin supplemented medium to

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select the putatively transformed shoots. Transformation frequency was found to be 0.34% in Dhaka-1 using pBI121GUS-NPTII construct.

Agrobacterium strain LBA4404 containing plasmid pCAMBIA2300enh35SAFP harboring *AFP* (antifungal protein) gene and *npt*II (neomycin phosphotransferase) (construct II) was used for the integration of gene of interest. De-embryonated cotyledon and single cotyledon attached decapitated embryo explants of variety Dhaka-1and BARI Chinabadam-8 were used for transformation experiments. Among the two explants best response was observed from de-embryonated cotyledon explants. In this case, transformation efficiency was found to be 1.52% in Dhaka-1 and 0.85% in BARI Chinabadam-8. A total 17 putatively transgenic plants (T_0) of Dhaka-1 were confirmed through PCR analysis. The integration of *npt*II gene was further confirmed by Southern blot hybridization in 10 T_0 peanut plants survived through acclimatization and developed till maturation. T_1 seeds were collected and raised following the Biosafety guidelines in the double layered insect proof net house for further investigation.

Peanut as a grain legume has been considered as recalcitrant towards *in vitro* plant regeneration as well as for genetic transformation. The results of both *in vitro* plant regeneration and *Agrobacterium*-mediated genetic transformation using marker as well as antifungal (gene of interest) gene has pave the way for developing fungal disease resistant peanut plants.

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1. INTRODUCTION

1. INTRODUCTION

Leguminosae or Fabaceae is a very important family of angiosperms comprising of many species in relation to human nutrition, pasture and fodder needs. Important protein rich seed bearing plants mostly herbaceous grain legumes are members of this family. Grain legumes play a significant role in the national economy, nutrition, food security and soil health of any country. They rank next to cereals in terms of human nutrition. There are several species and subspecies classified as food legumes. But, only few (15 to 20) genera are very important. Hundred of cultivars within these genera included in the agricultural practices, each having some selected attributes. Most important of these species are *Glycine max* L., *Arachis hypogaea* L., *Cicer arietinum, Lens culinaris* Medik, *Pisum sativum* L., *Lathyrus sativus* L., *Cajanus cajan* (L.) Millsp., *Vigna radiate* (L.) R. Wilczek, *Vigna mungo* (L.) Hepper, *Vigna aconitifolia* Jecq., *Vigna umbellate* Thunb., *Vigna unguiculata* L., *Phaseolus vulgaris* L., *Macrotyloma uniflorum* Lam. etc. These species constitute over 80% of total food legume output (Sunkara et al. 2007).

Peanut (*Arachis hypogaea* L.) is an important food legume crops and is considered as one of the principal economic oilseed crops of the world (Cobb and Johnson 1973). The genus *Arachis* belongs to the subfamily Papilionaceae. The botanical name is derived from the Greek word *Arachis* meaning ‟ legume” and *hypogaea* meaning ‟ below ground”, are referring to the formation of pods in the soil (Pattee and Stalker 1995). The cultivated peanuts have been recognized around the world by an assortment of interesting names. While Americans call it peanut, it is also known as African nut, Chinese nut, Manila nut, kipper nut, hawks nut, jar nut, earth chestnut, monkey nut, goober pea, ground pea, ground bean etc. (Gregory et al. 1980). The genus *Arachis* comprises 69 species placed under 9 sections (Kaprovickas and Gregory 1994). Section *Arachis* contains cultivated peanut. Cultivated peanut is classified into two subspecies on the basis of their branching pattern (Gibbons et al. 1972): subspecies *hypogaea* with alternate branching and subspecies *fastigiata* with sequential branching. Each subspecies is again divided into two and four botanical varieties, respectively; subsp. *hypogaea* into var. *hypogaea* (Virginia) and var. *hirsuta* (Runner); and subsp. *fastigiata* into var. *fastigiata* (Valencia), var. *vulgaris* (Spanish), var. *peruviana*, and var. *aequatoriana* (Krapovickas and Gregory 1994). Each of these botanical varieties has different plant, pod and seed characteristics.

Peanut was originated in South America. Evidence for the earliest known use of peanuts comes from an archaeological site in Peru and dates to about 3500 years ago (Hammons 1994). Different species belonging to the genus *Arachis* have been described, but with the exception of some primitive land races in South America, only *A. hypogaea* L. is grown for human consumption. With the exploration of the New World beginning in the 16th century, peanut quickly spread from South America to Africa and Asia along with other new world crops such as bean, squash, potato, tobacco and corn (Hammons 1994). In India the entry of peanut is probably from several routes, like Philippines, China and Japan (Talawar 2004). Peanuts are grown in most tropical, subtropical and temperate countries, especially in Africa, Asia and North and South America between 40° North and 40° South of the equator where temperatures range from 25°C to 30°C (Weiss 2000). Peanut is an allopolyploid (4x=40) meaning that the plant was derived from distinct A and B genomes. Several *Arachis* spp. have been proposed as the donor of each genome. The most recent evidence suggests that a cross between *A. ipanesis* and *A. duranensis* is the most likely origin. There would have been a genome duplication after hybridization occurred to produce fertile offspring (Favero et al. 2006).

The peanut is one of the world's most popular and universal legume crops. It is ranked 13th among the food crops of the world (production utilized directly as food or in confections) and cultivated in more than 100 countries in all six continents (Nigam 2014).The geographical classification of peanut is delineated in six regions: the America, Africa, Asia, New East Asia, Europe and Oceania (Gregory et al. 1980). The production of peanut is mainly concentrated in Asia, Africa and America where the global production was 59.8%, 29.8% and 10.4% respectively (FAO 2015). Developing countries account for about 97% of the world's peanut area and about 94% of total production (Freeman et al. 1999). Peanut is currently grown on about 25.45 million hectares worldwide and global production was 45.23 million tons in 2013 (FAOSTAT 2015). According to United States Department of Agriculture (USDA) in 2012-13, China led world groundnut production with 42.4%, India 14.5%, Nigeria 7.8%, USA 4.4%, Burma 3.7%, Indonesia 3.1%, Argentina 2.6%, Sudan 2.2%, of global peanuts for that year. According to the Food and Agriculture Organization of the United Nations, in 2013, the top 20 peanut producing countries were: China, India, Nigeria, USA, Sudan (former), Myanmar, Indonesia, Argentina, United Republic of Tanzania, Senegal, Cameroon, Ghana, Viet

Nam, Chad, Malawi, Democratic Republic of the Congo, Brazil, Uganda, Niger, and Burkina Faso.

In Bangladesh peanut *(Arachis hypogaea* L.) has been considered as one of the most important crops. Peanut is cultivated either as a rabi or as a kharif crop in the 21 regions in the district of Noakhali, Faridpur, Dinajpur, Dhaka, Pabna, Kishoreganj, Rangpur, Barisal, Tangail, Chittagong, Sylhet, Comilla, Jessore, Patuakhali, Bogra, Jamalpur, Mymensingh, Kushtia, Rajshahi, Chittagong HT and Khulna (Bulletin of Tropical Legumes 2012). In 2014 the area of peanuts cultivation in Bangladesh was in about 31,000 hectares and the annual production was about 50,000.00 tones. The average yield was recorded as 16,129 hg/ha. (FAO 2015).

Cultivated peanut (*Arachis hypogaea* L.) is a self-pollinated, tropical annual legume. Despite its name and appearance, the peanut is not a nut, but rather a legume. It is an annual herb that grows to about 60 cm tall. The stems and branches are sturdy and hairy. The leaves are opposite, pinnate with four leaflets that are egg-shaped, 2 to 10 cm long, 1 to 3 cm wide and sometimes hairy. The flowers are like a typical pea flower in shape, 2 to 4 cm across and yellow in colour. The pods, usually 25 to 50 mm long, bearing two or three seeds are oblong, roughly cylindrical with rounded ends, are contracted between the seeds and have a thin, netted, spongy shell. The seeds are oblong or square with wrinkled surfaces and there is diversity in seed coat color ranging from whitish to dark purple, but mahogany red rose and salmon predominate. Peanut is self-pollinating (Smith 1950) although natural cross-pollination due to the action of bees has been reported at a levels ranging from 0 to 3.9% depending on the season, genotype and location (Culp et al. 1968; Leuck and Hammons 1969; Gibbons and Tattersfield 1969). After pollination, the flower withers and a peg carrying the fertilized ovule is formed at the base of the flower. The growth of the peg is positively geotropic and upon contact with the soil, the tip starts to develop into a characteristic pod (Ntare 2007). Figure 1 shows the diagrammatic sketch of a typical peanut plant.

Deep well-drained sandy loam, or sandy clay loam soil with a pH of 6.5 - 7.0 and high fertility are ideal for peanut cultivation (Singh and Oswalt 1995). Such soil is preferred since it is usually loose and friable, permitting easier penetration of roots and pegs, better percolation of rainfall and easier harvesting. The optimum temperatures for growing peanut range from 25°C to 35°C. The early maturing small-seeded peanut varieties require 300-500 mm while the medium to late maturing large-seeded varieties need 1000-

1200 mm rainfall (Ntare et al. 2007). Although, they are relatively drought resistant, are sensitive to salinity and high soil acidity (pH<5). Saline soils are not suitable since peanut has a very low salt tolerance (Weiss 1983).

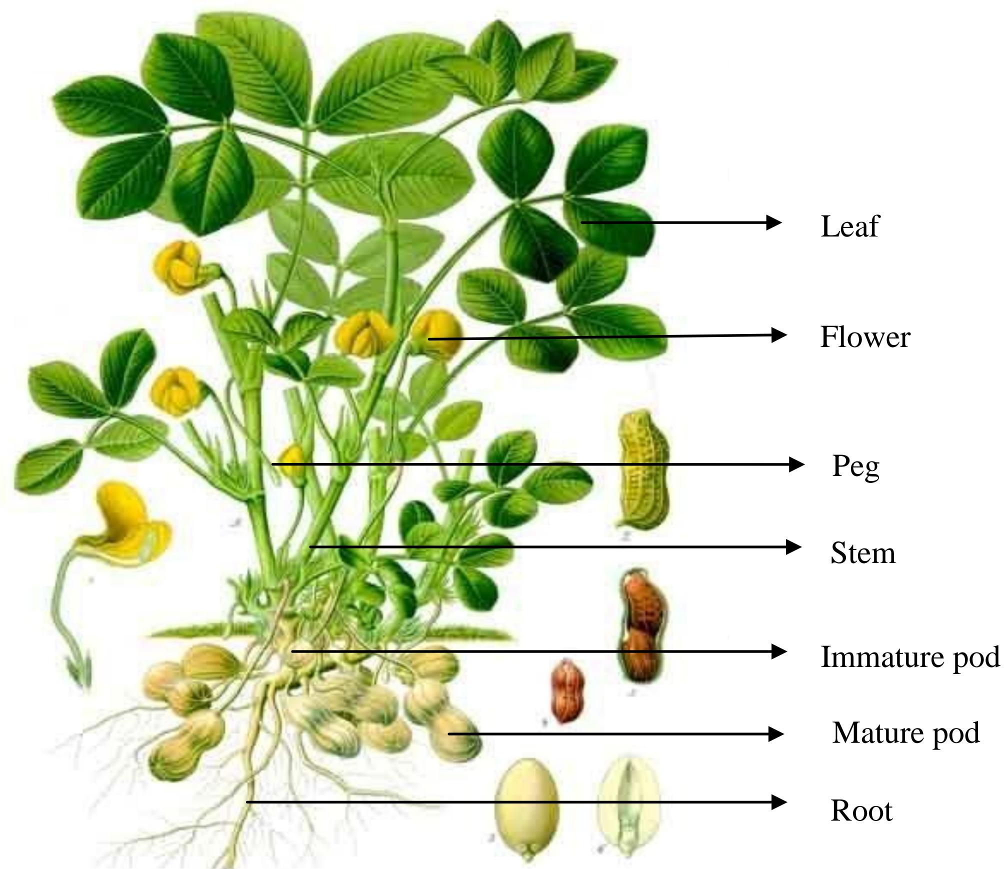

Fig. 1: A typical peanut (*Arachis hypogaea* L.) plant (www. Metafro .be/ prelude /view _ country.cc=SL).

Peanut is a primary source of edible oil and has high oil (45% - 50%), fat (44%-54%), and a reasonable amount of digestible protein (25%-30%) (Arntzen 1994). A pound of peanuts is high in food energy and provides approximately the same energy value as 2 pounds of beef, 1.5 pounds of Cheddar cheese, 9 pints of milk, or 36 medium-size eggs (Woodroof JG 1983). It is the richest plant source of thiamine and also in niacin, which is low in cereals. Peanut is a valuable source of vitamins E, K and B (Arntzen 1994). Vitamin E (a-tocopherol) is a powerful lipid soluble antioxidant which helps to maintain the integrity of cell membrane, by protecting it from harmful oxygen free radicals. In addition, peanut oil contains high proportion of unsaturated fatty acids especially oleic acid (46.8%), linoleic acid (33.4%) and palmitic acid (10.0%) (Gunstone 2002). Oleic acid is a common monounsaturated fat in human diet. Monounsaturated fat consumption

has been associated with decreased low-density lipoprotein (LDL) cholesterol or "bad cholesterol" and possibly increased high-density lipoprotein (HDL) cholesterol or "good cholesterol”. It is an excellent source of resveratrol (another polyphenolic antioxidant) which has been found to have protective function against cancers, heart disease, degenerative nerve disease, Alzheimer's disease and viral/fungal infections. Moreover, it also contributes to brain health, brain circulation and blood flow, as it contains 85% of RDI of niacin (www.nutrition-and-you.com/peanuts.html). Peanut butter is free from *trans*-fat. Current nutritional interest in *trans*-fatty acid, major US brand of peanut butter were examined for the presence of *trans*-fatty acids but no *trans*-fat were detected in any samples using an analytical system with a detection limit of 0.01% (Sanders 2001).

Peanut cultivation is more profitable, because it is a good soil conditioner. Due to symbiotic nitrogen fixation by *Rhizobium, Bradyrhizobium*, etc. nodule forming bacteria peanut offers an alternative way to reduce nitrogen fertilization. The residue of peanut crop also provides nitrogen for following crops such as rice, cassava, sugarcane and field maize. Consequently, the cost of crop production is reduced, soil fertility is recovered and sustainable agriculture can be obtained (Phudenpa et al. 2004). Symbiotic nitrogen fixation depends on an interaction between the *Rhizobium* strain, host plant genome and environment (Nambiar and Dart 1980). It has been observed that, in a year approximately 72-124 kg nitrogen could be benefited from only one hectare of peanut crop cultivated land (Silva and Uchida 2000). It is also an effective cover crop for land exposed to soil erosion.

All parts of the peanut plant can be used. It is grown primarily for human consumption, as whole seeds that or is processed to make peanut butter, oil, peanut flour and other products. Peanut is consumed mostly as roasted nut and as a confectionary item in our country. Unlike its superiority to mustard oil, peanut is less popular as cooking oil in Bangladesh. Peanut shells are an important fodder for cattle particularly in drought-prone areas. The pods or shells serve as high fiber roughage in livestock feed, fuel, mulch and are used in manufacturing particle board or fertilizer.

Bangladesh is primarily an agricultural country and peanut occupies third place among oilseed crops (mustard first and sesame second) regarding to its area and production (Reddy et al. 1992), although it is consumed almost entirely as a confectionery product. According to FAO 2015, during 2014 in Bangladesh 31,000 ha area were used for peanut production and 50,000 tonnes of peanut was produced annually. Where in 2013,

production was 51,400 tonnes and area harvested was 32,000 ha. On the other hand in 2014 the average production of peanut in China the largest peanut producing country was 16.5 million metric tons and in the second greatest peanut producing country in India it was 6.6 million metric tons according to area harvested 4.63 million ha and 4.68 million ha respectively (FAO 2015). This production data of our country shows that in 2014 both harvested area as well as production of peanut was decreased compared to harvested area and production of 2013 in Bangladesh. Though, Bangladesh had negative growth in area and positive growth in yield of peanut in the 1990s and in the 2000s, Bangladesh had positive growth in both area and yield which indicate that peanut had expanded to the areas suitable for this crop (Deb and Soumitra 2015). But the production of the crop is still too low compare to other countries. It is clearly depicts that, the average peanut production in our country is not up to the mark and to obtain desired performance improvement of this crop is essential. Improvement of this crop cannot be achieved without the incorporation resistant genes against various biotic and abiotic stresses (Agrios 1997).

From the above discussion it is clear that there is an obvious need to improve the production of this crop as the yielding ability of existing peanut varieties is not adequate. There is a need to increase productivity, enhance the nutritional value and other desired agronomic quality of this crop. This cannot be done by increasing the sowing area only, as the cultivated peanut varieties of Bangladesh are characterized by low yield potential. The production and quality of peanut are fluctuates severely due to a combination of biotic stress like diseases as well as abiotic stresses like climatic factors, etc. The biotic stress including diseases, insects and pests are highly responsible for the production and quality degradation of peanut. Peanut is associated with various diseases mainly caused by fungus, virus, nematodes, mycoplasma etc. The major constraints of peanut production in our country as well as in many countries of the world are the fungal foliar diseases (Talukdar 1974). Among them early and late leaf spot disease (commonly known as 'Tikka') caused by *Cercospora arachidicola* S. Hori. and *Cercosporidium personatum* renamed *Phaeoisariopsis personata* (Berk & Curtis) respectively. They are the most destructive and widely distributed diseases of peanut in Bangladesh which may cause about 30 - 40% yield loss (Mondal and Wahhab 2001). Not only yield loss but also these diseases reduce seed quality by decreasing seed size and oil content (Ghuge et al. 1981). Early leaf spot appears at early stage (around 35 days) at the upper surface of leaves with

irregularly circular lesions which are light brown in colour. Late leaf spot appears at later stage (around 50-60 days) at lower surface of leaves with nearly circuler spots and are deep brown to black in color. Late leaf spot is more destructive. Rust disease caused by *Puccinia arachidis* Speg. is one of the most important diseases of peanut in Bangladesh. It appears at the later stage of plant growth and may cause 20-30% yield loss (Mondal and Wahhab 2001). Stem rot disease caused by *Sclerotium rolfsii* Sacc. and causes more than 27% yield loss (Hossain and Hossain 2011). Aflatoxin contamination caused by the mould *Aspergillus* affects peanut production and is toxic to humans and animals and may lead to low market values (Abdallah et al. 2005).

Therefore, to overcome the aforementioned constraints of peanut production, the improvement of this crop is a vital task. In the past several attempts have been made using conventional breeding techniques for the transfer of desirable characters to the cultivated species. However, conventional breeding programs did not yield remarkable success for the development of disease resistant peanut lines. Peanut improvement has been limited due to the lack of integration of resistance to many diseases and pests from wild species of peanut (Stalker and Moss 1987). In this case, because of high degree of self pollination and non availability of desired traits in the available germplasm of peanut the success of conventional breeding were not up to the mark. In addition, many potentially important gene transfer through inter-specific crosses are restricted by genetic incompatibilities (Miller et al. 1992). Moreover, conventional breeding programmes are also time consuming.

For the above cited reasons it is proven that conventional breeding methods alone are not adequate for the improvement of peanut. Beside the sexual breeding techniques, there are some other ways of creating variability including induction of somaclonal variation trough tissue culture, somatic hybridization and genetic engineering. Tissue culture techniques can play a significant role for the enrichment of genetic variability which give rise to variation at an unexpectedly high rate and may be a novel source of genetic variability in many plant species (Scowcroft et al. 1987, Larkin 1987). In this regard the recalcitrant nature of leguminous tissue *in vitro* eluded any notable progress for a long time. However, there are several reports of successful *in vitro* regeneration of peanut (Mc Kently et al. 1989, 1991; Durham and Parrott 1992; Baker and Wetzstein 1992, Cheng et al. 1992, Gill and Saxena 1992, Oizas-Akins et al. 1992, Pestana et al. 1999, Little et al. 2000, Sarker and Islam 2000). In spite of playing an important role for the achievement of

genetic variability tissue culture technique alone contribute a little in the production of disease resistant plants as well as plants of better agronomic characters in peanut (Mroginski et al. 1981, Narasimhulu and Reddy 1983, Atreya et al. 1984, Bose 1991, Bhuiyan 1992).

Therefore, advanced biotechnological approaches have been using to introduce suitable agronomically useful traits into established cultivars. Now a day plant genetic engineering technology is being considered as one of the most useful biotechnological methods. Due to the spectacular development of gene transfer technology it is now possible to introduce specific gene from any source (related or unrelated plant species or even from microbes) into existing elite plant lines (Gardner 1993). Thus genetic transformation provides a prospective way to enhance productivity increasing resistance to disease, pests and environmental stress which is not possible through breeding or tissue culture alone. Under these circumstances modern techniques of biotechnology, commonly known as “genetic transformation”, can be applied for the improvement of the crop. Genetic transformation, a comparatively new and exciting tissue culture base technology, has opened a new avenue for the use of recombinant DNA technology and useful in completing the conventional breeding programs.

Such crops evolved by this transgenic technique are called genetically modified or GM crops. This technology is regarded as a pre-breeding method that can provide a solution to certain constraints that limit crop production and quality. Since 1987 numerous potentially useful transgenic plants of cotton, maize, potato, tobacco, rapeseed, raspberry, soybean, pea, tomato, rice, Hawaiian papaya, squash (Zucchini), sugar cane, sugar beet, etc. have been generated (Fisk and Dandekar 1993). Biotech crop hectares amazingly increased by, 100-fold, from 1.7 million hectares in 1996, to 179.7 million hectares in 2015 (James 2015). There are several different methods involved in transformation of higher plants (Gasser and Fraley 1989). Transformation through non-oncogenic *Agrobacterium* strain is the most efficient method in dicotyledonous species (Lindsey 1992). *Agrobacterium tumefaciens*, a common soil bacterium naturally causes gall formation on a wide range of plant species, including most dicotyledonous and some monocotyledonous species (Van Larebeke et al. 1974). Large Ti (Tumour inducing) plasmid present in the virulent strains of *Agrobacterium tumefaciens* plays role in this tumour induction. Actually a part of this Ti plasmid, called ‘Transfer DNA’ is transferred to the plant nuclear genome during transformation and become integrated there. In these

transformation experiments specific reporter genes along with the gene of interest are integrated with the T-DNA. In several experiments GUS-A (β-glucuronidase) gene and neomycin phoshotranferase-II (*npt* II, Kanamycin resistant) gene have been used for this purpose as marker genes.

Advocates have argued that biotechnology will lead the next revolution in agriculture production and sustainable economic resources are being based to bring this vision about. Biotechnology industry has used the issue of world hunger as a cornerstone and legumes are playing one of the main roles to minimize world hunger (White et al. 2004). However, comparatively little success in gene transformation has been reported in legumes mainly because of their recalcitrant nature in culture (Nisbet and Webb 1990), although considerable advancements in tissue culture system for large seeded legumes have been achieved. The production of transgenic plant with *A. tumefeciens* as a gene vector was reported in pea (Schroeder et al. 1993, Kathen and Jacobson 1990), soybean (Hinchee et al. 1988, Zhang et al. 2016), alfalfa (Deak et al. 1986), white clover (White and Greenwood 1987) and Sub-clover (Khan et al. 1994), Chick pea (Kar et al. 1996, Indurker et al. 2010, Mehrotra et al. 2011, Tripathi et al. 2013), mungbean, (Jaiwal et al. 2001), Lentil (Sarker et al. 2003). Nevertheless, there are some more reports on transgenic plant production from leguminous crops, namely, *Glycine max* L. Merrill (Hinchee et al. 1988), *Pisum sativum* L., (Kathen and Jacobson 1990, Puonti-Kaerlas et al. 1990, Schroeder et al. 1993); *Vigna radiata* L. (Wilczek) (Jaiwal et al. 2001).

Successful integration of transgene or foreign genes in groundnut was first reported by Lacorte et al. (1991). But they were not able to generate plants from the transformed tissues. Eapen and George (1994) first reported regenerated groundnut plants following *Agrobacterium*-mediated genetic transformation. Since then, many successful genetic transformation protocols have been reported in peanut via *Agrobacterium*-mediated genetic transformation method based on tissue culture (Cheng et al. 1996, 1997; Li et al. 1997; Sharma and Anjaiah 2000; Sarker et al. 2000; Anuradha et al. 2006; Beena et al. 2008; Tiwari et al. 2008; Iqbal et al. *2011,* 2012; Prasad et al. 2013; Manjulatha et al. 2014; Singh et al. 2014; Tiwari et al. 2015 and Chen et al. 2015). Transformed plants of peanut have also been obtained by non-tissue culture based approach or in planta transformation by inoculating injured embryo axes with *A. tumefaciens* (McKently et al. 1995; Rohini and Rao 2000; Manjulatha et al. 2014; Pandurangaiah et al. 2014). Physical method like microprojectile bombardment mediated transformation or Biolistic method of

transformation also used for development of transformed peanut plants (Ozias-Akins et al. 1993, Singsit et al. 1997; Livingstone and Birch 1999; Athmaram et al. 2006; Chu et al. 2013; Yang et al. 1998, Bhatnagar et al. 2010 and Asif et al. 2011). Padua et al. 2000 have been developed transgenic plants from embryonic leaflet explants of peanut by electroporation method.

Agrobacterium-mediated genetic transformation is widely accepted technology in peanut transformation in comparison to other techniques. This transformation was initially based on tissue culture method and takes less time (usually 4–5 months) to obtain primary putative transgenic plants (Garladinne et al. 2016). Various strains of *Agrobacterium* such as LBA4404, EHA105, EHA101, C58 and A281 were used for the transformation of peanut. *Agrobacterium*-mediated transformation protocols have been standardized using different explants including leaf sections, zygotic embryos, cotyledonary nodes, embryo axes, leaflets, de-embryonated cotyledon and hypocotyls (Li et al. 1997; Venkatachalam et al. 2000, Anuradha et al. 2008, Tiwari and Tuli 2012; Prasad et al. 2013). First fertile transgenic plants were developed in New Mexico with Valencia variety in which leaflet explants were infected with *Agrobacterium* strain EHA105 and showed 10 % regenerated on selection medium as gus positives and also these plants showed stable integration of transgenes (uid A and *npt* II) at T1 generation with 0.2-0.3 % of transformation frequency (Cheng et al. 1996). Among the various explants cotyledon and de-embryonated cotyledon explants are mostly preferred in transformation *via* organogenesis and somatic embryogenesis (Krishna et al. 2015, Garladinne et al. 2016). Even the recovery of transformed multiple shoots was (~10) higher in de-embryonated cotyledon explants when it was compared to immature embryos (Mehta et al. 2013). Another *Agrobacterium* mediated transformation of peanut was reported by Tiwari and Tuli (2012) using EHA101 carrying 35S Intron-uidA construct with de-embryonated cotyledon explants of variety JL-21 where 81 % of transformation efficiency was recorded. Bhatnagar-Mathur et al. 2007, Sharma and Anjaiah 2000, reported *Agrobacterium* (C58) mediated transformation of peanut using marker gene *npt*II and *uid*A from cotyledon explants. Transformation of peanut was reported by using zygotic embryo axes of matured seed via *Agrobacterium* EHA101 carrying binary vector with a-glucuronidase (uidA) and *npt*II genes (McKently et al. 1995; Hood et al. 1986). Cotyledonary nodal explants were infected with *Agrobacterium* strain LBA4404 and GV2260 carrying uid A and *npt*II, which resulted in

58 % of regeneration frequency and 3.54 % of transformation frequency which were reported by Venkatachalam et al. 1998 and Anuradha et al. 2006 respectively.

Fungal diseases are predominantly affected the production and quality of peanut in both the tropic and semi-arid tropic regions (Krishna et al. 2015). Several genes were introduced into peanut through transgenic approaches for providing resistance against fungal diseases. Leaf spot disease is one of the major concerns in peanut, because of its potential devastating impact on crop yield. Chitinases and glucanases are hydrolytic enzymes which degrade the fungal cell wall and spore formation and these enzymes are attractive candidate genes for development of fungal resistant plants. Tobacco chitinase gene showed resistance in peanut against leaf spot or tikka disease caused by fungal pathogen *C. arachidicola* (Rohini and Rao 2001). Over expression of rice chitinase gene in peanut showed fungal resistance against *Cercospora arachidicola* and good correlation was observed between chitinase activity and fungal resistance at the laboratory level (Iqbal et al. 2012; Rohini and Rao 2001; Prasad et al. 2013). Defensin gene such as RsAFP-2 (*Raphanus sativum* antifungal protein-2) was transferred into peanut and the resulting transgenic peanut plants showed enhanced resistance against the pathogens, *Pheaoisariopsis personata* and *Cercospora arachidicola*, which jointly cause serious late leaf spot disease (Anuradha et al. 2008).

Peanut is cultivated mostly in rain fed regions, where higher temperature prolonged the drought conditions recur which causes the limited productivity in peanut. Development of drought tolerant varieties is needed to mitigate the environmental stresses (Bhatnagar-Mathur et al. 2007; Chu et al. 2008a). Manjulatha et al. 2014 reported that they introduced PDH45 (pea DNA helicase homologous) gene into the peanut genome, which showed abiotic stress tolerance and improved peanut productivity at T_3 generation under field conditions.

From the above observations it indicates that the introduction of suitable genes for resistance through genetic transformation may be a feasible option in combating the diseases in peanut. In Bangladesh several attempts have been made to develop transgenic peanut plants through *Agrobacterium*-mediated genetic transformation using marker gene/s (Sarker et al. 2000). Through these studies they were able to transfer GUS and *npt*II marker genes into locally grown peanut variety Tridana badam (DM-1). Although some work have been done using marker gene/s (Sarker and Islam 1999, Sarker et al. 2000, Sarker and Naher 2003) only a very few reports were obtained towards the

preliminary research on the transformation of peanut using fungal disease resistance genes in our country. At present, no transgenic peanut cultivars have been released for commercial production in Bangladesh or elsewhere. Many important traits could be incorporated into the cultivated genome and transformation technologies will become increasingly important for peanut breeding as more genes with agronomic potential are isolated.

Considering the importance of developing disease resistant peanut lines the present investigation was designed with the following objectives:

1. Optimization of transformation compatible as well as reproducible *in vitro* regeneration system in selected varieties of peanut.
2. Development of an efficient *Agrobacterium*-mediated genetic transformation protocol using marker gene/s like *GUS* (β-glucuronidase) and *npt*II (neomycin phosphotransferase).
3. Development of fungal disease resistant peanut lines through *Agrobacterium*-mediated genetic transformation using *AFP* (anti fungal protein) genes.
4. Characterization of putative transgenic peanut plants through molecular analysis.

2. MATERIALS

2. MATERIALS

2.1 Plant materials

The following three varieties of peanut were used in the present investigation:

(i) Dhaka-1
(ii) BARI Chinabadam-8
(iii) Binachinabadam-4

2.1.1 Source

Seeds of Dhaka-1 and BARI Chinabadam-8 were collected from Oil Seed Division of Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur and seeds of Binachinabadam-4 was collected from Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. The materials were maintained in the Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Dhaka. Figure 2 represents the pods and seeds of peanut varieties used in the present investigation.

2.1.2 Descriptions

Important characteristics of these varieties are described below:

(i) Dhaka-1:

Spanish type; also known as “Maizchar Badam”; widely grown recommended cultivar; erect; plant height is 30 - 40 cm; leaves are pale green in colour; flowers are found in the main stem and branches. Pod contains 1 to 2 seeds; seeds are light brown in colour; non-dormant; plants are thermo-sensitive and drought resistant to some extent. Matures in 140 - 150 days in winter and 130 - 140 days in summer; size of pods and seeds are comparatively smaller; testa colour light brown; 100-seed weight is about 30 - 35g; shelling percentage is 72 - 75%; highly susceptible to *Cercospora*; average yield 1850 - 2050 kg/ha in ‘Rabi’ and 1600 - 1800 kg/ha in ‘Kharif’ seasons.

(ii) BARI Chinabadam-8:

The height of the plant is 35 - 42 cm.; leaf deep green; seeds grow in clusters; veins are not conspicuous; two seeds per pod; every plant contain 20 - 25 pod; seeds are large in size; non-dormant; shell of the nuts is smooth and soft; testa colour reddish; life cycle is 140 - 150 days in 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*; average yield is 2500 kg/ha.

(iii) Binachinabadam-4:

Plants are dwarf; stem mostly upright than the mother variety Dhaka-1. Leaves lanceolate, light green in colour; pods are shiny, without constriction and strong venation; maximum pod yield potential is 2600 kg/ha in winter and 2500 kg/ha in summer; life cycle is 140 - 150 days in rabi season and 100 - 120 days in kharif season; seeds contain 48.6% oil and 27.5% protein; moderately resistant to collar rot, *Cercospora* leaf spot and rust diseases.

2.1.3 Explants

Four different types of explants, namely, immature leaflet (IL), decapitated half embryo (HE), De-embryonated cotyledon (DEC), and single cotyledon attached decapitated embryo (SCAE) were used in the present investigation. Various types of explants have been presented in Fig. 3. Immature leaflet explants were collected from 7 - 8 days germinated seeds and rest of the explants were collected from overnight soaked seed and used for *in vitro* regeneration.

Fig. 2 (a - f): Pods and seeds of three varieties of peanut (*Arachis hypogaea* L.) used in the present study. (a) Pods of Dhaka-1; (b) Seeds of Dhaka-1; (c) Pods of BARI Chinabadam-8; (d) Seeds of BARI chinabadam-8; (e) Pods of Binachinabadam-4; (f) Seeds of Binachinabadam-4.

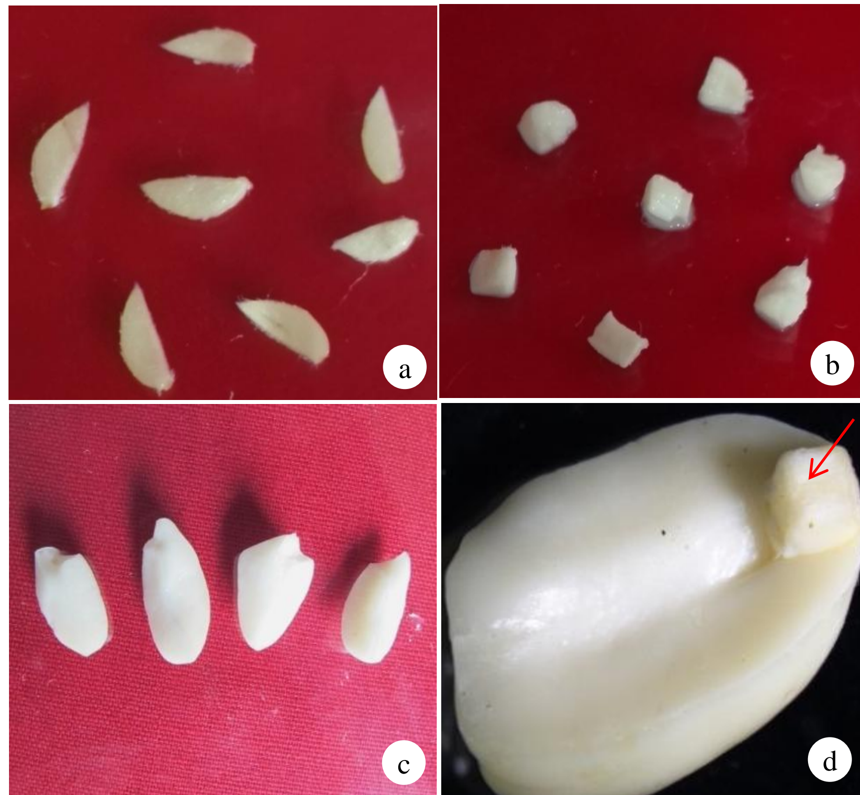

Fig. 3 (a - d): Different type of explants used in the present investigation. (a) Immature leaflet (×3); (b) Decapitated half embryo; (c) De-embryonated cotyledon; (d) Stereomicroscopic view of single cotyledon attached decapitated embryo (arrow) (×6).

2.2 *Agrobacterium* strain and vector plasmids

Two different genetically engineered constructs were used in transformation experiments. These are-

1. pBI121GUS-NPTII
2. pCAMBIA2300enh35SAFP

Agrobacterium strain namely LBA4404 was used for the transformation experiments.

2.2.1 Constract I (pBI121GUS-NPTII)

Agrobacterium tumefaciens strain LBA4404 contains plasmid pBI121 (binary vector). This binary vector have two genes within the right border (RB) and left border (LB) region of the gene construct (Fig. 4a):

(i) The *udi*A gene (Jefferson et al. 1987) encoding GUS (β-glucuronidase), driven by CaMV 35S promoter and NOS terminator. This reporter gene can be used to assess the efficiency of transformation. The *npt*II gene (Herrea-Estrella et al. 1983) encoding neomycin phosphotransferase II conferring kanamycin resistance, driven by NOS promoter and NOS terminator.
The reporter gene *GUS* can be used to assess the efficiency of transformation. The *GUS* gene has an intron in the coding sequence which can express GUS activity in the plant cell but not in the cells of *Agrobacterium.* In this strain, the transformed tissue as well as transgenic plant can be selected by using kanamycin.

2.2.2 Construct II (pCAMBIA2300enh35SAFP)

Agrobacterium tumefaciens strain LBA4404 contains plasmid pCAMBIA2300enh35S AFP. This binary vector contains *npt*II and *AFP* genes within the right border (RB) and left border (LB) region of the construct (Fig. 4b). This strain was kindly provided by Dr. VS Reddy, Plant Transformation group, ICGEB. The *AFP* gene was cloned into pCAMBIA2300 and driven by 35S promoter. The insertion was confirmed by PCR using AFPcaBam intron and AFPC2RS specific primers, and *Eco*RI + *Hind*III digestion of pCAMBIA2300enh35SAFPF plasmid. The construct was further transformed into *Agrobacterium* strain LBA4404.

2.3 Chemicals used for various purposes

2.3.1 Component of culture medium

Substance	Molecular weight	Company
MS Basal Salt Mixture	476	DUCHEFA, Netherlands
B5 Vitamin	484.47	DUCHEFA, Netherlands
Plant Agar		DUCHEFA, Netherlands
Sucrose	180	SIGMA, USA
Gelrite		SIGMA, USA

2.3.2 Antibiotics

Substance	Molecular weight	Company	Solvent
Combactam	582.6	Pfitzer, USA	ddH_2O
Ticarcillin	428.4	DUCHEFA, Netherlands	ddH_2O
Cefatoxime	455.47	ALKEM, India	ddH_2O
Kanamycin	484.49	DUCHEFA, Netherlands	ddH_2O
Streptomycin	1457.4	DUCHEFA, Netherlands	ddH_2O
Rifampicin	822.94	DUCHEFA, Netherlands	ddH_2O

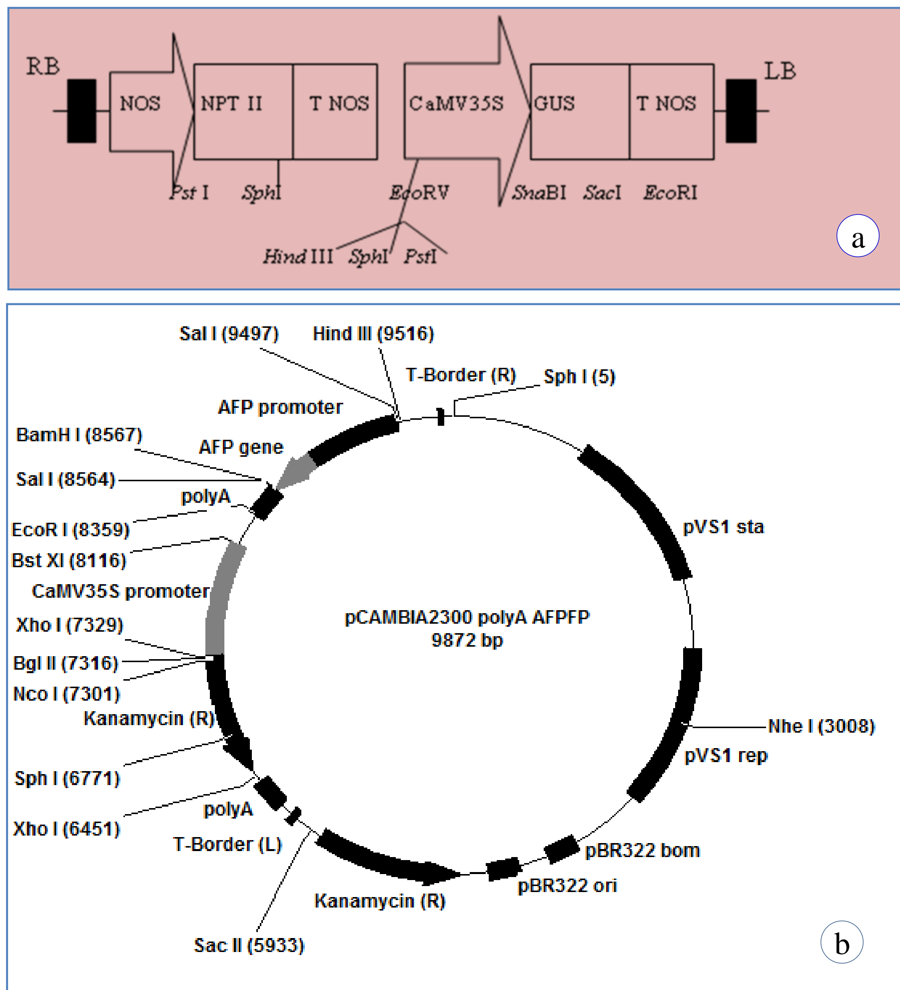

Fig. 4: Diagrammatic representation of gene construct of *Agrobacterium tumefaciens*. (a) T-DNA region between left (LB) and right (RB) border of *Agrobacterium tumefaciens* strain LBA4404 containing pBI121GUS-NPTII construct; (b) Plasmid pCAMBIA2300enh35S AFP in *Agrobacterium tumefaciens* strain LBA4404.

2.3.3 Plant hormones and additives

Substance	Molecular weight (MW)	Company	Solvent
BAP	225.3	DUCHEFA, Netherlands	1[N] NaOH
Kinetin	215.2	DUCHEFA, Netherlands	1[N] NaOH
IAA	175.18	DUCHEFA, Netherlands	1[N] NaOH
NAA	186.2	DUCHEFA, Netherlands	1[N] NaOH
2,4-D	221.6	DUCHEFA, Netherlands	1[N] NaOH
IBA	203.2	DUCHEFA, Netherlands	1[N] NaOH

2.3.4 GUS-assay buffer

(i) 100 mM sodium phosphate buffer (pH 7.0)

(ii) 0.5 mM potassium ferrocyanide

(iii) 10 mM EDTA

(iv) 1 mM (0.5 mg/ml) X-GLUC (dissolved in DMSO before adding it to Gus buffer)

2.3.5 DNA markers

DNA marker	**Concentration**	**Company**
Gene Ruler™ 100 bp DNA ladder	0.5 mg/ml	MBI Fermentas
Gene Ruler™ 1 kbp DNA ladder	0.5 mg/ml	MBI Fermentas

2.3.6 Solvent, sterilizers and others

Compound	Company
Dimethyle sulfoxide (DMSO)	SERVA, Germany
KOH	Carl Roth, Germany
NaOH	Carl Roth, Germany

2.3.7 Primers

Primer	Sequence (5'-3')	Product	Company
GUS	CCT GTA GAA ACC CCA ACC CG	750 bp	Macrogen, South Korea
	TGG CTG TGA CGC ACA GTT CA		
SFKan-F	GAA AAA CTC ATC GAG CAT CA	700 bp	Macrogen, South Korea
SFKan -R	TTG TCC TTT TAA CAG CGA TC		
AFP-F	CGC GGA TCC ATG GCG AGG TGT GAG AAT TTG GCT	190 bp	Invitrogen, USA
AFP-R	GAG TCC TCG AGA CAA TTT TTG GTG CAC CAA CAA C		
Kan-F	TCG ACC ATG GGG ATT GAA CAA GAT GG	700 bp	Invitrogen, USA
Kan-R	ATT CGA GCT CTC AGA AGA ACT CGT CAA GAA GGC		
PicA-F	ATG CGC ATG AGG CTC GTC TTC GAG	800 bp	Invitrogen, USA
PicA-R	GAC GCA ACG CAT CCT CGA TCA GCT		

2.4 Materials for isolation of plant DNA

All the solutions were made with deionised, sterile water and autoclaved.

(a) CTAB-buffer:

3	%	CTAB
1.4	M	NaCl
0.2	%	β-Mercaptoethanol
20	mM	EDTA
100	mM	Tris-HCl pH 8.0
0.5	%	PVP- 40 (soluble)

Add CTAB and β-Mercaptoethanol after autoclaving

(b) 24:1 CI Mix

24	ml	Chloroform
1	ml	Isoamylalcohol

(c) Wash buffer (WB)

76 %	Ethanol Abs.
10 mM	Ammonium acetate

(d) RNAs A: 10µg/µl Stock sol. in ddH_2O

(e) 7.5 M NH_4-Acetate

(f) 0.5 M EDTA (pH 8)

(g) TE-buffer + RNAseA

10 mM Tris-HCl, pH 8.0

1 mM EDTA

10 µg/ml RNAseA.

The volume was adjusted to 100 ml and stored at room temperature.

2.5. Materials for polymerase chain reaction (PCR)

Following the components were required for the PCR amplification reaction:

a) PCR Reaction Flexi Buffer (5x Green Go Taq)

i. 500 mM KCl.

ii. 100 mM Tris-HCl (pH-8.3 at room temperature).

iii. 0.1% Gelatin.

b) 25 mM MgCl in water for PCR.

c) 10 mM of dTTP, dATP, dCTP & dGTP (pH-7.35)

d) Taq DNA polymerase.

e) DMSO (20%)

20% DMSO was prepared by mixing 20 ml of DMSO in 80 ml of deionized sterile water and stored at -20°C.

f) DNA template

g) TE Buffer (Ref. 2.5): Buffer was filter sterilized and kept at -20°C for PCR use.

All the chemicals used for the preparation of these reagents were of Molecular biology grade and water used was ultra-pure.

2.6. Agarose gel electrophoresis

(a) Electrophoresis buffer (50X TAE) 500ml

Tris base	121 g
Acetic acid	26.8 ml
0.5 M EDTA, pH 8.0	50 ml

(b) Ethidium-bromide

10 mg/ml stock solution, store at 4°C

3. METHODS

3. METHODS

The *in vitro* plant regeneration and transformation experiments using marker as well as antifungal genes were carried out in the Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Dhaka. The different procedures used in the present study have been described under the following heads:

3.1 Media used

Different culture media used in the present investigation for various purposes were as follows:

3.1.1 Seed germination

Surface sterilized seeds were soaked overnight in sterilized distilled water to be used as a source of half embryo, de-embryonated cotyledon and single cotyledon attached embryo explants. For rapid seed germination and subsequent seedling development seeds were placed on cotton bed soaked with autoclaved sterile distilled water and subsequently leaflet explants ware collected from the germinated seedlings.

3.1.2 Media for shoot regeneration and elongation

For shoot initiation and their further development MS (Murashige and Skoog 1962) medium and MS medium with B_5 (Gamborg et al. 1968) vitamins (MSB medium) supplemented with various combinations of hormones such as BAP (6-Benzyl aminopurine), Kn (Kinetin), 2,4-D (2,4-Dichlorophenoxyacetic acid), IAA (Indole-3-acetic acid) and GA_3 (Gibberallic acid) were used during the present experiment.

3.1.3 Media for root induction

For the induction of roots at the base of the *in vitro* regenerated shoots, half strength of MS medium (half strength of macro, micro, organic nutrients and iron source of MS medium) supplemented with IAA (Indole-3 acetic acid), IBA (Indole-3 butyric acid) or NAA (α- naphthalene acetic acid) were used. Gelrite (Duchefa, Netherlands) was used as the solidifying agent for the rooting media, whereas agar was used in all others experiments.

3.1.4 Media for *Agrobacterium* culture

Three types of bacterial culture media, namely, YMB (Yeast extract Mannitol Broth), YEP (Yeast Extract Peptone) and LB (Luria Broth) with appropriate antibiotics were used to grow genetically engineered *Agrobacterium tumefaciens*. Liquid YEP, YMB and LB were used to grow *Agrobacterium tumefaciens* which were used as bacterial suspension for genetic transformation. Solid YEP and LB were used as maintenance media for different strains of *Agrobacterium*.

3.1.5 Co-culture medium

For the purpose of co-cultivation, shoot regeneration medium as well as MS media without hormonal supplement were used.

3.1.6 Selection media

For the selection of transformed tissues/plantlets kanamycin (Duchefa, Netherlands) was used, as the *Agrobacterium* construct used in the present investigation contained marker gene *npt*II (kanamycin resistance). The prepared regeneration medium after autoclaving was cooled down to 50˚C and appropriate antibiotics were added at a desired rate to a particular regeneration medium inside laminar flow cabinet. Medium was then poured into suitable culture vial and allowed to solidify. Apart from the above antibiotics some other antibiotics such as ticarcillin, combactam and cefatoxime were used to control the overgrowth of bacteria.

3.1.7 Antibiotics

Peanut explants were infected by genetically engineered *Agrobacterium* strains for transformation. To prevent the excessive growth of this microbe in the media after co-cultivation, it was essential to add appropriate antibiotics in the media . For this purpose any one of the following antibiotics were used.

(i) Ticarcillin (Duchefa, Netherlands)

(ii) Combactam (Duchefa, Netherlands)

(iii) Cefatoxime (Duchefa, Netherland)

3.2 Preparation of stock solutions for different culture media and growth regulators

Different stock solutions were prepared as the first step for the preparation of medium for ready use during the preparation of medium.

3.2.1 Preparation of stock solutions for MS medium

Since different constituents were required in different concentrations for the preparation of MS medium (Murashige and Skoog 1962), separate stock solutions for macro- and micro-nutrients, vitamins, plant growth regulators, etc. were prepared.

3.2.1.1 Stock solution A (Macro nutrients) for MS medium

This stock solution was made in such a way that its strength was 40 times more than the final strength of the medium in 1.0 liter distilled water. For this purpose, 40 times the weight of different salts required for 1.0 liter of medium were weighed accurately and were sequentially dissolved one after another in a 1.0 liter volumetric flask with 600 ml of distilled water. The last two salts are dissolved in 100 ml of distilled water separately and added serially at last. The final volume of the solution was made up to 1.0 liter by further addition of distilled water. The solution was filtered through Whatman No.1 filter paper to remove all the solid contaminants like dust, cotton, etc. and was poured into a clean plastic container. After labeling, the solution was stored in a refrigerator at 4˚C for several weeks.

3.2.1.2 Stock solution B (Micro nutrients) for MS medium

For this constituent of the medium two separate stock solutions were prepared. These are as follows:

Stock solution B_1 (all micro-nutrients except iron)

This part of the stock solution was made with all the micro-nutrients except $FeSO_4.7H_20$ and Na_2-EDTA. This was made 100 times the final strength of necessary components in 500 ml of distilled water as described for the stock solution A. The solution was filtered through Whatman No.1 filter paper to remove all the solid contaminants like dust, cotton, etc. and was poured into a clean plastic container and stored at 4˚C for future use.

Stock solution B_2 (iron chelate solution)

The second solution was made 100 times the final strength of $FeSO_4.7H_20$ and Na_2-EDTA in 500 ml distilled water in a conical flask and heated slowly at low temperature until the salts were dissolved completely. Finally the solution was filtered and stored in a refrigerator at 4˚C for future use.

3.2.1.3 Stock solution C (Organic constituents) for MS medium

It was also made 100 times the final strength of the medium in 500 ml of distilled water. This solution was also filtered and stored at 4˚C for future use.

3.2.1.4 Stock solution D (B_5 vitamin) for MSB medium

For the preparation of B_5 vitamin following components were added in distilled water.

Components	Amount needed for 1000 ml medium
Myo inositol	10.0 gm
Thiamin	1.0 gm
Nicotinic Acid	0.1 gm
Pyridoxin HCl	0.1 gm

This was made 100 times the final strength of necessary components in 500 ml of distilled water as described in the section 3.2.1.1. The solution was filtered and stored at 4˚C.

3.2.2 Stock solutions for growth regulators

The following different growth regulators and supplements were used in the present investigation.

I. Auxins

Auxins induce cell division and formation of callus. It causes cell division, cell elongation, swelling of tissues and the formation of adventitious roots. The auxins are:

(i) Indole-3-acetic acid (IAA)
(ii) α- naphthalene acetic acid (NAA)
(iii) Indole-3-butyric acid (IBA)

II. Cytokinins

Cytokinins concerned with cell division and modification of shoot differentiation in tissue culture. The most frequently used cytokinins are

(i) 6-benzyl amino purine (BAP)
(ii) 6-furfuryl amino purine (Kinetin/Kn)

III. Gibberallic acid (GA_3)

The growth regulators, their solvents, and molecular weight are listed below (Sigma Plant Cell Culture Catalogue, 1992)

Growth regulators	Solvent	Molecular weight
IAA	1N NaOH	175.2
IBA	1N NaOH	203.2
NAA	1N NaOH	186.2
BAP	1N NaOH	225.3
Kinetin	1N NaOH	215.2
2,4-D	Ethanol	221.04

To prepare any of the above mentioned hormonal stock solution, 10 mM stock solutions of each hormones were prepared . For this purpose certain amount of the hormone was weighted and dissolved in required amount of appropriate solvent and then the final volume of the solution was made up 50 ml by addition of distilled water. The solution was then filtered and poured into clean plastic container/another 50 ml in sterilized falcon tube and stored in a refrigerator at 4ºC for future use.

3.2.3 Preparation of stock solutions of antibiotics

Different types of antibiotics were used for different bacterial and plant regeneration media in transformation experiments.

a) Kanamycin (Duchefa, Netherland)
b) Ticarcillin (Duchefa, Netherland)
c) Combactam (Duchefa, Netherland)
d) Cefatoxime (Duchefa, Netherland)

For the preparation of kanamycin, ticarcillin and combactam stock solutions, 1 gm of each antibiotic was separately dissolved in 10 ml of deionized water. After micro filter sterilization, these solutions are stored in 1.5 ml Eppendorf tubes at - 20ºC in the dark as stock.

3.3 Preparation of medium for *in vitro* culture of explants using MS/MSB stocks solutions

To prepare one liter of medium the following steps were carried out successively

(i) For the preparation of MS/ MSB medium, 30 g of sucrose was dissolved in 500 ml of distilled water in a liter of volumetric flask.

(ii) 25 ml of stock solution A, 5 ml of stock solution B and 5 ml of stock solution C were added to this 500 ml distilled water and mixed well. Stock solution D was added instead of stock solution C for the preparation of MSB medium.

(iii) Required amount of hormonal supplements were taken from the stock solution of hormone and added to the medium either individually or in combinations and were mixed thoroughly. Since each of the hormonal stock solution contained 10 mM of the chemical in 50 ml of solution, the addition of 100 µl of any hormonal stock solution will make 1 litre of medium resulted in 1µM concentrations of that hormonal supplement. Different concentrations of hormonal supplements were prepared by adding required amount of the stock solution to the medium following the similar procedure described earlier.

(iv)The whole mixture was then volume up to 1litre with distilled water.

(v) pH of the medium was adjusted to 5.8 with a digital pH meter (JENWAY, Japan) with the help of 1[N] NaOH and 1[N] HCL. Before that, the pH meter was calibrated with two buffer solution having pH 4.01 and 6.86, respectively.

(vi) To solidify either 8.0 g (at 0.8%) of phytoagar (Duchefa, Netherlands) or 2.0 g (at o.2%) of phytagel (Sigma, USA) was added to the medium. To dissolve solidifying agent, the whole mixture was heated in a microwave oven (Butterfly, China).

(vii) To make liquid medium the last step (vi) of media preparation was omitted.

3.4 Preparation of MS medium for plants regeneration using MS powder

Occasionally commercially available powdered MS medium (Duchefa Biocheme, Netherlands) containing all the constituents of MS medium (inorganic salts, vitamins and amino acids) except sucrose and agar were used to prepare medium for *in vitro* regeneration of plants. Appropriate amount of media powder is dissolved in distilled

water (10% less than the final volume of the medium), and after adding sucrose (3%), the pH was adjusted to 5.8. Normally 0.8% agar was added to prepare agar solidified media.

3.5 Preparation of *Agrobacterium* culture medium

Liquid YMB, YEP and LB were used to grow the genetically engineered *Agrobacterium tumefaciens* strain which were later used to obtain bacterial suspension for transformation. Solid YEP, YMB and LB were used as maintenance media for different strains.

3.5.1 Preparation of YMB (Yeast Extract Mannitol Broth) medium

For the growth of *Agrobacterium tumefaciens* strain, YMB medium was prepared in the following manner:

Components	Amount needed for 100 ml medium
Mannitol	1.0 g
Yeast extract	0.04 g
$MgSO_4.H_2O$	0.02 g
NaCl	0.01 g
KH_2PO_4	0.05 g

All of these ingredients of the medium were added in 50 ml distilled water and mixed properly. Then the final volume was made up to 100 ml by adding distilled water. The pH was adjusted at 7.0 - 7.2.

For the preparation of working culture medium (to make bacterial suspension), liquid medium was used. For preparing maintenance medium 1.5 gm agar was added to the medium before autoclaving.

Liquid medium was stored at 4˚C for future use. Filter sterilized antibiotic kanamycin (50 mg/l), streptomycin (25 mg/l) and rifampicin (25 mg/l) was added to the autoclaved liquid medium (working) prior to bacterial inoculation and to the autoclaved maintenance medium when the medium was cooled down enough. The medium was then poured into Petri plates. After solidification, the media were ready for bacterial culture. When required these culture plates were stored at 4˚C for further use.

3.5.2 YEP medium was prepared in the following manner

Components	Amount needed for 100 ml medium
Bactopeptone	1.0 g
Bacto Yeast extract	1.0 g
NaCl	0.5 g

Preparation procedure for YEP media was same as above media (section 3.5.1).

3.5.3 LB medium was prepared in the following manner

Components	Amount needed for 100 ml medium
Bactotryptone	1.0 g
Yeast extract	0.5 g
NaCl	1.0 g

Preparation procedure for LB media was same as above media (section 3.5.1).

3.6 Preparation of co-culture medium

For co-cultivation (infection of peanut explants together with *Agrobacterium*), shoot regeneration medium as well as without hormonal supplement i.e. solidified MS media with a pH 5.8 was prepared.

3.7 Sterilization

Fixed volume of the medium was dispensed into culture vessels (i.e. test tubes, bottles or conical flasks) or 500 ml screw capped bottles. The culture vessels were plugged with non-absorbent cotton or covered with aluminium foil and marked with the help of a glass marker to indicate the specific media with hormonal supplements. The culture vessels were then autoclaved (HA-300MN, Hirayama, Japan) at 15 lbs/sq inch pressure at 121˚C temperature for 20 minutes.

3.8 Preparation of selection medium for putatively transformed plantlets

For the selection of transformed shoots antibiotics, namely, kanamycin (Duchefa, Netherlands) was used, as the *Agrobacterium* construct used in the present investigation contained *npt*II (kanamycin resistance) genes as selectable marker gene. After preparation the regeneration medium was autoclaved. The medium was cooled down to 50˚C and

appropriate antibiotics were added at a desired rate to a particular regeneration medium inside laminar flow cabinet. Medium was then poured into suitable culture vial and allowed to solidify. Apart from these other antibiotics such as ticarcillin, combactam or cefatoxime was also used to control the overgrowth of bacteria.

3.9 Precaution to ensure aseptic condition

All inoculation and aseptic manipulations were carried out in a laminar airflow cabinet (Labtech Co. Ltd., Korea, Forma Scientific, USA, Heraeus, Germany). The cabinet was switched 'on' for at least half an hour before use and cleaned with 70% alcohol to make it free from surface contaminants. The instruments like scalpels, forceps, inoculation loop, Petri dishes and materials like cotton wool, filter papers, Microcentrifuger tubes, etc. were sterilized by steam sterilization methods. During the entire period of work in the cabinet, the scalpels, forceps, and inoculation loop were kept immersed into absolute alcohol containing in a glass jar inside the cabinet. At the time of inoculation and subculture these were sterilized by flaming method from time to time in the cabinet. Both the hands were rinsed with 70% alcohol. All measures were taken to obtain maximum contamination free condition during the preparation of explants. After autoclaving bacterial media were poured inside the laminar airflow cabinet to avoid contamination. Antibiotics were filter sterilized with a micro-filter inside the laminar flow hood and stored in sterile Eppendorf tubes. Antibiotics were also added to the medium inside the laminar flow cabinet. After each transformation experiment, used *Agrobacterium* suspension and contaminated Petri dishes, cotton wool, filter papers, instrument, glass cuvettes were autoclaved to destroy genetically engineered *Agrobacterium* as a part of 'biosafety' requirement. Any contaminants and old bacterial cultures were also autoclaved before discarding them.

3.10 Culture techniques

The following culture techniques were employed in the present investigation

(i) Axenic culture
(ii) Explant culture (Inoculation)
(iii) Subculture
(iv) Rooting
(v) Transplantation
(vi) *Agrobacterium* culture
(vii) Infection and incubation
(viii) Co-culture

3.10.1 Axenic culture

To reduce the level of surface organisms the peanut seeds were washed first with running tap water for 3-5 times. The floating seeds were discarded. The seeds were then deepened in 70% alcohol for 1 minutes followed by washing with distilled water. After transferring the seeds in autoclaved flask, final surface disinfection was done with 0.1% $HgCl_2$ solution for 10-15 minutes inside the laminar flow cabinet. During this period, the flask was agitated. Then the seeds were washed four to five times with sterilized distilled water. The surface sterilized seeds were then kept in sterilized distilled water for overnight and sometimes sterile cotton soaked bottle for the collection of explants.

3.10.2 Explants culture (Inoculation)

Immature leaflet (IL), Half embryo (HE), de-embryonated cotyledon (DEC) and single cotyledon attached decapitated embryo (SCAE) explants were used in this investigation. Immature leaflets were excised from aseptically grown 7-8 days old seedlings. For the culture of decapitated half embryo, de-embryonated cotyledon and single cotyledon attached decapitated embryo explants surface sterilized seeds were soaked overnight in sterilized distilled water. The explants were then cultured on MS/MMB media supplemented with different concentrations and combinations of BAP, Kn, 2,4-D, NAA, for *in vitro* regeneration of shoots.

3.10.3 Subculture

After two weeks, regenerated shoots were transferred to fresh medium. Cultures were sub-cultured regularly, at an interval of 21-28 days for maintenance and were routinely examined for different morphogenic developments.

3.10.4 Rooting

2.5 to 4 cm long shoots were separated and cultured on freshly prepared half strength of MS medium containing different combinations and concentrations of IAA, IBA and NAA for root induction.

3.10.5 Transplantation

The plantlets with sufficient root system were taken out from the culture vessels and the roots were washed under running tap water. The plantlets were then transplanted to small pots containing sterilized soil. Pots were then covered with transparent perforated polythene bags. Inner side of these bags was moistened with water to prevent desiccation.

To reduce sudden shock, the pots were kept in growth room for two weeks, of which polythene covers were maintained for the first week and without cover for the second week. These plantlets were exposed to environment for 2-8 hours daily and again placed in growth room for another week. Three weeks after transplantation, when the regenerated plants were fully established in the small pots, then they were transferred to larger pots for further growth and to get seeds from those regenerated plants.

3.11 Preparation of *Agrobacterium* culture for transformation

As it has been mentioned earlier, two kinds of culture media were needed for the bacterial strain, one for maintaining *Agrobacterium* stock and the other for infection of explants.
For maintenance, one single colony from previously maintained stocks was streaked into freshly prepared Petri dish containing YMB or YEP or LB medium having appropriate antibiotics. The Petri dish was sealed with Para film and kept in incubator at 28˚C temperature for at least 48 hours. This was then kept at 4˚C to check the growth. The culture was sub cultured regularly every week in freshly prepared media to maintain the stock.
For infection of explants, bacterial suspension was prepared. For this purpose, single colony from the bacterial stock was taken in an inoculation loop and was inoculated in a conical flask containing liquid medium with required antibiotic. This culture was allowed to grow at 28˚C overnight on a rotary shaker to get optimum population for infection and co-cultivation of explants.

3.11.1 Infection and incubation

The overnight grown *Agrobacterium* culture was centrifuged for 10 minutes at 5000 rpm and the pellet was resuspended with liquid MS medium (pH 5.8) to make the *Agrobacterium* suspension. This *Agrobacterium* suspension was used for infection and incubation. Prior to this "Optical Density" or OD of the bacterial suspension was determined at 600 nm with the help of a spectrophotometer (Shimadzu, Japan). Following the determination of density, to get suitable and sufficient infection of the explants, cut explants were dipped in bacterial suspension for different incubation periods before transferring them to co-cultivation medium. Before each cut the scalpel blade was dipped in the *Agrobacterium* suspension. Sometimes overnight grown *Agrobacterium* culture were directly used for the infection and incubation of explants.

3.11.2 Co-culture

Following infection and incubation, the explants were co-cultured on regeneration medium or hormone free MS medium. Prior to transfer of all explants to co-cultivation media they were soaked in filter papers for a short period of time to remove excess bacterial suspension. All the explants were maintained in co-culture medium for 2 - 4 days.

3.11.3 Culture of *Agrobacterium* infected explants

Co-cultured explants were washed with distilled water three - four times until no opaque suspension was seen, then washed for 10 minutes with distilled water containing 300 mg/l ticarcillin /cefotoxime. Then explants were gently soaked with a sterile Whatman filter paper and transferred to regeneration medium containing 100 mg/l ticarcillin. After 12 - 15 days, the regenerated shoots were then sub-cultured in selection medium containing 50 mg/l kanamycin and 100 mg/l ticarcillin. Regenerated shoots were sub-cultured regularly with an interval of 15 - 21 days and the concentration of selection agents was gradually increased up to 200 -250 mg/l in case kanamycin on selection medium. Shoots survived on selection medium were sub-cultured on root induction medium for fully developed plantlets. As control, non-infected explants were cultured in normal regeneration medium. 7 - 10 days old shoots were sub-cultured in selection medium to detect the effect of selection agents on this control shoots. These controls were maintained with each set of transformation experiments to perform various comparative studies.

3.12 GUS (β-glucuronidase) histochemical assay

Agrobacterium strain LBA4404/pBI121GUS-NPTII used in the present study contains *gus* (*uid A*) reporter gene. The product of GUS gene is β-glucuronidase enzyme that reacts with a substrate 5-bromo-4-chloro-3- indolyl β-D-glucuronide or X-Gluc (Jefferson 1987) and gives an insoluble indigo blue colour at the site of GUS enzyme activity. Thus, it allows transformed tissues to be screened histochemically.

3.12.1 Reagents for histochemical GUS assay

(i) 0.5 M morphinoethane sulphonic acid (MES), pH 5.6:

9.76 gm of MES was dissolved in 80 ml ddH_2O. pH was adjusted to 5.6 with NaOH and volume was made.

This was stored in room temperatures.

(ii) Fixation solution, pH 5.6:

Component	Stock concentration	Final concentration
Formaldehyde (40%)	0.75% (v/v)	0.3%
0.5 M MES	0.002% (v/v)	10 mM
Manitol	5.46% (w/v)	0.3 M

This was stored at room temperature.

(iii) 50 mM solution of Na_3PO_4, pH 7.0:

Stock solutions:

A. 50 mM solution of $NaH_2PO_4.2H_2O$ (0.78 gm in 100 ml)
B. 50 mM solution of Na_2HPO_4 (0.71 gm in 100 ml)

For the preparation of 100 ml 50 mM solution of Na_3PO_4 (pH 7.0), 39 ml of stock solution A and 61.0 ml of B was mixed and the pH was adjusted to 7.0.

(iv) GUS -Buffer / X-Gluc solution:

For the preparation of 10 ml X-gluc solution, 10 mg of X-gluc (5- Bromo-4- Chloro-3-indolyl β-D glucuronide) was dissolved in DMSO (dimethyl sulphonic acid) in a pyrex tube. The final volume was done with 50 mM solution of Na_3PO_4. This was stored at -20˚ C.

3.12.2 GUS (β-glucuronidase) histochemical assay of transient expression in explants

Following each transformation experiment, randomly selected co-cultured tissues were examined for GUS- histochemical assay. Co-cultured explants and tissues and parts of putative transformed plantlets were immersed in X-gluc (5- Bromo-4- Chloro-3- indolyl-β-D glucuronide) solution. For this purpose explants were dipped in histochemical reagent, i.e. GUS buffer followed by several washes (3 times) and were incubated at 37˚C overnight. A characteristic indigo blue color would be the expression of GUS (β-

glucuronidase) gene in the plant tissue. In each experiment non-transformed explants were used as control. After X-gluc treatment, explants were transferred to 70% alcohol for decolorization. Decolorized explants were observed under stereomicroscope (Olympus, Japan). For confirmation slides were prepared with transformed tissue and were observed under microscope. Following each subculture some randomly selected plant parts (leaves, shoots) were also subjected to GUS histochemical assay according to the above mentioned method. Plant parts of the transformed plantlets were also examined through this method.

3.13 Anatomical study

Anatomical studies were carried out following maceration of the plant tissue to observe the expression of GUS gene within the explants tissue as well as with the different parts of putatively transformed plantlets.

3.13.1 Maceration of plant tissues

Explants showing GUS expression following X-gluc treatment were transferred to 70% alcohol for decolorization. After decolorization, blue colour containing explants were transferred to 1N NaOH into fresh glass vessels. These glass vessels were incubated for 20 mins at 60°C for adequate softening of the tissue. After cooling these materials were taken on a clean and oil free slide having a drop of 50% aqueous solution of glycerol (v/v) and clean cover slip was placed on each material and gentle pressure was given on the cover slip with a needle to spread the tissue uniformly. These slides were observed under microscope (Nikon Eclipse, Japan).

3.14 Plant genomic DNA isolation from putatively transformed plantlets

3.14.1 Collection of plant sample

To extract genomic DNA, *in vitro* regenerated putatively transient plantlets and their corresponding control plantlets were collected. The materials were washed in distilled water and dried on fresh tissue paper to remove any components of medium nutrients.

3.14.2 Preparation of stock solutions and working solutions used for DNA isolation

The following chemicals were used for plant genomic DNA isolation.

3.14.2.1 1 M stock solution of Tris HCl pH 8.0 (100 ml)

12.14 gm of Tris base was dissolved in 75 ml of distilled water. The pH of this solution was adjusted to 8.0. The volume of the solution was adjusted to a total of 100 ml with de-ionized distilled water. Then it was sterilized by autoclaving and stored at 4º C.

3.14.2.2 0.5 M stock solution of EDTA pH 8.0 (100 ml)

18.61 gm of EDTA was added to 75 ml of distilled water and stirred vigorously with a magnetic stirrer. Approximately 2 gm of NaOH pellets was added to adjust the final pH to 8.0. Final volume was adjusted to 100 ml with sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4º C.

3.14.2.3 5 M stock solution of NaCl (100 ml)

29.22 gm of sodium chloride (NaCl) was dissolved in 75 ml of distilled water. Then it was volume up to 100 ml with distilled water. The solution was sterilized by autoclaving and stored at 4º C.

3.14.2.4 β-Mercaptoethanol

β - Mercaptoethanol was available at strength of 14.4 M solution and was stored in a dark bottle at room temperature.

3.14.2.5 Ribonuclease A stock solution

10 mg RNAase A was dissolved in 1 ml of deionized distilled water and was store in -20º C.

3.14.2.6 Chloroform:Isoamyl alcohol (24:1) (100 ml)

24 ml of Chloroform and 1 ml of Isoamyl alcohol were mixed properly using vortex mixture. Chloroform: Isoamyl alcohol mixture is caustic and produces fumes. So, was used only in a fume hood wearing gloves and eye protection.

3.14.2.7 70% Ethanol (100 ml)

30 ml double distilled water was added in 70 ml absolute ethanol.

3.14.2.8 Stock solution of TE (Tris-HCl EDTA) buffer pH 8.0 (100 ml)

1 ml of 1 M Tris-HCl was added with 0.2 ml (200 µl) of 0.5 M EDTA. The final volume was adjusted to 100 ml with sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4° C.

3.14.2.9 7.5 M Ammonium acetate pH 5.2 (100 ml)

57.81 g of ammonium acetate was dissolved in 70 ml of ddH_2O and was adjusted to a volume of 100 ml with the addition of ddH_2O and was sterilized by autoclaving.

3.14.2.10 Extraction Buffer (Homogenization buffer)

Following components were used for preparing DNA extraction buffer.

Name of the chemical	Molecular Weight	Stock con.	Reference con./working con.	Working Volume	
				100 ml	1000 ml
CTAB			2%	2 g	20 g
NaCl	58.44	5 M	1.4 M	28 ml	280 ml
EDTA (pH 8)	372.24	0.5 M	20 mM	4 ml	40 ml
Trisbase (pH 8)	121.1	01 M	100 mM	10 ml	100 ml
β-Mercaptoethanol		14.4 M	100 mM	700 µl	7 ml

The following steps were performed for the preparation of Extraction Buffer (100 ml):

(i) 10 ml of 1 M Tris-HCl (autoclaved, pH 8.0) was taken in a 250 ml conical flask.

(ii) 28 ml of 5 M NaCl (autoclaved) was added to it.

(iii) 4 ml of 0.5 M EDTA (autoclaved, pH 8.0) was added next.

(iv) Then 0.5 gm PVP and 2/3 gm CTAB was added freshly and kept it at 60º C preheated water bath to dissolve the PVP.

(v) 200 µl β-mercaptoethanol was added prior to use the buffer and mixed it properly by shacking.

3.14.2.11 Isolation of genomic DNA using CTAB method

Plant genomic DNA was isolated according to Doyle and Doyle (1990). The steps of DNA isolation are as follows:

(i) 200 - 250 mg leaf materials of required source were taken and grind in liquid nitrogen (sometimes directly) using pestle and mortar.

(ii) 800 µl of extraction buffer was added and grinded the leaf until it became homogenous paste.

(iii) The paste was transferred to 2 ml centrifuge tube and incubated at 60º C water bath for 30 min.

(iv) 700µl of chloroform:Isoamyl alcohol (24:1) was added to the extract and centrifuged at room temperature for 10 min at 13000 rpm. Sometimes this process was repeated twice to obtain a clear sample.

(v) The supernatant was collected and DNA was precipitated with 2/3 volume chilled Isopropanol and kept the sample overnight in - 20º C.

(vi) Then the suspension was centrifuged for 10 min at 13000 rpm at room temperature.

(vii) Supernatant discarded and the pellet was washed with 70% ethanol for 3 times.

(viii) The DNA was then resuspended in 40 - 50µl of TE buffer.

3.14.3 Quantification of Isolated DNA

Measurement of isolated DNA concentration can be done by comparing DNA with the standard DNA on agarose gel electrophoresis or by estimating the absorbance of DNA by spectrophotometer at 260 nm. Both the methods were carried out during this investigation.

3.14.3.1 Measurement of DNA concentration and quality by Agarose Gel Electrophoresis

3.14.3.1.1 Preparation of stock solutions used for Gel Electrophoresis

3.14.3.1.1.1 50 × TAE Buffer (pH8.3) (1 litre)

242 g Tris base was dissolved into 900 ml of sterile de-ionized distilled water. Then 57 ml glacial acetic acid was added to the solution. Finally, 100 ml 0.5 EDTA (pH 8.0) was added in it. The pH of the solution was adjusted with concentrated HCl at pH 8.3. The final volume of the solution was adjusted to 1000 ml.

3.14.3.1.1.2 10 × Loading Dye

For the preparation of 10 ml of 10 × loading dye, 40 mg of bromophenol blue (i.e., the final concentration was 0.4%), 40 mg of xylene cyanol FF (i.e., the final concentration was 0.4%) and 5 ml of 98% glycerol (i.e., the final concentration was 50%) were added to 4 ml of sterile de-ionized distilled water. The final volume was adjusted to 10 ml with sterile de-ionized distilled water and was stored at 4º C.

3.14.3.1.1.3 Ethidium Bromide Solution

For 1ml solution, 10 mg of Ethidium Bromide was dissolved in 1 ml of sterile de-ionized distilled water. It was mixed by hand shaking. The solution was transferred to a dark bottle and was stored at room temperature.

3.14.4 Agarose Gel Electrophoresis

The standard method used to separate, identify and purify DNA fragments through electrophoresis was followed according to the method described by Sharp *et al.* 1973.

(i) 1.0/0.8 gm of agarose was melted into 100 ml of TAE buffer, ethidium bromide was added (10 µg/ml) and poured into gel-tray fixed with appropriate combs.

(ii) After the gel was solidified it was placed into gel-running kit containing 1× TAE buffer.

(iii) Digested plant DNA solutions were loaded with 6× gel loading dye and elecetrophoresis was continued until DNA fragments were separated well.

3.15 Polymerase Chain Reaction

3.15.1 Preparation of the Master Mixture

Master mixture was prepared by mixing all of the PCR component e.g. reaction buffer, dNTPs, Primer- F & R, Taq DNA polymerase etc. except the component template DNA. In each reaction, the volume of PCR buffer was used $\frac{1}{10}$ th of the total reaction volume which was 25µl. After thorough mixing and momentary spin of the master mixture, it was transferred to different PCR tubes. The final volume was made 25µl by adding varying amounts of sterilized ultra-pure water. Template DNA was added afterwards. DNA polymerase was added just before the start of the reaction. Finally, the tubes were

subjected to momentary spin and transferred to thermo cycler for the amplification reaction (Eppendorf Mastercycler gradient).

3.15.2 Thermal Cycling Profile used in PCR

The working concentration of the template DNA was 100 ng for each sample. The PCR condition was used for gene amplification as described below:

The PCR condition for GUS and *npt*II gene

Step	Temperature	Time	No. of Cycle
Initial denaturation	94°C	5 min	1
Denaturation	94°C	1 min	
Annealing	54°C	1 min	35
Elongation	72°C	1 min	
Final elongation	72°C	5 min	1

The PCR condition for Kanamycin and *AFP* gene

Step	Temperature	Time	No. of Cycle
Initial denaturation	94°C	4 min	1
Denaturation	94°C	30 sec	
Annealing	50°C	30 sec	35
Elongation	72°C	30 sec	
Final elongation	72°C	5 min	1

The PCR condition for *picA* gene

Step	Temperature	Time	No. of Cycle
Initial denaturation	94°C	5 min	1
Denaturation	94°C	1 min	
Annealing	60°C	1 min	35
Elongation	72°C	1 min	
Final elongation	72°C	5 min	1

3.15.3 Analysis of PCR product

3.15.3.1 Visualizing the PCR Product

3 µl of DNA dye was added to the PCR amplified DNA. After a momentary spin the PCR products were loaded in wells of 1.0 - 1.2% agarose gel containing ethidium bromide (0.05µl/ml). Electrophoresis was accomplished at 40 volts and the PCR products were visualized under UV transilluminator and photographs were taken for documentation by gel documentation (Bio.Sci. Tech.Gelsccan, 6.0, professional, Germany).

3.16 Southern blot analysis

Plant genomic DNA (10 µg) was digested with appropriate enzymes (*Hind*III) for overnight. Digested DNA fragments were separated on the 0.8% agarose gel in TAE buffer, containing ethidium bromide (0.5 µg/ml) at 30 volt for 16 hours. After visualization in gel doc the gel was destained/washed twice with autoclaved MQ H2O for 15 minutes. The gel was treated twice with 0.2 N/ 4ml HCl by gentle agitation for 15 minutes each for depurination/denaturation of the DNA. After which, gel was washed twice by gentle agitation (15 minutes each) with combination of NaOH 0.5 M + NaCl 1.5 M. Following, the gel was neutralized the denaturing solution by washing twice (10 minutes each) with ammonium acetate (0.5 M) and transferred onto nylon hybridization membrane. The gel was placed upside down on a glass plate covered by Saran wrap. The nylon membrane was cut to the exact size, placed onto gel. Three sheets of Whatman paper No.1 were soaked in ammonium acetate (0.5 M), was placed onto membrane. The filter paper towels 10 $\times$ 15 cm in size were placed onto Whatman papers. Following, glass plate was placed on the top. 400 g weight was placed on glass plate. Transfer of digested DNAs from gel to membrane was left for overnight, fixed on to membrane by UV- cross linker and stored until required (Sambrook et al. 1989).

Composition of the buffers and solutions used for Southern Hybridization

1. 10× TBE		
a. Tris	10 g	Total volume was made up to 1000 ml with distilled water and autoclave
b. 0.5 M EDTA (pH-8)	40 ml	
c. Boric Acid	55g	
2. Denaturation Solution		
a. Nacl (1 M)	29.22 g	Total volume was made up to 500 ml with distilled water. Autoclave and store at room temperature
b. NaOH (0.5 N)	10 g	
c. Distilled water	400 ml	
3. Neutralisation Solution		
a. Nacl (1.5 M)	43.83 g	Dissolve NaCl and Tris in 350 ml distill water. The final pH was adjusted to 7 with concentrated HCl and then final volume was made up to 500 ml.
b. Tris (0.5 M)	30.28 g	
c. Distilled water	350 ml	
4. 20× SSC		
a. NaCl (3 M)	348 g	The final pH was adjusted to 7.0 with concentrated HCl and then final volume was made up to 2L
b. Na Citrate (0.3 M)	176.4 g	
5. Maleic Acid buffer (10×)		
a. 0.1 M maleic acid	11.60 g	Final volume was made up to 1000 ml with NaOH (solid) to pH 7.5. Autoclave and store at room temperature.
b. 0.15 M Nacl	8.766 g	
6. Washing buffer		1× maleic acid buffer pH 7.5; 0.3% (v/v) Tween 20.

4. RESULTS

4. RESULTS

The present investigation was conducted in order to established plant regeneration systems for three varieties of peanut (*Arachis hypogaea* L.), namely, Dhaka-1, BARI Chinabadam-8 and Binachinabadam-4 towards the development of a reliable protocol for *Agrobacterium*-mediated genetic transformation for two varieties of peanut, namely, Dhaka-1 and BARI Chinabadam-8 with an aim of developing biotic stress tolerant peanut lines.

This study was carried out in two phases. In the first phase, several experiments were conducted to obtain an efficient *in vitro* regeneration system for the above mentioned two varieties of peanut using various explants, namely, immature leaflet, decapitated half embryo, de-embryonated cotyledon and single cotyledon attached decapitated embryo. In the second phase, a series of experiments were conducted for genetic transformation using two different genetically engineered construct in an *Agrobacterium tumefaciens* LBA4404. They were namely LBA4404 harboring plasmid pBI121GUS-NPTII (considered as construct I) and LBA4404 harboring plasmid pCAMBIA2300enh35SAFP (considered as construct II). For this purpose two varieties such as Dhaka-1 and BARI Chinabadam-8 were used. Construct pBI121GUS-NPTII was mainly used for the development of an efficient *Agrobacterium*-mediated genetic transformation protocol of peanut. This construct I was used for development of transformation protocol since it contained two marker genes namely, *GUS* (β - glucoronidase) as screenable marker and *npt*II (neomycin phosphotransferase) as selectable marker gene. On the other hand, LBA4404 with plasmid pCAMBIA2300enh35SAFP (construct II) contained two genes conferring defensin gene *AFP* (antifungal protein as a gene of interest) and *npt*II gene as selectable marker. Construct pCAMBIA2300enh35SAFP was used for the integration of antifungal gene into peanut genome with an aims to develop fungal disease resistant peanut lines. For the establishment of an efficient *Agrobacterium*-mediated transformation protocol further experiments were carried out using construct I for optimization of various parameters required for *Agrobacterium*-mediated genetic transformation including the optical density of *Agrobacterium* suspension, suitable incubation and co-cultivation periods required for effective transformation. Beside these experiments the concentration of selectable agent (kanamycin) was optimized to obtaining required transformed plantlets. Experiments were also conducted to regenerate

plantlets from *Agrobacterium* infected explants. Through proper selection procedures the transformed shoots were selected and rooted properly and then the plantlets were established in soil following optimum hardening. Further molecular characterization of transformed plantlets was carried out through Polymerase chain reaction (PCR) and Southern blot analyses. The results of the present study obtained from different experiments are presented in the following sections.

4.1 *In vitro* regeneration of plantlets

A transformation compatible *in vitro* regeneration system is a prerequisite for the development of successful genetically engineered plants. The success of *in vitro* regeneration is greatly influenced by the age of tissue or organ that is used as initial explant. Explants taken from juvenile plant tissues, particularly from seedlings are highly responsive. In the present study, immature leaflet, decapitated half embryo, de-embryonated cotyledon and single cotyledon attached decapitated embryo explants were used to develop transformation compatible *in vitro* regeneration system. Leaflet explants were obtained from aseptically germinated seeds. The other three explants such as decapitated half embryo, de-embryonated cotyledon and single cotyledon attached decapitated embryo explants were collected from over-night soaked seeds. Data was recorded by estimating the number of shoots per explants, number of responsive explants, as well as the days required for initiation of shoots from explants. After successful shoot development, the *in vitro* raised shoots were cultured for *in vitro* root induction. Rooted plantlets were consequently acclimatized in soil following proper hardening and allowed to grow under field conditions.

4.1.1 *In vitro* regeneration of shoots

Various explants of three varieties of peanut, namely Dhaka-1, BARI Chinabadam-8 and Binachinabadam-4 were used for *in vitro* regeneration of shoots on MS medium supplemented with different concentrations and combinations of auxins and cytokinins. The results of these experiments are described under the following headings.

4.1.1.1 Preparation of seed derived explants for *in vitro* regeneration

Seeds were surface sterilized with 70% alcohol and 0.1% $HgCl_2$ solution was used to maintain the aseptic condition and to avoid unwanted contamination of explants. Healthy seeds were dipped into 70% alcohol for one minute and then sterilized with 0.1% $HgCl_2$

solution for 8 - 10 minutes. Afterwards the seeds were rinsed with sterilized distilled water for 4 - 5 times to remove the traces of $HgCl_2$. For obtaining immature leaflet explants surface sterilized seeds were germinated in conical flasks containing autoclaved cotton soaked with sterile distilled water. Under this condition germination of seeds required 2 - 4 days (Table 1). Leaflet explants were collected from 6 - 8 days old seedlings (Fig. 6). Decapitated half embryo, de-embryonated cotyledon and single cotyledon attached decapitated embryo explants were prepared from surface sterilized overnight soaked seeds. Occasionally, de-embryonated cotyledon explants were prepared from seeds soaked with sterile distilled water for two to four hours.

Table 1. Germination of seeds of two varieties of peanut on sterile cotton bed soaked with sterile distilled water.

Peanut varieties	No. of seeds inoculated	No. of seeds germinated	% of germination	Days required for seed germination
Dhaka-1	50	49	98	2 – 3
BARI Chinabadam-8	50	48	96	3 – 4

4.1.1.2 Effect of age of immature leaflet explants towards *in vitro* regeneration

It was observed that the age of the immature leaflet explants played vital role towards *in vitro* regeneration of shoots. In case of regeneration of shoots from leaflet explants, it was found that in most cases shoot regeneration was seriously influenced by the age of leaflet. Both the varieties of peanut seeds took 2 - 4 days for germination (Table 1). It was found that leaflet explants collected from early germinating seeds (4 - 5 days) was not suitable for initiation of shoots (Fig. 5). On the other hand it was also noticed that leaflet explants collected from delayed germinated seeds (10 - 12 days) were also not suitable for *in vitro* regeneration. The leaflet explants collected from longer period of germinated seeds produced more callus rather than initiation of shoots. Leaflets explants collected from 7 days old germinated seed of Dhaka-1 showed best result towards shoots regeneration (Fig. 6b). But in case of BARI Chinabadam-8, it took 8 days to collect the most responsive leaflet explants from the germinated seeds (Fig. 6c). Results of these experiments have been shown in Fig. 5. It was also noticed that the best responsive young leaflet explants were characterized with light red or purple mid rib. Different stages of

germinated seeds of peanut in collecting the leaflet explants are presented in Fig. 6 (a, b and c).

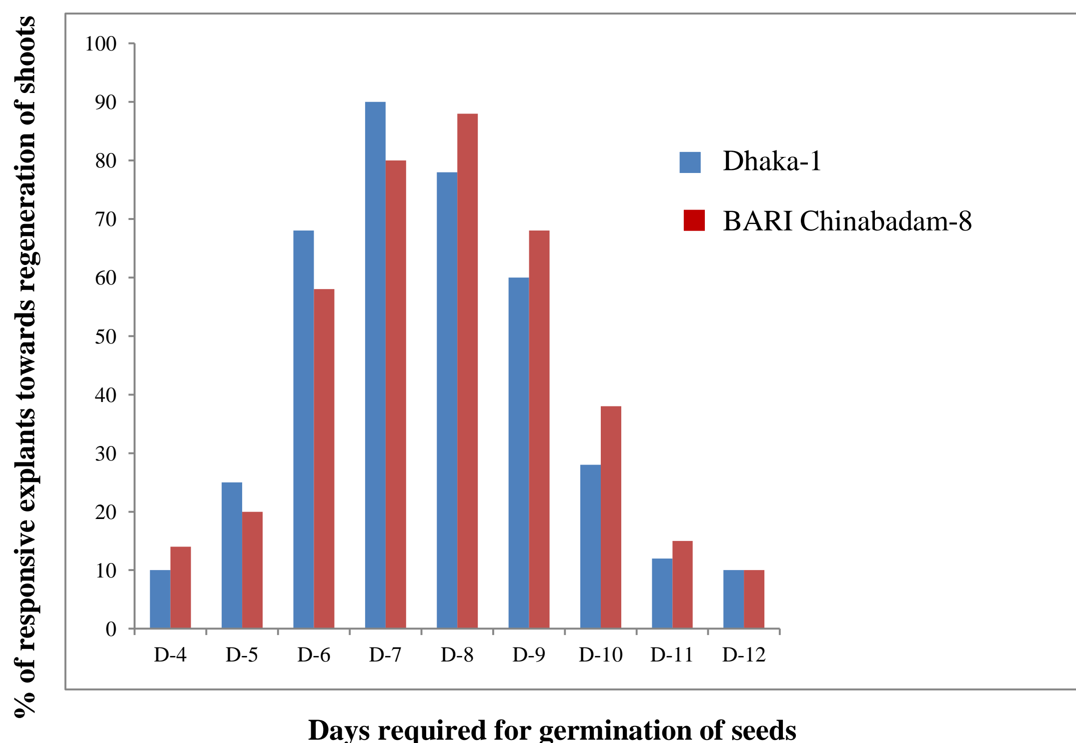

Fig. 5: Effect of germination period of peanut seeds in collecting the immature leaflet explants towards regeneration of shoots.

4.1.1.3 Effect of culture medium and hormonal supplements towards regeneration of shoots from different explants

Each type of explants requires specific hormonal supplements for the initiation of organogenesis from *in vitro* grown cultures. Composition of culture medium is always considered as an important factor for the successful *in vitro* regeneration from any explant. In the present investigation, MS medium with different concentrations and combinations of hormonal supplements were used for induction and elongation of shoots. For this purpose, MS medium with three different hormonal supplements such as BAP, Kn and NAA were used to obtain the proper initiation of shoots from immature leaflet, decapitated half embryo, de-embryonated cotyledon and single cotyledon attached decapitated embryo explants. The explants of immature leaflet and decapitated half embryo produced adequate responses towards the initiation and development of shoots (Table 2, 3, 4, 5 and 10). In the present set of experiments it was noticed that de-embryonated cotyledon explants did not show any response towards regeneration on MS

Fig. 6 (a - c): Collection of immature leaflet explants. (a) Various stages of seed germination (in days); (b) Immature leaflet explants (arrow) collected from 7 days old germinated seeds of Dhaka-1 (Bar = 2.5 mm); (c) Leaflet explants (arrow) collected from 8 days old germinated seeds of BARI Chinabadam-8 (Bar = 2.91 mm).

medium containing the above mentioned hormonal supplements (Table 12). The single cotyledon attached decapitated embryo explants also did not produced adequate responses for shoot development on MS with different hormonal supplements. For this reason MSB medium was used for the regeneration of shoots from the explants of de-embryonated cotyledon and single cotyledon attached decapitated embryo. MSB medium used in this study consisted of MS macro and micro nutrients (Murashige and Skoog, 1962) and vitamin of Gamborg's B5 medium (Gamborg, 1968). MSB medium supplemented with BAP alone and in combination of BAP and 2,4-D were used towards direct and indirect regeneration of shoots from both the de-embryonated cotyledon and single cotyledon attached decapitated embryo explants. The hormonal supplements used for shoot induction was considered as shoot induction medium (SIM) and the hormonal supplements used for shoot elongation was considered as shoot elongation medium (SEM).

4.1.1.3.1 Effect of various concentrations of BAP on regeneration of shoots from immature leaflet explants of Dhaka-1.

MS medium supplemented with different concentrations of BAP (4.4, 11.1, 22.2, 33.3 and 44.4 μM) were employed to examine their effects on initiation of regeneration and development of shoots from immature leaflet explants of Dhaka-1. The results of experiment have been presented in Table 2.

Form immature leaflet explants regeneration was initiated through the intervention of callus (Fig. 7a). Induction of callus was initiated after 9 - 16 days of inoculation of leaflet explants in MS medium supplemented with BAP. Occasionally leaflet explants produced shoots directly without the formation of callus (Fig. 7b). The best regeneration response via callus formation was observed on MS medium with 22.2 μM BAP. In most of the cases 10 -15 days were required for the induction of callus regeneration. Shoot buds were found to initiate within the developed callus on the same hormonal supplements. Numerous shoot buds as well as elongation of previously formed shoots were found to initiate from the induced calli within 105 days of culture (Fig. 7c and 7d). Highest mean number of shoots (9.0) was obtained on MS media supplemented with 22.2 μM BAP. MS medium containing 33.3 μM BAP also produced higher number multiple shoots. However, most of the regenerated shoots were stunted in growth and did not elongate properly in this hormonal combination of BAP (Fig. 7e). The lowest response of shoot regeneration was observed on MS with 4.4 μM BAP (Table 2, Fig. 7f).

4.1.1.3.2 Effect of different concentrations of BAP towards regeneration of shoots from immature leaflet explants of BARI Chinabadam-8

MS medium with different different concentrations of BAP (4.4, 11.1, 22.2, 33.3 and 44.4 µM) were employed to examine their effects on initiation of regeneration and development of shoots from immature leaflet explants obtained from BARI Chinabadam-8. Similar to Dhaka-1 multiple shoot buds initiated from the callus of the leaflet explants. The results of this experiment are presented in Table 3. The best regeneration responses were observed on MS medium with 22.2 µM BAP where 12 - 16 days were required for initiation of callus. Multiple shoots were found to initiate from the developed callus on the same media. Numerous shoot buds and elongation of previously formed shoots were initiated within 105 days of culture. In this concentration of BAP 73.33% explants were responded towards shoot initiation from the callus and highest mean number of shoots per explants were 8.5 in case of BARI Chinabadam-8. Different stages of shoot regeneration on this hormonal combination of BAP are shown in Fig. 8 (a - c). MS medium containing 33.3 µM BAP also produce higher number of multiple shoots where mean number of shoots per explants were 8.2. In this concentration of BAP rate of elongation of shoots was comparatively better than that of Dhaka-1 (Fig. 8d). When the explants were cultured on MS medium containing 11.1 µM BAP, 75% explants were responded towards shoot initiation but in this combination mean number of shoots per explants was 5.8. Mean number of shoots per explants was 4.25 and 5.60 when the explants were cultured on MS medium containing 4.4 µM BAP and 44.4 µM BAP, respectively.

4.1.1.3.3 Combined effect of different concentrations and combinations of BAP and Kn towards shoot regeneration from immature leaflet explants of Dhaka-1

MS medium supplemented with different concentrations and combinations of BAP (11.1 and 22.2 µM) and Kn (0.46 and 2.3 µM) were employed to examine their effects on callus induction and shoot development from immature leaflet explants from two varieties of peanut. In this experiment a total of four different combinations of BAP and Kn were tested. Results of these experiment have been presented in Table 4.

Table 2. Response of immature leaflet explants towards multiple shoot regeneration using different concentrations of BAP in MS medium in case of Dhaka-1.

Conc. of BAP (µM)	No. of explants inoculated	% of responsive explants towards callus formation	Days required for initiation of callus	% of callus producing explants initiating shoots	Mean no. of shoots/ explant after 3.5 months of culture
4.4	45	86.66	10 - 15	66.66	5.16
11.1	45	88.88	9 - 15	60.00	6.4
22.2	**45**	**91.11**	**10 - 15**	**80.00**	**9.0**
33.3	45	93.33	10 - 15	75.00	8.5
44.4	45	91.11	11 - 16	60.00	6.0

Table 3. Effect of different concentrations of BAP containing MS medium for multiple shoot regeneration from immature leaflet explants in case of BARI Chinabadam-8.

Conc. of BAP (µM)	No. of explants inoculated	% of responsive explants towards callus formation	Days required for initiation of callus	% of callus producing explants initiating shoots	Mean no. of shoots/ explant after 3.5 months of culture
4.4	45	93.33	10 - 16	60.00	4.25
11.1	45	86.66	10 - 16	75.00	5.8
22.2	**45**	**88.88**	**12 - 16**	**73.33**	**8.5**
33.3	45	91.11	11 - 16	62.50	8.2
44.4	45	88.88	12 - 17	53.33	5.6

The best regeneration responses towards callus formation as well as shoot regeneration was observed when leaflet explants were cultured on MS medium supplemented with 22.2 µM BAP and 2.3 µM Kn. In this hormonal combinations of BAP and Kn, 96% explants produced callus and from the callus producing explants 72.22% was found to initiate shoots within 60 - 75 days of culture. The mean number of shoots/explant was 8.0 on 22.2 µM BAP and 2.3 µM Kn supplemented media after 3.5 months of culturing of the explants. Different stages of regeneration (shoot bud formation, proliferation and multiplication of shoots) of shoots using the combination of 22.2 µM BAP and 2.3 µM Kn on MS medium are shown in Fig. 9 (a - c). Regenerated shoots were sub-cultured on the same medium containing the same hormonal supplements for their multiplication and elongation at regular intervals of four weeks. In each subculture newly developed shoot buds were observed along with the elongated shoots. MS medium supplemented with 11.1 µM BAP and 2.3 µM Kn also showed good response towards multiple shoots formation (Fig. 9d and e). In this hormonal combination of 11.1 µM BAP and 2.3 µM Kn 66.66% explants was found to initiate shoots within 60 - 75 days of culture and the mean number of shoots/explant was 7.4. Further it was noticed that MS medium supplemented with 11.1 µM BAP and 0.46 µM Kn showed 55% shoot regeneration response with 6.0 mean number of shoots/explant. The percentage of shoot regeneration responsive of explants was 65.5 and the mean number of shoots/explant was 6.5 in the combination of MS with 22.2 µM BAP and 0.46 µM Kn (Table 4, Fig. 9f).

Table 4. Combined effect of different combination of BAP and Kn towards multiple shoot regeneration using immature leaflet explants of Dhaka-1.

Conc.of hormones (µM) BAP + Kn	No. of explants inoculated	% of responsive explants towards callus induction	Days required for initiation of callus	% of callus producing explants initiating shoots	Mean no. of shoots/ explant after 3.5 months of culture
11.1 + 0.46	50	94	10 - 16	55.0	6.2
11.1 + 2.3	50	92	14 - 18	66.66	7.4
22.2 + 0.46	50	90	12 - 17	65.5	6.5
22.2 + 2.3	**50**	**96**	**10 - 15**	**72.22**	**8.0**

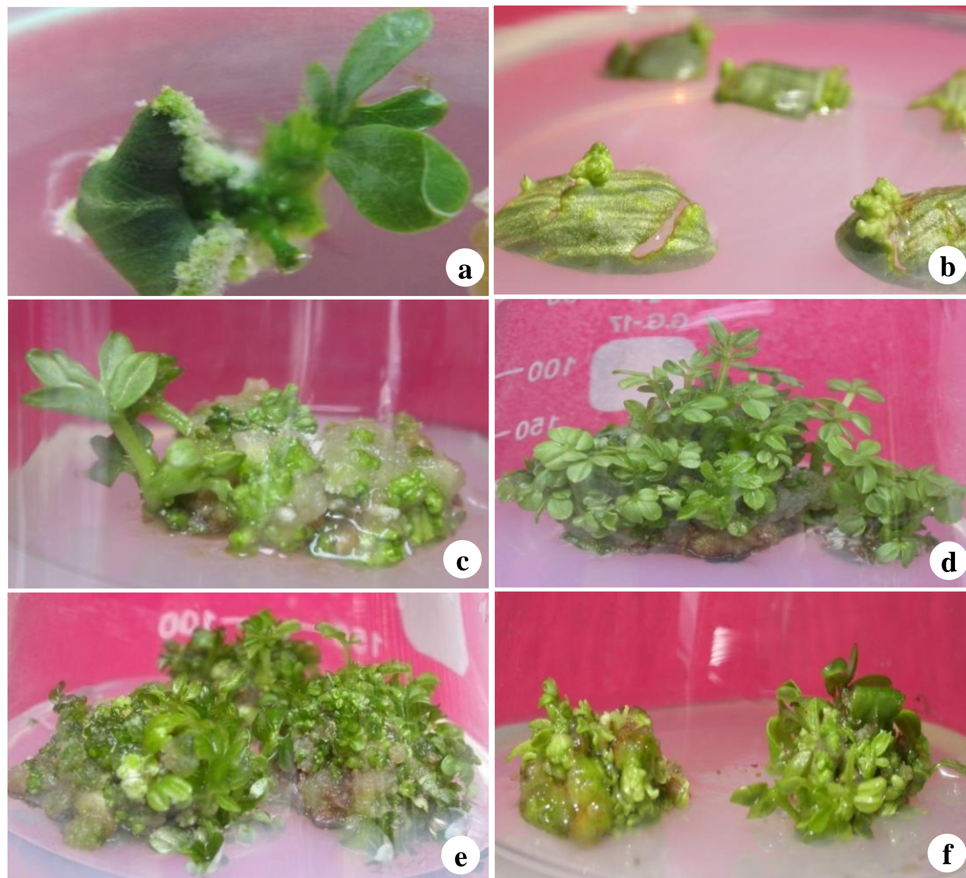

Fig. 7 (a - f): Different stages of shoot formation from immature leaflet explants of Dhaka-1 on MS medium supplemented with different concentrations of BAP. (a) Initiation of shoots via callus formation from leaflet explants on MS supplemented with 22.2 µM BAP; (b) Shoot initiated directly from leaflet explants on the same medium as mentioned in Fig. 7a; (c) Multiple shoots formation on MS medium containing 22.2 µM BAP; (d) Elongated multiple shoots on the same medium mentioned in Fig. 7c; (e) Shoot bud along with multiple shoots developed on MS medium containing 33.3 µM BAP; (f) Multiple shoots formation on MS medium containing 4.4 µM BAP.

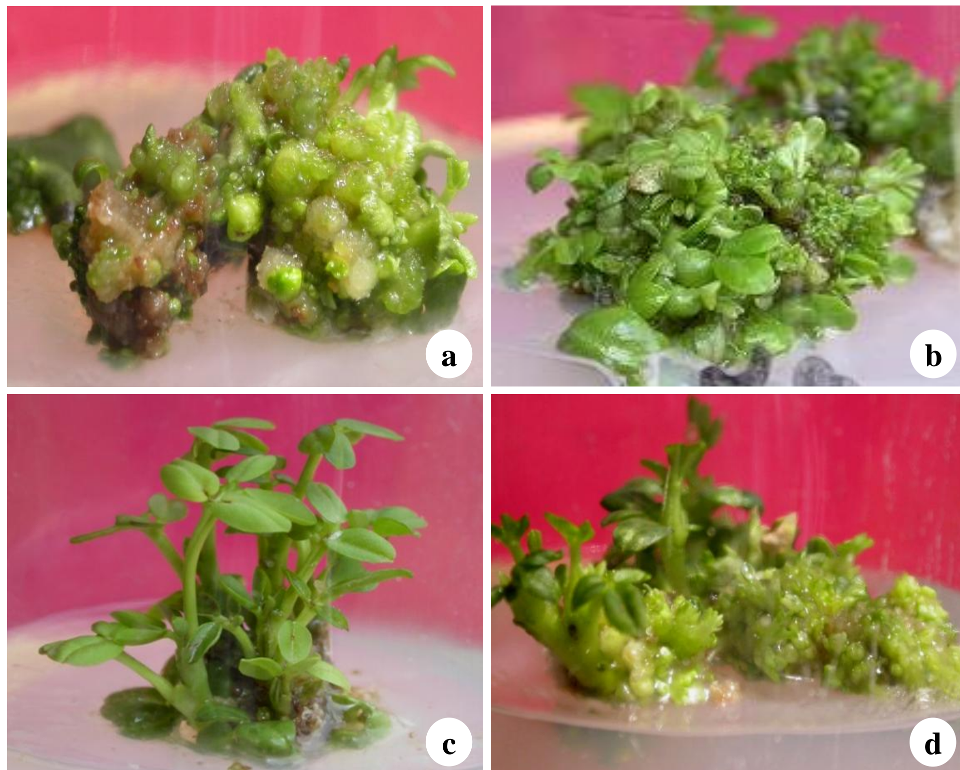

Fig. 8 (a - d): Various stages of shoot regeneration from immature leaflet explants of BARI Chinabadam-8 on MS medium supplemented with various concentrations of BAP. (a) Initiation of multiple shoots on MS medium containing 22.2 µM BAP; (b) Development of multiple shoots on the same medium as mentioned in Fig. 8a; (c) Elongation of multiple shoots on the same medium as mentioned in Fig. 8a; (d) Multiple shoot formation and elongation of shoots on MS medium containing 33.3 µM BAP.

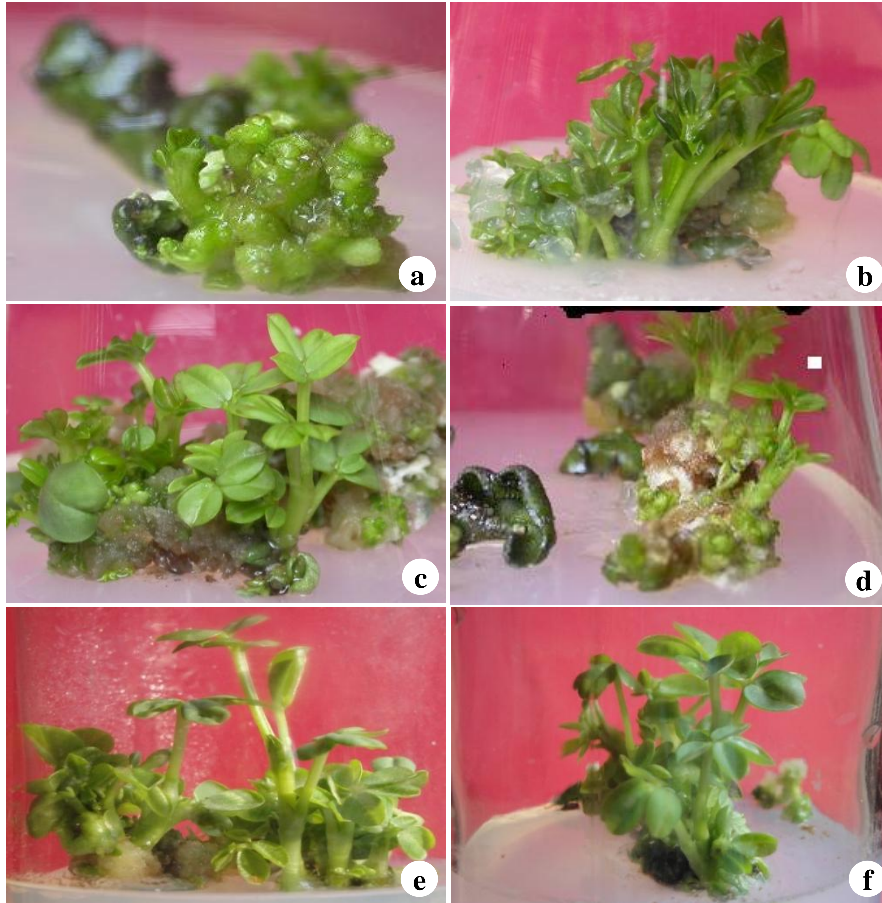

Fig. 9 (a - f): Various stages of shoot development from immature leaflet explants of Dhaka-1 on MS medium supplemented with various concentration of BAP and Kn. (a) Shoot bud formation on MS medium supplemented with 22.2 µM BAP and 2.3 µM Kn; (b) Elongated multiple shoots developed on the same medium as mentioned in Fig. 9a; (c) Multiple shoots formation via callus formation on medium as mentioned in Fig. 9a; (d) Multiple shoot bud formation along with elongated shoots via callus formation on MS medium supplemented with 11.1 µM BAP and 2.3 µM Kn; (e) Elongation of shoots on medium as mentioned in Fig. 9d; (f) Elongated shoots developed on MS medium with 22.2 µM BAP and 0.46 µM Kn.

4.1.1.3.4 Combined effect of BAP and Kn towards multiple shoot formation from immature leaflet explants of BARI Chinabadam-8

In case of BARI Chinabadam-8, MS medium supplemented with different concentrations and combinations of BAP (11.1 and 22.2 µM) and Kn (0.46 and 2.3 µM) were used to observed their effects on induction of callus as well as initiation of shoot from immature leaflet explants. Results of this set of experiment have been presented in Table 5. More or less 88 - 94% of cultured leaflet explants showed response towards callus initiation depending on different concentrations of hormonal supplements. Afterwards shoots were initiated from the induced callus depending on the nature of hormonal supplements. Among the various concentrations, the best regeneration response towards shoot formation was observed on MS medium supplemented with 22.2 µM BAP and 2.3 µM Kn. In this hormonal combination 75% explants showed shoot initiation and mean number of shoots/explant was recorded as 7.2 after 3.5 months of culture (Fig. 10a).

Regenerated shoots were sub-cultured on the same medium with same hormonal combinations for their multiplication and elongation at regular intervals of four weeks (Fig.10b and 10c). Moreover from the Table 5 it is observed that in the hormonal combination of 11.1 µM BAP and 2.3 µM Kn, 62% explants showed shoot initiation within 65 - 75 days of culture and the mean number of shoots/explant was 7.0. MS medium supplemented with 11.1 µM BAP and 0.46 µM Kn as well as MS with 22.2 µM BAP and 0.46 µM Kn showed 58% and 78% shoot regeneration responses, respectively where mean number of shoots/explant was 6.0 in both the combinations (Fig. 10d).

Table 5. Effect of different concentrations of BAP and Kn in MS medium for multiple shoot regeneration in case of BARI Chinabadam-8 from immature leaflet explants.

Conc.of hormones (µM) BAP + Kn	No. of explants inoculated	% of responsive explants	Days required for initiation of callus	% of callus producing explants initiating shoots	Mean no. of shoots/ explant after 3.5 months of culture
11.1 + 0.46	50	90	12 - 18	58	6.0
11.1 + 2.3	50	90	12 - 16	62	7.0
22.2 + 0.46	50	88	12 - 16	78	6.0
22.2 + 2.3	**50**	**94**	**10 - 16**	**75**	**7.2**

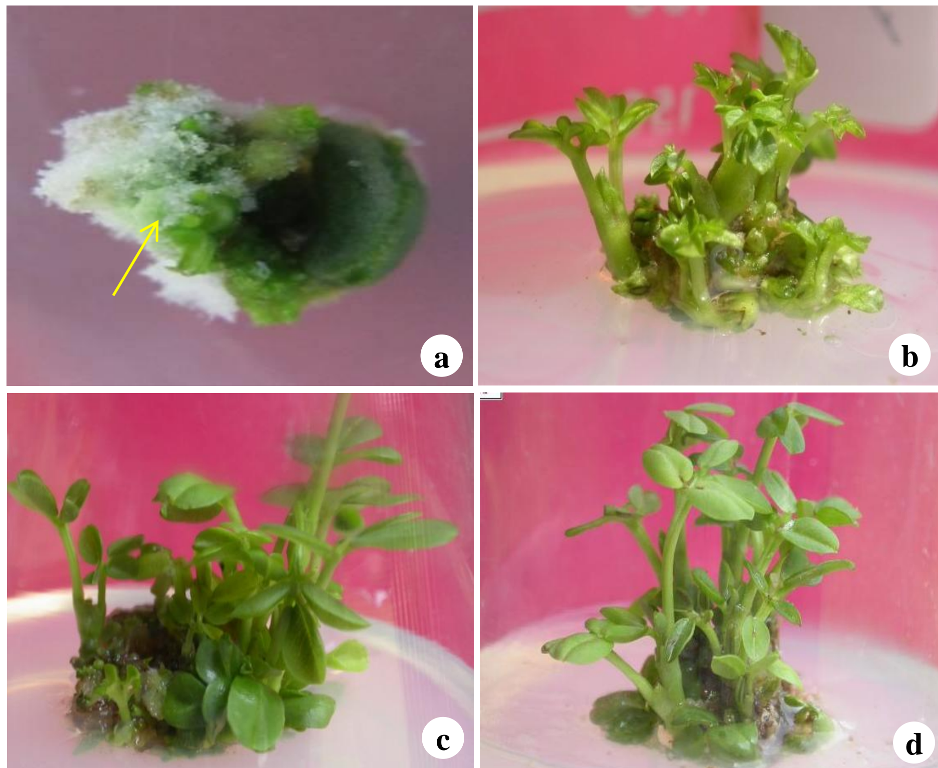

Fig. 10 (a - d): Various stages of shoot formation from immature leaflet explants of BARI Chinabadam-8 on MS medium supplemented with different concentration of BAP and Kn. (a) Shoot formation from the callus (arrow) on MS medium supplemented with 22.2 µM BAP and 2.3 µM Kn; (b) Multiple shoot formation on the same medium as mentioned in Fig 10a; (c) Elongated multiple shoots developed on the same medium as mentioned in Fig 10a; (d) Fully developed elongated shoots on MS medium with 22.2 µM BAP and 0.46 µM Kn.

4.1.1.3.5 Effect of different concentration and combinations of BAP and NAA on regeneration of shoots from immature leaflet explants of Dhaka-1

To evaluate the combined effect of BAP and NAA towards initiation of shoots from the immature leaflet explants of Dhaka-1, six different hormonal combinations were applied. For this purpose MS medium with different concentrations and combinations of BAP (22.2, 44.4 and 66.6 µM) and NAA (2.6 and 5.4 µM) were used to induce callus and consequent development of shoots. Table 6 is presented to elaborate the results of this experiment. In the present experiment it was noticed that all leaflet explants showed early response towards callus induction but percentage of shoot formation was low in all the combinations of BAP and NAA compare to BAP alone (Table 2) and in combination of BAP and Kn (Table 4). About 6 - 14 days were required for callus induction at various concentrations of BAP and NAA. Among the various combinations tested maximum response towards shoot regeneration was observed on MS medium supplemented with 44.4 µM BAP with 2.6 µM NAA (Fig. 11a and 11b). In this combination 38.88% explants showed shoot initiation response where mean number of shoots per explants were 4.0. The mean number of shoots per explant was found to vary from 2.0 to 4.0 when explants were cultured on the other BAP and NAA supplemented medium (Fig. 11c and Table 6). It was observed that in many cases when the explants were cultured on MS with various BAP and NAA supplemented medium each explants produced large amount of callus rather than formation of shoots compared to other combination of BAP alone and in combination of BAP and Kn (Fig. 11d).

Table 6. Response of immature leaflet explants towards multiple shoot regeneration using different concentrations of BAP and NAA in MS medium in case of Dhaka-1.

Conc.of hormones (µM) BAP+ NAA	No.of explants inoculated	% of callus producing explants	Days required for initiation of callus	% of callus producing explants initiating shoots	Mean no. of shoots/ explant after 3.5 months of culture
22.2 + 2.6	45	97.77	6 - 9	26.66	2.4
22.2 + 5.4	45	95.55	7 - 9	20.00	2.6
44.4 + 2.6	**45**	**88.88**	**8 - 12**	**38.88**	**4.0**
44.4 + 5.4	45	95.55	8 - 13	33.33	3.0
66.6 + 2.6	45	91.11	8 - 14	38.88	2.5
66.6 + 5.4	45	93.33	9 - 14	30.00	2.0

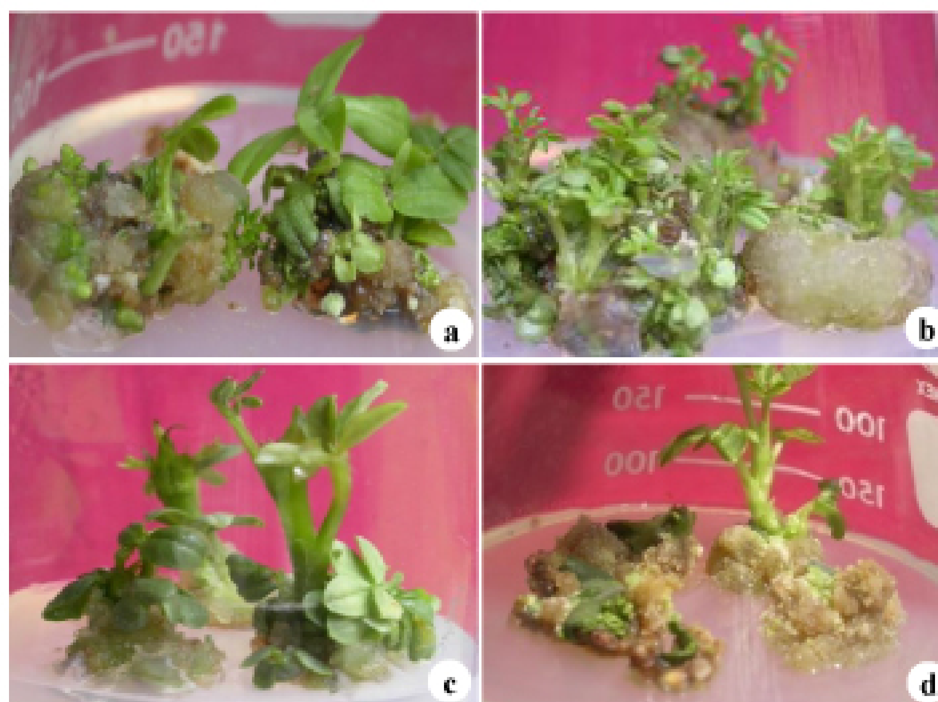

Fig. 11 (a - d): Different stages of shoot formation from immature leaflet explants of Dhaka-1 on MS medium with different concentration of BAP and NAA. (a) Initiation of multiple shoots on MS medium supplemented with 44.4 μM BAP and 2.6 μM NAA; (b) Elongated multiple shoots along with callus on the same medium as mentioned in Fig 11a; (c) Multiple shoots formation on MS medium with 22.2 μM BAP and 2.6 μM NAA; (d) Induction of profuse callus from leaflet explants on MS medium with 66.6 μM BAP and 5.4 μM NAA.

4.1.1.3.6 Effect of various combinations of BAP and NAA on shoot regeneration from immature leaflet explants of BARI Chinabadam -8

In case of BARI Chinabadam-8, MS medium supplemented with different concentrations and combinations of BAP (22.2, 44.4 and 66.6 µM) and NAA (2.6 and 5.4 µM) were applied to observed their effects towards initiation of shoots using immature leaflet explants. Six different combinations of above mentioned hormonal supplements were employed to induce callus as well as regeneration of shoots. Results of this experiment has been shown in Table 7. It was noticed that in all the hormonal combinations of BAP and NAA, all leaflet explants produced very poor number of shoots. This variety required 6 - 16 days for initiation of callus at various concentrations of BAP and NAA. Among the various combinations maximum shoot regeneration was observed on MS medium supplemented with 22.2 µM BAP and 2.6 µM NAA (Fig. 12a and 12b). In this combination of BAP and NAA 40% explants showed initiation of shoot and mean number of shoots per explants was recorded to be 4.0. It was observed that when explants were cultured on MS with higher BAP and NAA supplemented medium the explants produced large amount callus instead of producing shoots (Fig. 12c and 12d).

Table 7. Response of immature leaflet explants from BARI Chinabadam-8 towards multiple shoot regeneration using different concentrations of BAP and NAA in MS medium.

Conc. of hormones (µM) BAP+NAA	No. of explants inoculated	% of explants formed callus	Days required for initiation of callus	% of callus producing explants initiating shoots	Mean no. of shoots/ explant after 3.5 months of culture
22.2 + 2.6	**45**	**95.55**	**6 - 8**	**40.00**	**3.8**
22.2 + 5.4	45	95.55	7 - 10	40.00	3.5
44.4 + 2.6	45	93.33	8 - 11	33.00	2.0
44.4 + 5.4	45	93.33	10 - 14	30.00	3.0
66.6 + 2.6	45	95.55	11 - 16	25.00	1.75
66.6 + 5.4	45	88.88	10 - 16	20.00	2.25

4.1.1.3.7 Response of decapitated half embryo explants of Dhaka-1 towards multiple shoots regeneration on MS medium with different concentrations of BAP

In case of Dhaka-1, MS medium supplemented with different concentrations of BAP (4.4, 11.1, 22.2, 33.3 and 44.4 µM) were used to examine its effect on initiation of shoot regeneration and their development from decapitated half embryo explants. About 90 - 95% of cultured explants were responded towards regeneration of shoots depending on different concentrations of hormonal supplements. The results of these observations are presented in Table 8. In this experiment, a total of five different hormonal combinations were tested. Among these combinations of BAP maximum shoot regeneration was observed on MS medium supplemented with both 22.2 µM as well as 33.3 µM BAP (Table 8). Initiation of shoots started within 5-10 days of inoculation on MS supplemented with 22.2 µM BAP as well as MS with 33.3 µM BAP (Fig. 13a). Mean number of shoot per explant was 4.0 after 40 days of inoculation on MS with BAP supplements (Fig. 13b and 13c). From the Table it is noticed that MS medium with 4.4 µM BAP produced lower number of shoots per explants (Fig. 13d).

Table 8. Effect of different concentrations of BAP in MS medium towards multiple shoot regeneration using decapitated half embryo explants of Dhaka-1.

BAP (µM)	No. of explants inoculated	Days required for initiation of shoot regeneration	% of explants showed shoot initiation	Mean no. of shoots/ explant after 40 days of culture
4.4	40	8 - 10	90	1.5
11.1	40	6 - 12	94	3.2
22.2	**40**	**5 - 10**	**94**	**4.0**
33.3	**40**	**6 - 10**	**95**	**4.0**

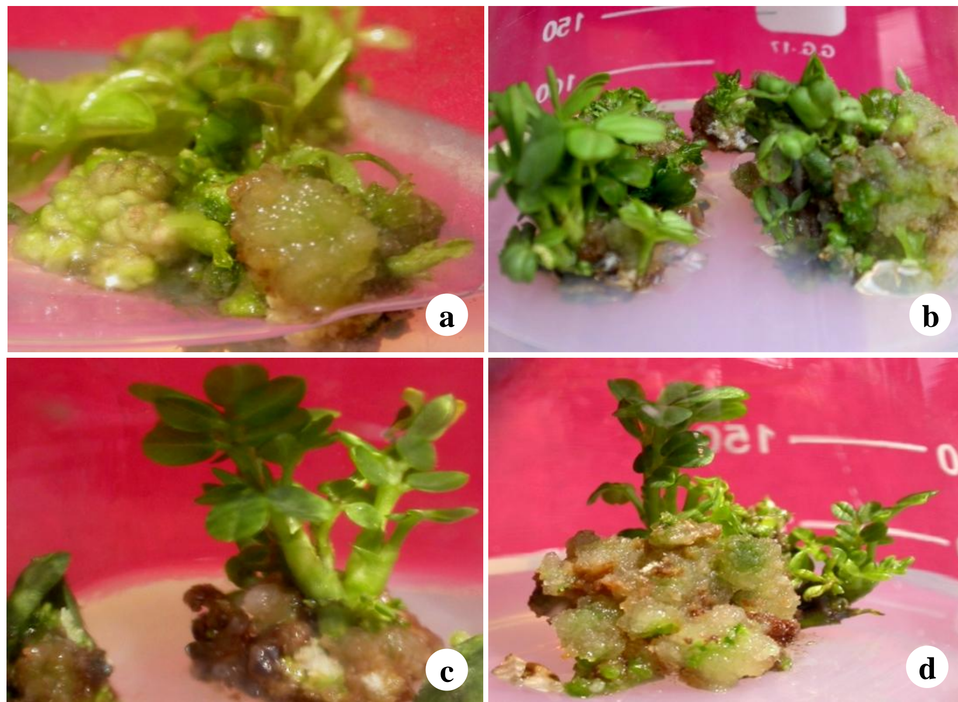

Fig. 12 (a - d): Regeneration of shoot from immature leaflet explant of BARI Chinabadam-8 on MS medium supplemented with BAP and NAA. (a) Multiple shoot formation on MS medium supplemented with 22.2 µM BAP and 2.6 µM NAA; (b) Elongated multiple shoots along with callus on the same media composition as mentioned in Fig. 12a; (c) Shoot regeneration from the callus on MS medium with 44.4 µM BAP and 2.6 µM NAA; (d) Induction of profuse callus on MS medium containing 66.6 µM BAP and 5.4 µM NAA.

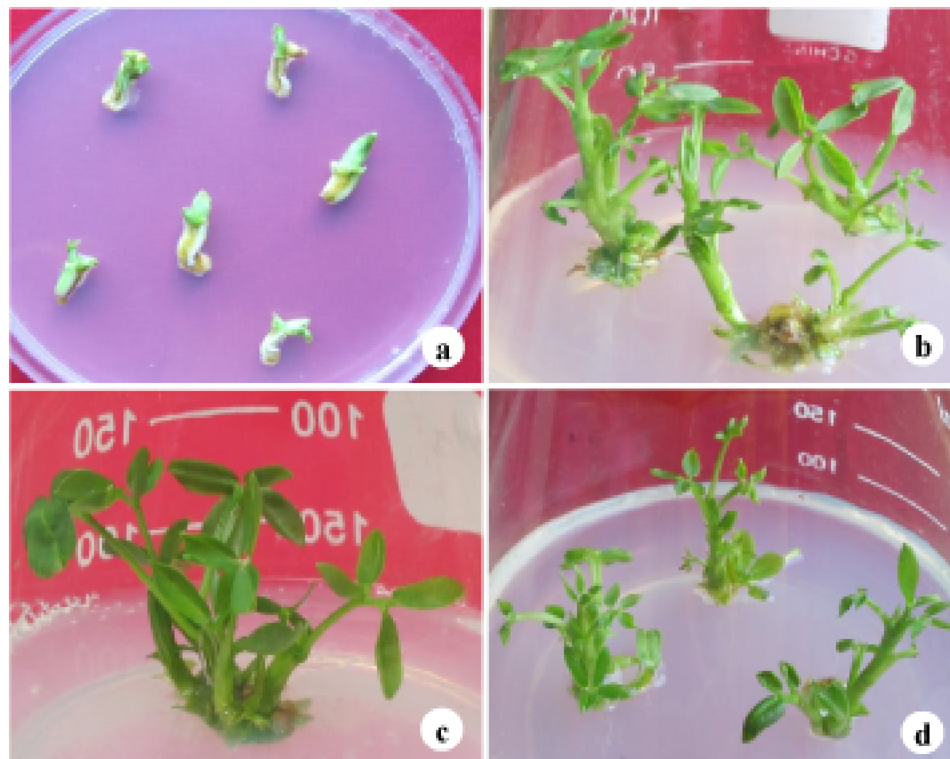

Fig. 13 (a - d): Various stages of shoot formation from decapitated half embryo explants from Dhaka-1 on MS medium supplemented with various concentration of BAP. (a) Initiation of shoots from decapitated half embryo explants; (b) Multiplication and elongation of shoots on MS medium containing 22.2 µM BAP; (c) Multiplication of shoots on MS medium containing 33.3 µM BAP; (d) Shoot regeneration on MS medium containing 11.1 µM BAP.

4.1.1.3.8 Effect of different concentrations of BAP on MS medium towards regeneration of shoots from decapitated half embryo explants of BARI Chinabadam-8

Half embryo explants of BARI Chinabadam-8 were inoculated on MS with different concentrations of BAP (4.4, 11.1, 22.2, 33.3, 44.4 µM) to show their proper shoot initiation response. About 92 - 96% explants showed regeneration of shoots on the above mentioned hormonal supplements. The results of these observations are described in Table 9. The best regeneration was observed on MS medium supplemented with 22.2 µM BAP. In this concentration of BAP 8 - 10 days were required for shoot initiation (Fig. 14a) and mean no of shoots/explant was 4.2 (Fig. 14b). However, it took about 7 - 12 days for initiation of shoots on MS medium containing other concentrations of BAP supplemented media (Table 9). MS medium with 4.4 µM BAP produced lower mean number of shoots (2.0) compared to other concentrations of BAP (Table 9, Fig. 14c). 94% explants showed initiation of shoots on both MS with 11.1 µM and 33.3 µM BAP supplemented media and the mean number of shoots/explant was 3.0 and 3.8, respectively (Fig. 14d).

Table 9. Response of decapitated half embryo explants towards multiple shoot using different concentrations of BAP in MS medium of BARI Chinabadam-8.

BAP (µM)	No. of explants inoculated	Days required for initiation of regeneration	% of explants showed shoot initiation	Mean no. of shoots/ explant after 40 days of culture
4.4	40	8 - 12	92	2.0
11.1	40	7 - 12	94	3.0
22.2	**40**	**8 - 10**	**96**	**4.2**
33.3	40	9 - 12	94	3.8

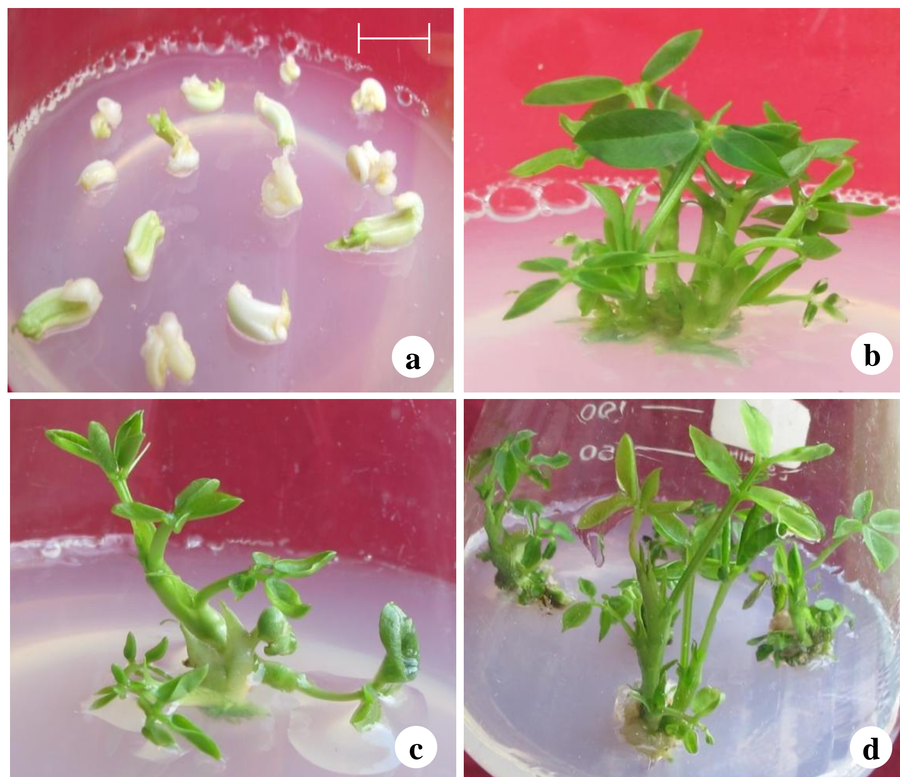

Fig. 14 (a - d): Various stages of development of shoots from decapitated half embryo explant of BARI Chinabadam-8 on MS medium supplemented with BAP. (a) Shoot initiation from decapitated half embryo explants on MS medium containing 22.2 µM BAP (Bar = 2 mm); (b) Development of multiple shoots on the same media as mentioned in Fig. 14a; (c) Shoot regenerated on MS medium containing 4.4 µM BAP; (d) Shoot developed on MS medium containing 33.3 µM BAP.

4.1.1.3.9 Effect of different combinations and concentrations of BAP and Kn on regeneration of shoots from decapitated half embryo explants of Dhaka-1

In this experiment four different combinations of BAP and Kn were tested to examine their effects on shoot initiation and shoot development from decapitated half embryo explants of Dhaka-1. The results of these observations are presented in Table 10. Shoot regeneration was observed from decapitated half embryo explants in all the combinations of BAP and Kn although number of shoots/explant varied in different combinations. Among the different combinations best shoot regeneration was observed on MS medium supplemented with 22.2 µM BAP and 2.3 µM Kn. In this combination 100% explants responded towards regeneration and mean number of shoots/explant was 5.0. Different stages of regeneration of shoots on this concentration are shown in Fig.15 (a - c). From the Table 10 it is observed that MS medium containing 11.1 µM BAP and 2.3 µM Kn also showed good response towards initiation of multiple shoots (96%) and mean number of shoots/explant was 4.8 (Fig. 15d). Occasionally formation of callus was noticed at the base of explants in the hormonal combinations of 22.2 µM BAP and 0.46 µM Kn in MS medium (Fig. 15e and 15f).

Table 10. Response of decapitated half embryo explants towards multiple shoot regeneration using different concentrations of BAP and Kn in MS medium from Dhaka-1.

BAP + Kn (µM)	No. of explants inoculated	Days required for initiation of regeneration	% of explants showed shoot initiation	Mean no. of shoots/ explants after 40 days of culture
11.1 + 0.46	50	9 - 11	96	4.5
11.1 + 2.3	50	9 - 10	96	4.8
22.2 + 0.46	50	9 - 11	92	4.0
22.2 + 2.3	**50**	**8 - 10**	**100**	**5.0**

Fig. 15 (a - f): Various stages of shoot regeneration from decapitated half embryo explants of Dhaka-1 on MS medium supplemented with BAP and Kn. (a) Shoot initiation from decapitated half embryo explants on MS with 22.2 µM BAP and 2.3 µM Kn; (b) Multiple shoots regenerated on the same media combination as mentioned in Fig. 15a; (c) Elongated shoots on the same medium as mentioned in Fig. 15a; (d) Multiple shoots along with elongated shoots on MS medium containing 11.1 µM BAP and 2.3 µM Kn; (e) Multiple shoots as well as formation of callus (arrow) on MS medium containing 22.2 µM BAP and 0.46 µM Kn; (f) Multiple shoots regenerated on MS medium containing 22.2 µM BAP and 2.3 µM Kn.

4.1.1.3.10 Combined effect of different combination of BAP and Kn in MS medium towards multiple shoot regeneration using decapitated half embryo explants of BARI Chinabadam-8

The regeneration ability of decapitated half embryo explants was tested to find out their shoot induction ability on MS medium supplemented with different concentration of BAP (11.1 and 22.2 µM) and Kn (0.46 and 2.3 µM). In all the BAP and Kn supplemented MS medium 92 - 100% explants exhibited response to induction of shoots. The mean number of shoots/explant was varied in different combinations of BAP and Kn in MS medium. Among the different combinations of BAP and Kn tested, best response towards regeneration of shoots was observed on MS medium supplemented with 11.1 µM BAP and 2.3 µM Kn. At this concentration 8 - 11 days were required for initiation of shoots where 100% explants responded and mean number of shoots/explant was 4.2 (Fig. 16a). In this hormonal combination multiple shoots along with callus formation was also observed (Fig. 16b). MS medium with 22.2 µM BAP and 0.46 µM Kn as well as 22.2 µM BAP and 2.3 µM Kn also showed 100% regeneration response but mean number of shoots/explant was recorded as 3.7 and 4.0, respectively (Fig. 16c and 16d). The results of these observations are presented in Table 11.

Table 11. Effect of different concentrations of BAP and Kn on MS medium using decapitated half embryo explants of BARI Chinabadam-8 towards multiple shoot regeneration.

BAP + Kn (µM)	No. of explants inoculated	% of explants showed shoot initiation	Days required for initiation of regeneration	Mean no. of shoots/ explant after 40 days of culture
11.1 + 0.46	50	92	8 - 11	3.2
11.1 + 2.3	50	100	8 - 11	4.2
22.2 + 0.46	50	100	9 - 10	3.7
22.2 + 2.3	50	100	8 - 10	4.0

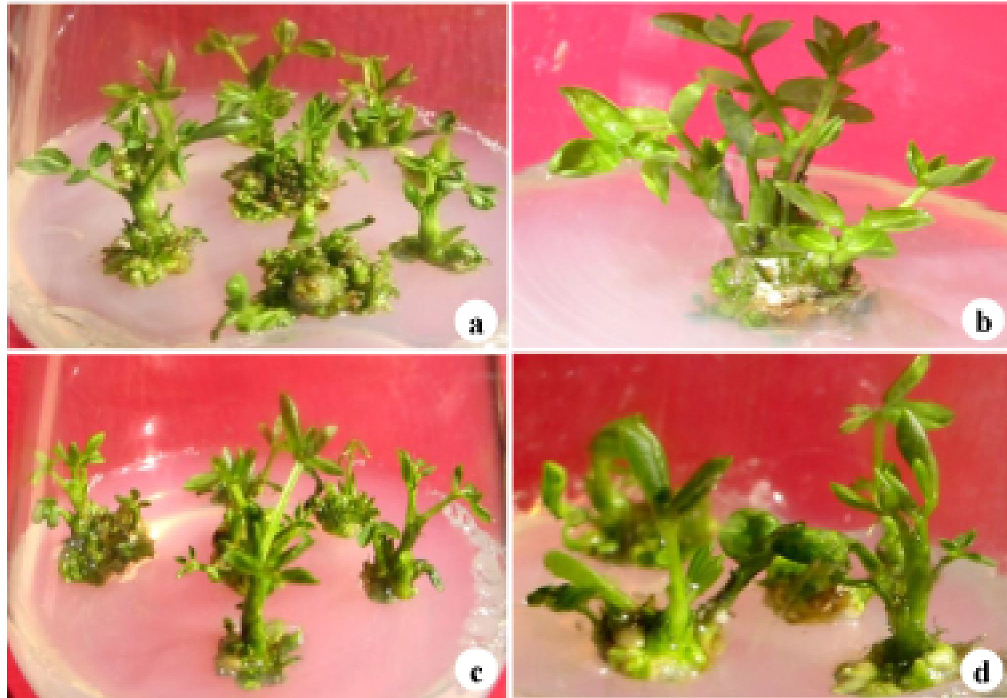

Fig. 16 (a - d): Different stages of shoot regeneration from decapitated half embryo explants of BARI Chinabadam-8 on MS medium supplemented with BAP and Kn. (a) Formation of multiple shoots on MS medium containing 11.1 µM BAP and 2.3 µM Kn; (b) Multiple shoot along with callus formation on the same media as mentioned in Fig. 16a; (c) Shoot regeneration on MS medium containing 22.2 µM BAP and 0.46 µM Kn; (d) Shoot regeneration on MS medium containing 22.2 µM BAP and 2.3 µM Kn.

4.1.1.3.11 Effect of MS medium with BAP alone and in combination of BAP and Kn from de-embryonated cotyledon and single cotyledon attached decapitated embryo from Dhaka-1 and BARI Chinabadam-8

A set of experiments were conducted using de-embryonated cotyledon and single cotyledon attached decapitated embryo explants from both the Dhaka-1 and BARI Chinabadam-8 on MS medium with BAP alone and in combination of BAP and Kn. These results have been presented in Table 12. In these experiments, it was found that no de-embryonated cotyledon explants showed any response towards callus induction as well as initiation of shoots on MS with any hormonal combinations (Table 12). In case of single cotyledon attached decapitated embryo explants all showed responses towards multiple shoots formation with lower number of shoots/explant. It was noticed that among the two media combinations best response was observed on MS with 22.2 µM BAP and 2.3 µM Kn from single cotyledon attached decapitated embryo explants of Dhaka-1.

Table 12: Response of de-embryonated cotyledon and single cotyledon attached decapitated embryo explants from Dhaka-1 and BARI Chinabadam-8 on MS medium with BAP and Kn.

Media combination.	Variety	Explants	No. of explants inoculated	Days required for shoot initiation	% of responsive explants	Mean no. of shoots/ explant after 40 days of culture
MS + 22.2 µM BAP	Dhaka-1	DEC	40	-	-	No response
		SCAE	40	5-10	62	3.5
	BARI Chinabadam-8	DEC	40	-	-	No response
		SCAE	40	7-10	60	3.2
MS + 22.2 µM BAP + 2.3 µM Kn	Dhaka-1	DEC	40	-	-	No response
		SCAE	40	5-9	75	4.2
	BARI Chinabadam-8	DEC	40	-	-	No response
		SCAE	40	6-10	75	4.0

DEC= De-embryonated cotyledon; SCAE= Single cotyledon attached decapitated embryo.

4.1.1.3.12 Effect of BAP and 2,4-D on MSB (MS salts and Gamborg's B_5 vitamins) medium towards shoot regeneration from de-embryonated cotyledon (DEC) explants of Dhaka-1.

From the previous experiments (section, 4.1.3.11) it was observed that de-embryonated cotyledon (DEC) explants did not show any regeneration response on MS media with various hormonal supplements. For this reason MSB (MS salts and Gamborg's B_5 vitamins) media was used for the initiation of regeneration from the de-embryonated cotyledon explants. The response of de-embryonated cotyledon explants towards shoot initiation via callus induction was examined on MSB medium supplemented with different concentrations of BAP and 2, 4-D. The results of the observation have been presented in Table 13. For this purpose de-embryonated cotyledon explants (Fig. 17a) were inoculated on shoot induction medium (SIM) which contained various concentration of BAP and 2, 4-D. Afterwards the explants with induced shoot buds were transferred on to the shoot elongation medium (SEM) which contained various lower concentrations of BAP. For SIM, MSB medium supplemented with various concentrations of BAP (10 µM and 20 µM) and 2, 4-D (10 µM and 20 µM) were used for induction of shoots through callus formation. It was observed that among the two combinations, MSB medium supplemented with 20 µM BAP and 10 µM 2,4-D showed the best responses towards shoot initiation. It was observed that after 4 - 5 days cotyledon turned green in colour (Fig. 17b) and within 8-10 days on SIM, callus formation was initiated from the explants. After 11 - 15 days shoot bud formation was observed on MSB with 20 µM BAP and 10 µM 2,4-D supplemented medium (SIM) (Fig. 17c). It may be mentioned here that it was necessary to embed the cut ends of the cotyledon explants within the medium otherwise callus induction and shoot bud formation was found to hamper significantly. In this situation most of the de-embryonated cotyledon explants were not turned green in colour, as initiation of callus and shoots was started from large green cotyledon.

After two weeks, the cotyledon explants were transferred to shoot elongation medium (SEM) which contained MSB supplemented with 2 - 4 µM BAP. Maximum response towards multiple shoot bud initiation was observed on MSB with 4 µM BAP. It was observed that when DEC explants incubated for 2 weeks on SIM, the proximal end (de-embryonated portion), distal ends (opposite of de-embryonated portion) and cut surfaces differentiated into callus. Upon subculture the explants on shoot elongation medium, multiple shoot buds were started to initiate from the proximal end of cotyledon (Fig. 17c), while compact undifferentiated calli was formed on the rest of the sites (Fig. 17d). In

Dhaka-1, 68.57% explants showed shoot initiation response on MSB with 2 µM BAP whereas 74.28% shoot initiation was observed on MSB containing 4 µM BAP. The mean number of shoots/explant was 5.5 and 6.5 when cotyledon explants were cultured on MSB with 2 µM BAP and MSB with 4 µM BAP supplemented media, respectively (Fig. 17e, Table13). It was noticed that when the cotyledon explants were kept on shoot induction medium more than 16 days formation of roots was observed from the callus of proximal end.

On the other hand about 33.33% and 35.0% of cotyledon explants responded towards shoot regeneration on various concentration of SEM when the explants were initially cultured on MSB with 10 µM BAP and 20 µM 2,4-D (Table 13). In this case mean number of shoots per explant was 1.5 and 2.5 when cotyledon explants were cultured on SEM such as MSB with 2µM BAP and MSB with 4µM BAP, respectively. In MSB with 10 µM BAP and 20 µM 2,4-D supplemented media (SIM) explants produced profuse callus with slow initiation of shoots (Fig. 17f).

Using this explants it was observed that for proper development of shoots it was necessary to subculture whole cotyledon with the multiple shoots containing proximal parts. It was noticed that development and elongation of shoots was delayed when part of the de-embryonated cotyledon explants were subcultured on SEM.

4.1.1.3.13. Effect of BAP and 2,4-D on shoots regeneration from de-embryonated cotyledon (DEC) explants of BARI Chinabadam-8 and Binachinabadam-4

Following the observation obtained in case of Dhaka-1 (section 4.1.1.3.12), the present set of experiments were conducted using the de-embryonated cotyledon (DEC) explants of BARI Chinabadam-8 and Binachinabadam-4. For shoot initiation MSB supplemented with 20 µM BAP and 10 µM 2,4-D (considered as shoot induction medium, SIM) were used. And MSB with 4 µM BAP (considered as shoot elongation medium, SEM) showed optimum elongation of shoots. In BARI Chinabadam-8, 62.5% explants responded towards callus induction on SIM and among the callus 72% showed regeneration of shoots on MSB with 4 µM BAP (SEM).The mean no of shoots/explant was 5.5 in SEM. The results of these observations are presented in Table 14. It was observed that if explants were not sub-cultured in shoot elongation medium within 16 days, formation of profuse callus were formed rather than shoot initiation. Similar to Dhaka-1 this variety also showed the formation of roots from the compact green callus from the proximal end

of the de-embryonated cotyledon explants. In case of BARI Chinabadam-8, it was also observed that development and elongation of shoots was delayed when de-embryonated cotyledon explants were subcultured partially. Often it took 8 - 12 weeks for the elongation of shoots. Various stages of regeneration are presented in Fig. 18 (a - f).

When de-embryonated cotyledon explants of Binachinabadam-4 were cultured on MSB supplemented with 20 µM BAP and 10 µM 2,4-D (considered as SIM) 70% explants responded towards callus formation. When they were transformed on SEM (MSB with 4 µM BAP) 71% callus explants responded towards regeneration of shoots and mean no of shoots/explant was 4.2. The results of these observations are presented in Table 14. Various stages of regeneration are presented in Fig. 19 (a - d). In case of Binachinabadam-4 formation of profuse callus were also observed rather than initiation of shoots if the cotyledon explants were not sub-cultured timely on 2,4-D containing shoot elongation medium.

Table 13. Response of de-embryonated cotyledon explants of Dhaka-1 towards shoot regeneration on MSB medium supplemented with different concentrations of BAP and 2,4-D.

Composition of SIM		No. of explants formed callus in SIM	Days required for callus formation	Com. of SEM (BAPµM)	Days required for shoot initiation from callus	% of explants showed regener ation	Mean no. of shoots/ explant after 45 days of culture
BAP (µM)	2,4-D (µM)						
20	10	35	8-10	2	11 - 15	68.57	5.5
20	10	35	8-10	4	11 - 16	74.28	6.5
10	20	30	7-10	2	18 - 24	33.33	1.5
10	20	30	7-10	4	18 - 22	35.0	2.5

Table 14. Response of de-embryonated cotyledon explants of BARI Chinabadam-8 and Binachinabadam-4 towards callus formation and multiple shoots regeneration on MSB medium supplemented with different composition of BAP and 2,4-D.

Cons. of SIM		Variety	% of explants formed callus in SIM	Days required for callus formation	Cons. of SEM (BAPµM)	% of explants showed shoot regeneration	Mean no. of shoots/ explant after 45 days of culture
BAP (µM)	2,4-D (µM)						
20	10	BARI Chinabadam-8	62.5	9 - 12	4	72	5.5
20	10	Bina chinabadam-4	70	7 - 11	2	74	4.2

4.1.1.3.14 Effect of BAP with MSB (MS salts and Gamborg's B_5 vitamins) medium towards multiple shoot regeneration from de-embryonated cotyledon explants of three peanut varieties

A set of experiments were carried out with de-embryonated cotyledon explants from three peanut varieties, namely, Dhaka-1, BARI Chinabadam-8 and Binachinabadam-4. For this purpose MSB medium with different concentrations of BAP were used to observe their effect on regeneration of shoots. Firstly de-embryonated cotyledon explants were inoculated on shoot induction medium (SIM). After shoot bud initiation they were transferred to the shoot elongation medium (SEM). Here two types of shoot initiation media (SIM1 and SIM2) were used. The SIM1 was composed MSB medium with 88.8 µM BAP whereas, the SIM2 was composed of MSB medium with 66.6 µM BAP. Explants were initially inoculated on SIM1 (MSB medium with 88.8 µM BAP) for 15 days and afterwards they were transferred to SIM2 (MSB with 66.6 µM BAP). On SIM1 explants turned green in colour and occasionally initiation of shoots was formed in this hormonal combination of BAP (Fig. 20b). Multiple shoots were started to initiate mainly on SIM2 when explants were cultured on this hormonal combination of BAP (SIM2) for the next 15 days. For further growth and elongation explants were cultured on SEM which contained MSB medium with 13 µM BAP. For proper shoot bud developments two more subcultures were required on SEM. Most of the cases direct organogenesis was occurred at the proximal part (de-embryonated portion) of explants on SIM2 (MSB with 66.6 µM BAP).

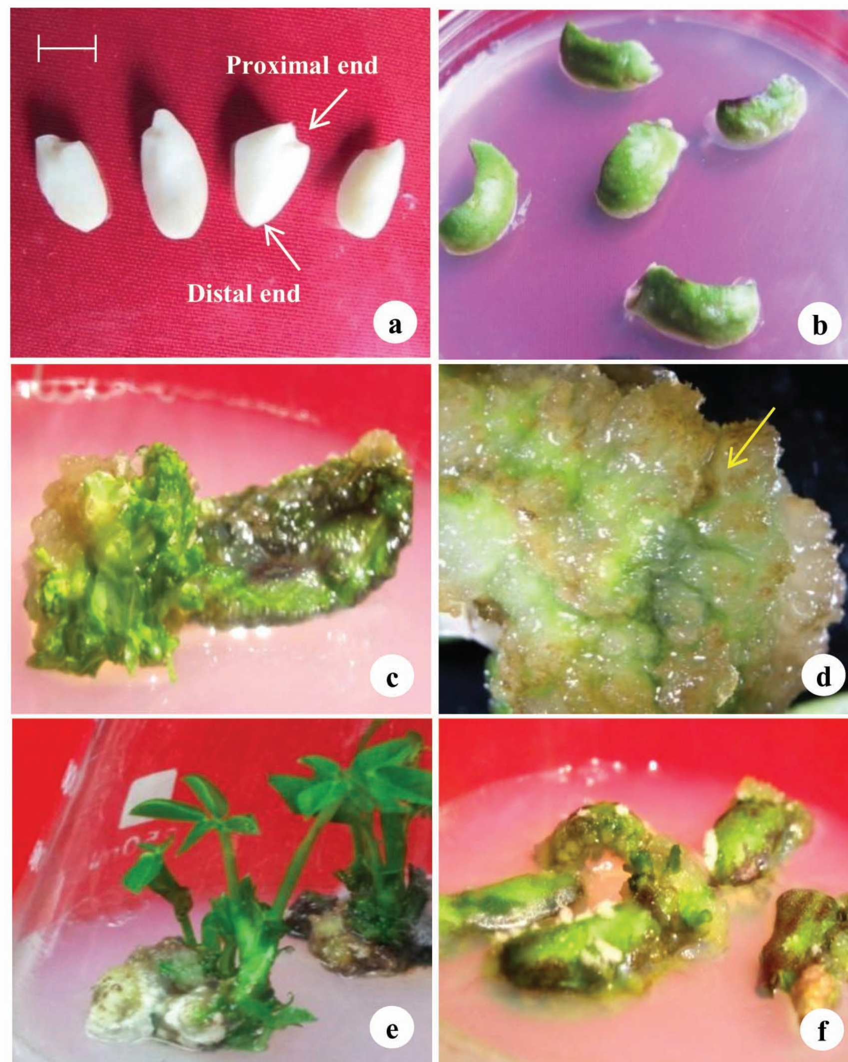

Fig. 17 (a - f): Different stages of shoot regeneration from de-embryonated cotyledon explant of Dhaka-1 on MSB medium supplemented with BAP and 2,4-D. (a) De-embryonated cotyledon explants showing position of proximal and distal ends (Bar = 1.5 mm); (b) Cotyledons became green on shoot induction medium, SIM (MSB with 20 µM BAP and 10 µM 2,4-D); (c) Callus along with shoot formation on cotyledon after 11-13 days of culture on SIM; (d) Stereomicroscopic view of formation of compact callus (arrow) in distal end instead of shoot formation on shoot elongation medium, SEM (MSB with 20 µM BAP); (e) Elongation of multiple shoots on SEM; (f) Initiation of shoots from the callus on the proximal end of cotyledon after 20-25 days of culture on MSB with 10 µM BAP and 20 µM 2,4-D.

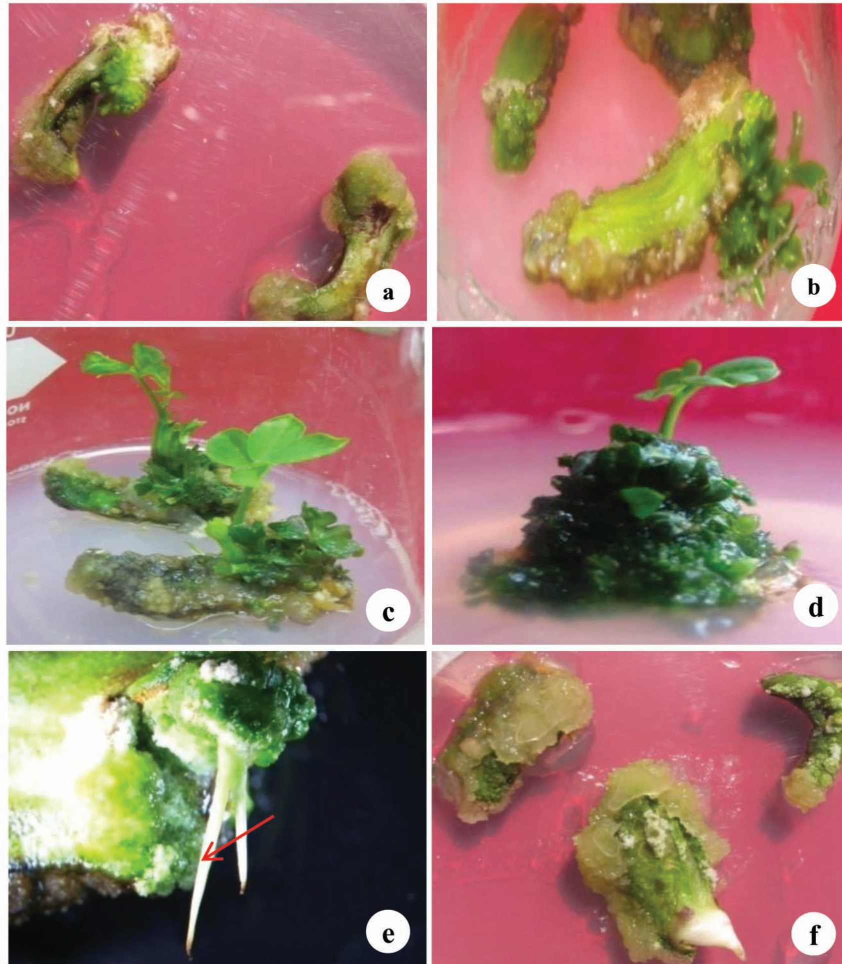

Fig. 18 (a - f). Different stages of shoot regeneration from de-mbryonated cotyledon explant of BARI Chinabadam-8 on MSB with BAP and 2,4-D. (a) Callus along with shoot formation in de-embryonated cotyledon after 11-13 days of culture on shoot induction medium, SIM (MSB with 20 µM BAP and 10 µM 2,4-D); (b) Proliferation of multiple shoots on shoot elongation medium, SEM (MSB with 4 µM BAP); (c) Elongation of multiple shoot on SEM; (d) Delayed development and elongation of shoots when part of the explants was sub-cultured; (e) Formation of adventitious roots from compact callus on SIM (arrow); (f) Formation of profuse callus when explants were culture in SIM for more than 16 days.

Fig. 19 (a - d): Various stages of shoot formation from de-embryonated cotyledon explant of Binachinabadam-4 on MSB with BAP and 2,4-D. (a) De-embryonated cotyledon explants; (b) Cotyledon explants became green on SIM (MSB supplemented with 20 µM BAP and 10 µM 2,4-D); (c) Stereomicroscopic view of initiation of multiple shoots from proximal part of cotyledon on shoot initiation medium ; (d) Formation of multiple shoots on shoot elongation medium.

Though most of the cases direct organogenesis was occurred but very occasionally initiation of shoots was started from the de-embryonated cotyledon explants via callus formation. Like BAP and 2,4-D supplemented medium shoot bud formation hampered in shoot induction medium (SIM1 and SIM2) if cut end of the explants were not embedded to the medium directly. For shoot bud development, full cotyledon was required to be attached with multiple shoots of proximal end. Main advantage of this combination of BAP was, multiple shoots elongated properly after 1 or 2 subculture in shoot elongation media (MSB with 13 µM BAP).

Among the three varieties Dhaka-1 showed best response towards multiple shoots formation on MSB with BAP supplemented medium. A total of 80.0% explants showed regeneration response in SIM1 (MSB with 88.8 µM BAP). Among the responded explants, 71.8% explants showed shoot regeneration response in SIM2 (MSB with 66.6 µM BAP) and 21 - 31 days were required for multiple shoot regeneration in this combinations of BAP in MSB. The results of this experiment are shown in Table 15. Mean number of shoots/explant on SEM (MSB with 13 µM BAP) was recorded as 8.0 in Dhaka-1. In case of Dhaka-1 formation of callus was observed occasionally in initial culture. Different stages of shoot regeneration in this variety are shown in Fig. 20 (a - f).

Binachinabadam-4 also showed better response towards regeneration on the media composition of BAP with MSB. The result of this experiment is presented in Table 15. In Binachinabadam-4, 77.7 % explants showed regeneration response on MSB with 88.8 µM BAP (SIM1) and 20 - 35 days were required for multiple shoot formation on MSB with 66.6 µM BAP (SIM2). In combination of BAP with MSB (SEM) mean number of shoots/explant was 7.5. Different stages of shoot regeneration in this variety are shown in Fig. 21 (a - f).

Among the three varieties, BARI Chinabadam-8 showed comparatively lower number of shoots/explant (7.0) compare to other two varieties. Here, 72.5% explants showed regeneration response on SIM1 and among them 62.0% explants responded on SIM2 where it took about 30 - 38 days for multiple shoot formation. In this variety callus formation observed along with multiple shoots on SEM. Different stages of shoot regeneration in BARI Chinabadam-8 are shown in Fig. 22 (a - d). The results of this experiment are shown in Table 15.

Table 15. Response of de-embryonated cotyledon explants of three peanut varieties towards regeneration of multiple shoots on MSB medium supplemented with BAP.

Composition of SIM (BAP µM)		Variety	No. of explants inoculated	% of explants formed callus in SIM	% of responsive explants formed multiple shoots in SIM2	Cons. of SEM (BAP µM)	Mean no. of shoots/ explants after 45 days of culture
88.8	66.6	Dhaka-1	40	80.0	71.8	13	8.0
88.8	66.6	BARI Chinabadam-8	40	75.0	62.0	13	7.0
88.8	66.6	Bina chinabadam-4	40	77.5	67.7	13	7.5

4.1.1.3.15 Effect of BAP and 2,4-D in MSB medium for the regeneration of shoots from single cotyledon attached decapitated embryo explants of Dhaka-1, BARI Chinabadam-8 and Binachinabadam-4

In this set of experiment, single cotyledon attached decapitated embryo explants of Dhaka-1, BARI Chinabadam-8 and Binachinabadam-4 were used to show their effects towards regeneration. The single cotyledon attached decapitated embryo explants were inoculated on MSB medium supplemented with 20 µM BAP and 10 µM 2,4-D which was denoted as shoot induction medium (SIM). Healthy multiple shoots were initiated directly from the embryos of all the varieties. Results of this experiment have been presented in Table 18. Occasionally formation of callus was noticed along with shoot regeneration on shoot induction medium MSB with 20 µM BAP and 10 µM 2,4-D. For shoot initiation, 5 - 8 days were required for Dhaka-1, 4 - 9 days for BARI Chinabadam-8 and 5 - 10 days required for Binachinabadam-4 (Table 16). 96% explants of Dhaka-1 as well as BARI Chinabadam-8 showed shoot regeneration response on the SIM (MSB with 20 µM BAP and 10 µM 2,4-D). The mean number of shoots/explant was recorded to be 6.2 in Dhaka-1 and 6.0 in both BARI Chinabadam-8 and Binachinabadam-4 variety when the explants were subcultured on SEM which contained MSB with 4 µM BAP. In the SEM (MSB with 4 µM BAP) *in vitro* flower formation was also observed. Various stages of regeneration of shoots is presented in Fig. 23 (a-f).

Fig. 20 (a - f): Stages of shoot regeneration from de-embryonated cotyledon explant of Dhaka-1 on MSB medium supplemented with BAP. (a) De-embryonated cotyledon explants (arrow shows the proximal end, Bar = 4.3 mm); (b) Cotyledons became Green on Shoot induction medium 1, SIM1 (MSB with 88.8 µM); (c) Direct multiple shoot regeneration on SIM2 (MSB with 66.6 µM BAP) after 25-30 days culture; (d) Proliferation of multiple shoots in shoot elongation medium, SEM (MSB with 13 µM BAP); (e) Elongation of multiple shoots on SEM; (f) Callus formation along with elongated multiple shoots after 60 to 70 days culture.

Fig. 21 (a - f): Different stages of shoot regeneration from de-embryonated cotyledon explant of Binachinabadam-4 on MSB medium supplemented with BAP. (a) De-embryonated cotyledon explants; (b) Multiple shoot formation on shoot induction medium 1, SEM1 (MSB with 88.8 µM); (c) Shoot initiation via callus formation on shoot induction medium 2, SIM2 (MSB with 66.6 µM); (d) Proliferation of multiple shoots in shoot elongation medium SEM (MSB with 13 µM BAP); (e) Multiple shoots along with elongated shoots after 50-60 days culture on SEM; (f) Elongation of multiple shoots on SEM.

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Fig. 22 (a - d): Different stages of shoot development from dembryonated cotyledon explants of BARI Chinabadam-8 on MSB with BAP. (a) Multiple shoots initiate on shoot initiation medium 2, SIM2 (MSB with 66.6 µM BAP); (b) Proliferation of multiple shoots along with callus in shoot elongation medium, SEM (MSB with 13 µM BAP); (c) Multiple shoots along with elongated shoots on SEM; (d) Elongated shoots developed on SEM .

Table 16. Effect of BAP and 2,4-D on MSB medium using single cotyledon attached decapitated embryo explants towards multiple shoot formation of Dhaka-1, BARI Chinabadam-8 and Binachinabadam-4.

Hormonal composition of SIM	variety	No. of explants inoculated	Days required for initiation of regeneration	% of explants showed shoot initiation	Comp. of SEM	Mean no. of shoots/ explant after 40 days in SEM
20 µM BAP + 10 µM 2,4-D	**Dhaka-1**	**50**	**5 - 8**	**96**	4 µM BAP	**6.2**
	BARI Chinabadam-8	50	4 - 9	96		6.0
	Bina chinabadam-4	50	5 - 10	94		6.0

4.1.1.3.16 Effect of BAP in MSB medium on regeneration of shoots from single cotyledon attached decapitated embryo explants of Dhaka-1, BARI Chinabadam-8 and Binachinabadam-4

Effect of BAP on MSB was observed on shoot regeneration from single cotyledon attached decapitated embryo explants of three varieties of peanut namely, Dhaka-1, BARI Chinabadam-8 and Binachinabadam-4. Single cotyledon attached decapitated embryo explants were inoculated on MSB medium with 88.8 µM BAP which also denoted as shoot induction medium (SIM1) and after 15 days explants were sub-cultured on MSB with 66.6 µM BAP contained media which was considered as SIM2. Shoot initiation was started on SIM1 (MSB medium with 88.8 µM BAP) within 4 - 8 days and multiple shoots were also initiated on SIM1 within 15 days of culture. Multiple shoots were proliferated in SIM2 (MSB with 66.6 µM BAP) and then explants were sub-cultured on shoot elongation medium (SEM) which contained MSB with 13 µM BAP. In this combination 92 - 94% explants showed shoot initiation in Dhaka-1 as well as in BARI Chinabadam-8 where mean no of shoots/explant was 7.0 for both the varieties. In case of Binachinabadam-4, 94% explants showed regeneration response where mean no of shoots/explant was 6.5. The data of the present study are described in Table17. Different stages of shoot regeneration are presented in Fig. 24 (a - f).

Table 17. Response of single cotyledon attached decapitated embryo explants towards multiple shoot formation using BAP on MSB medium of three peanut varieties.

Composition of SIM	variety	No. of explants inoculated	Days required for initiation of regeneration	% of explants showed shoot initiation	Com. of SIM	Mean no. of shoots/ explant after 40 days in SEM
88.8 µM BAP (SIM1) And 66.6 µM BAP (SIM2)	Dhaka-1	40	4 - 8	92	13 µM BAP	7.0
	BARI China badam-8	40	5 - 8	94		7.0
	Bina chinabadam-4	40	5 - 8	94		6.5

4.1.2 Effect of different growth regulators on induction of roots from regenerated shoots

Induction of root at the base of *in vitro* regenerated shoots is a vital step for the production of plantlets. Generally spontaneous formation of roots was not observed from the *in vitro* regenerated shoots of peanut. For the induction of roots various concentrations of three different auxins, namely, IAA, IBA and NAA were used in the present study. Approximately 2.5 - 4.0 cm long *in vitro* derived shoots were excised and cultured on half strength of MS medium containing various concentrations of the above mentioned auxins. Rooted plantlets from all three varieties were transplanted to soil in small plastic pot for their establishment.

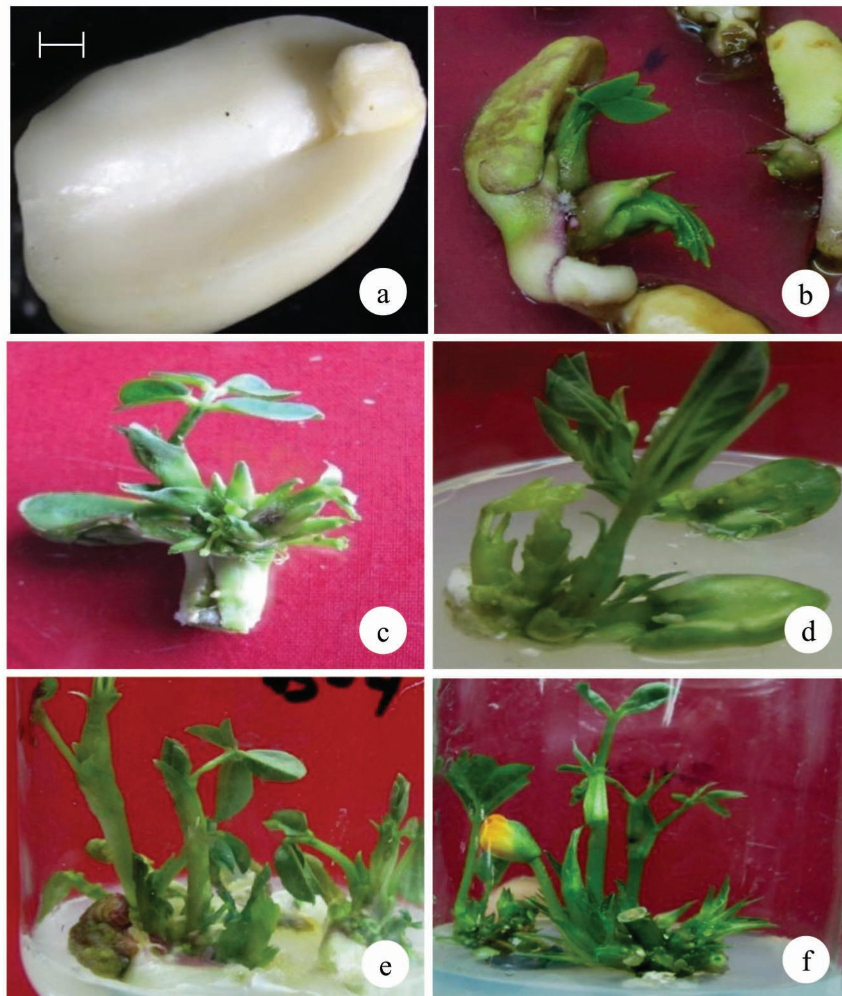

Fig. 23 (a - f): Different stages of shoot development from single cotyledon attached decapitated embryo (SCAE) explant of three varieties of peanut on MSB with BAP and 2,4-D. (a) Single cotyledon attached decapitated embryo (SCAE) explants (bar = 6 mm); (b) Shoots initiation from SCAE explant in Binachinabadam-4 on shoot induction medium, SIM (MSB with 20 µM BAP and 10 µM 2,4-D); (c) Multiple shoots formation in Dhaka-1 on SIM; (d) Multiple shoots formation in BARI Chinabadam-8 on SIM; (e) Elongation of multiple shoots in Dhaka-1 on shoot elongation medium, SEM (MSB with 20 µM BAP); (f) Shoot multiplications and *in vitro* flower formation in Dhaka-1 on SEM.

Fig. 24 (a – f): Different stages of shoot formation from single cotyledon attached decapitated embryo (SCAE) explant on MSB with BAP in three varieties of peanut. (a) Shoot initiation from SCAE explant in Dhaka-1 on shoot induction medium 1, SIM1 (MSB with 88.8 µM BAP); (b) Proliferation of multiple shoots of Dhaka-1 on shoot induction media 2, SIM2 (MSB with 66.6µM BAP); (c) Elongation of multiple shoots from Dhaka-1 on shoot elongation medium, SEM (MSB with 13 µM BAP); (d) Multiple shoots formation in BARI Chinabadam-8 on SIM2; (e) Formation of multiple shoots along with elongated shoots in Binachina badam-4 on SIM2; (f) Elongation of shoots in Binachinabadam-4 on SEM.

4.1.2.1 Effect of different concentration of IBA on half strength of MS for induction of roots from three varieties of peanut

Half strength of MS media with three different concentrations of IBA (1.0, 2.5 and 5.0 µM) were used to examine their effects for the induction of roots from the *in vitro* regenerated shoots of three peanut varieties. Results on root induction in three peanut varieties using different concentrations of IBA have been shown in Table 18. High frequency of induction of roots in both Dhaka-1 and BARI Chinabadam-8 was observed on half strength of MS medium containing 5.0 µM IBA (Fig. 25a and 25b). It was recorded that 85% regenerated shoots of Dhaka-1 and that of 80% of BARI Chinabadam-8 formed well developed roots on 5.0 µM IBA supplemented media. Root initiation was noticed within 8 - 20 days of culture while it took about 22 - 30 days were required to get fully developed roots in both the varieties. The number of roots/shoot was 7 - 14 in case of Dhaka-1 and that of 6 - 10 for BARI Chinabadam-8 (Fig. 25c). Initiation of roots in Binachinabadam-4 was started within 7 - 16 days of culture and maximum numbers of roots were found to form on both 2.5 µM and 5.0 µM IBA supplemented half strength of MS medium (Fig. 25d and 25e). It was observed that in IBA supplemented media roots were initiated directly at the base of cut end of *in vitro* regenerated shoots without the formation of any callus (Fig. 25e). Occasionallys callus was found to form at the cut end after 20 – 35 days of culture (Fig. 25f).

4.1.2.2 Effect of different concentrations of IAA on half strength of MS for the induction of roots in three varieties of peanut

Different concentrations of IAA (1.0, 2.5 and 5.0 µM) with half strength of MS were used to examine their effects on induction of roots in three varieties of peanut. Table 19 has been presented the effect of those concentrations of IAA. In case of Dhaka-1 best root induction was observed on half strength of MS medium containing 1.0 µM of IAA. Higher percentage (85%) of regenerated shoots showed initiation of roots within 10 - 15 days of culture while it took about 25 - 30 days to get fully developed roots. Number of roots /shoot was 8 - 14 when the shoots were cultured on 1.0 µM IAA supplemented media. In case of BARI Chinabadam-8 and Binachinabadam-4 best induction of roots (80%) was observed on half strength of MS medium containing 2.5 µM IAA. In BARI Chinabadam-8 roots were initiated within 10 - 22 days of culture while Binachinabadam-4 took about 8 - 16 days for the initiation of roots on 2.5 µM IAA supplemented media.

It was noticed that in various combinations of IAA supplemented media callus like structure produced from the cut end of shoots. Formation of roots on various combinations of IAA on half strength of MS in three varieties is shown in Fig. 26 (a - f).

Table 18. Effect of different concentration of IBA on half strength of MS towards formation of roots from regenerated shoots in case of three peanut varieties.

Varieties	Conc. of IBA (μM)	No. of shoot inoculated for root induction	% of shoots forming roots	Days required to initiate roots	Days required to get well developed roots	No. of roots/ shoot
Dhaka-1	1.0	20	60	10 - 22	22 - 30	3 - 7
	2.5	20	80	9 - 20	20 - 28	6 - 11
	5.0	20	85	8 - 20	22 - 30	7 - 14
BARI Chinabadam-8	1.0	20	55	9 - 18	20 - 30	5 - 6
	2.5	20	75	10 - 22	25 - 30	5 - 10
	5.0	20	80	9 - 20	25 - 30	6 - 10
Bina chinabadam-4	1.0	20	45	15 - 20	22 - 28	3 - 5
	2.5	20	80	8 - 16	20 - 32	4 - 7
	5.0	20	80	7 - 16	20 - 30	5 - 7

Table 19. Effect of different concentration of IAA on half strength of MS media for the induction of roots from regenerated shoots in three peanut varieties.

Varieties	Concentration of IAA (μM)	Number of shoot inoculated for rooting	% of shoots forming roots	Days required to initiate roots	Days required to get well developed roots	No. of roots/ shoot
Dhaka-1	1.0	20	85	10 - 15	25 - 30	8 - 14
	2.5	20	75	18 - 30	28 - 32	6 - 10
	5.0	20	75	18 - 25	28 - 30	5 - 10
BARI Chinabadam-8	1.0	20	70	12 - 20	30 - 32	4 - 10
	2.5	20	80	10 - 22	25 - 30	6 - 12
	5.0	20	70	14 - 24	25 - 28	4 - 10
Bina chinabadam-4	1.0	20	75	14 - 20	22 - 30	6 - 10
	2.5	20	80	8 - 16	25 - 30	8 - 21
	5.0	20	75	12 - 20	25 - 30	6 - 15

Fig. 25 (a - f): Formation on roots from the cut ends of the *in vitro* regenerated shoots on half strength of MS medium containing various concentrations of IBA in case of three varieties of peanut. (a) Initiation of roots from the shoots Dhaka-1 on medium containing 5µM IBA; (b) Same as Fig. 25a but in case of BARI Chinabadam-8; (c) Fully developed roots of Dhaka-1 on 5µM IBA containing medium; (d) Formation of roots in Binahinabadam-4 containing 5µM IBA; (e) Formation of fully developed roots at the base of cut end of BARI Chinabadam-8 on 2.5µM IBA supplemented media; (f) Formation of compact callus at the cut end in Dhaka-1.

Fig. 26 (a - f): Formation of roots from the cut ends of the regenerated shoots on half strength of MS medium containing various concentrations of IAA in case of three varieties of peanut. (a) Initiation of roots from callus like structure of Dhaka-1 containing 1µM IAA; (b) Initiation of multiple roots on 2.5 µM IAA in Binahinabadam-4; (c) Formation of multiple roots along with callus in Dhaka-1 on 2.5µM IAA; (d) Formation of roots in Binachinabadam-4 on 1µM IAA supplemented media; (e) Fully developed roots of BARI Chinabadam-8 on 2.5µM IAA supplemented media; (f) Fully developed roots in Dhaka-1 media containing 1µM IAA.

4.1.2.3 Effect of different concentrations of NAA on half strength of MS towards formation of roots in three peanut varieties

A set of experiments were carried out to investigate the effects of NAA on half strength of MS media towards formation of roots. For this purpose three different concentrations of NAA (1.0, 2.5 and 5.0 μM NAA) were used for induction of roots in three varieties of peanut. In this case profuse callusing was observed along with induction of roots on various combinations of NAA. The results of this experiment have been presented in Table 20. It has been observed that all three varieties showed maximum response towards root induction on half strength of MS medium containing 2.5 μM NAA. In the combination of 2.5 μM NAA, 75% of regenerated shoots were started to initiate roots within 9 - 20 days of culture in Dhaka-1 and that of 7 - 14 days of culture in Binachinabadam-4. It was also observed that 20 - 27 days were required to get fully developed roots in both the varieties of Dhaka-1 and Binachinabadam-4. In case of BARI Chinabadam-8 roots were started to initiate within 8 - 22 days and highest percentage of (70%) roots were induced on half strength of MS supplemented with 2.5 μM NAA. In this combination of NAA well developed roots were found to form within 25 - 30 days of culture. Fig. 27 (a - f) shows the results of root formation on various combinations of NAA with half strength of MS media in three varieties of peanut.

4.1.3 Transplantation of rooted plantlets and formation of pods

The plantlets with well developed roots of peanut varieties Dhaka-1, BARI Chinabadam-8 and Binachinabadam-4 were successfully transplanted into small plastic pots containing autoclaved soil (Fig. 28a and 29a,b). The transplantation procedure has been described in the section 3.10.5. Using this method the survival rate of the transplanted plantlets was found to be about 95% in case of Dhaka-1 and it was 90% in case of BARI Chinabadam-8 and Binachinabadam-4 (Table 21). For their further growth and establishment, the survived plantlets were transferred to earthen pots and finally in the field. Fig. 28 (b - e) and Fig. 29 (b - c) shows the plantlets which transferred to earthen pots and field. The peanuts were harvested from *in vitro* grown plantlets after 130 -140 days and each plant contained more or less 5 - 9 pods of peanuts (Fig. 28f and Fig. 29d, e).

Table 20. Effect of different concentration of NAA on half strength of MS media towards formation of roots from regenerated shoots in case of three varieties of peanut.

Variety	Conc. of NAA (µM)	Number of shoot inoculated for rooting	% of shoots forming roots	Days required to initiate roots	Days required to get well developed roots	No. of roots/ shoot
Dhaka-1	1.0	20	60	8 - 22	20 - 28	2 - 3
	2.5	20	75	9 - 20	22 - 27	3 - 10
	5.0	20	60	9 - 20	25 - 30	4 - 9
BARI Chinabadam-8	1.0	20	50	10 - 22	30 - 32	2 - 4
	2.5	20	70	8 - 18	25 - 30	5 - 12
	5.0	20	50	9 - 22	25 - 28	3 - 9
Bina chinabadam-4	1.0	20	55	9 - 16	20 - 28	1 - 5
	2.5	20	75	7 - 14	20 - 27	5 - 13
	5.0	20	60	8 - 16	22 - 27	7 - 13

Table 21. Transplantation of rooted plantlets in soil and their survival rate.

Varieties	No. of plants transplanted in soil	No. of survival plants in soil	% of survival plants in soil
Dhaka-1	10	9	95
BARI Chinabadam-8	10	8	90
Binachinabadam-4	10	9	90

Fig. 27 (a - f): Initiation and development of roots from the cut ends of the regenerated shoots in three varieties of peanut on half strength MS medium containing various concentration of NAA. (a) Initiation of roots in Dhaka-1 on 2.5µM NAA containing medium; (b) Initiation of roots along with callus formation on 2.5 µM NAA in case of BARI Chinabadam-8; (c) Formation of comparatively poor roots in BARI Chinabadam-8 on 1µM NAA; (d) Formation of multiple roots on 2.5µM NAA supplemented media in Binachinabadam-4; (e) Roots along with callus on 2.5µM NAA supplemented media of BARI Chinabadam-8 ; (f) Mature roots of Dhaka-1 on 1µM NAA.

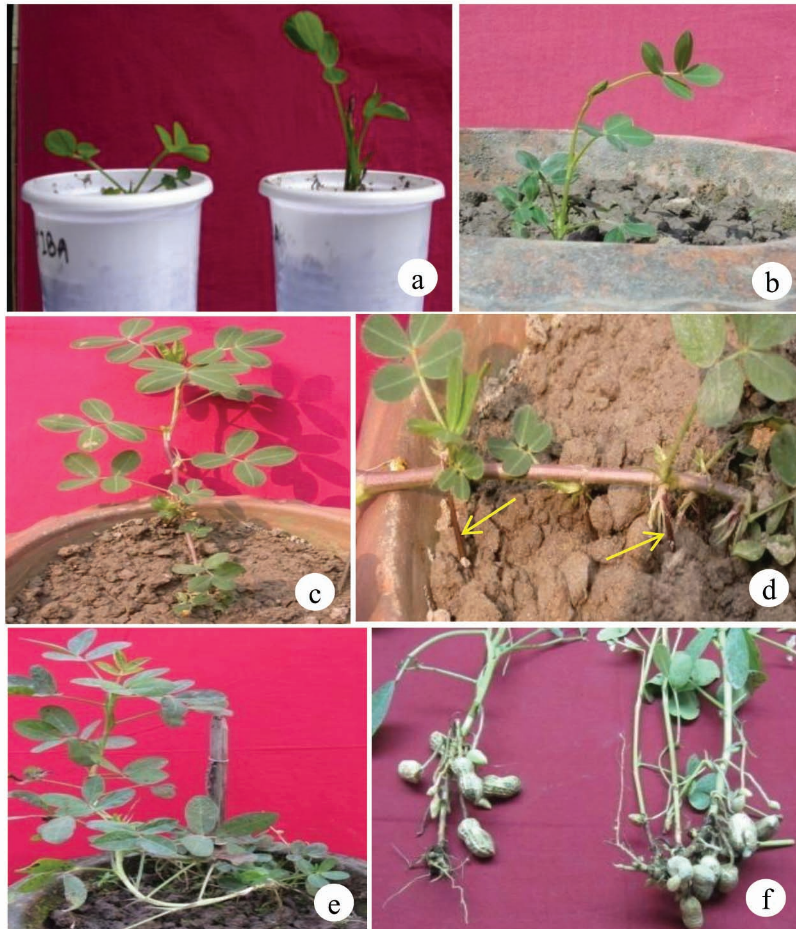

Fig. 28 (a - f): Transplantation as well as formation of pods from *in vitro* derived plantlets of Dhaka-1. (a) The regenerated plantlets transferred to soil in small pots; (b) The plantlets transfer in large earthen pot; (c) Young plantlets with flower in large earthen pots; (d) The peg (arrow) inserted into the soil; (e) Mature peanut plants before harvest; (f) Harvested peanut plants (Dhaka-1) with pods.

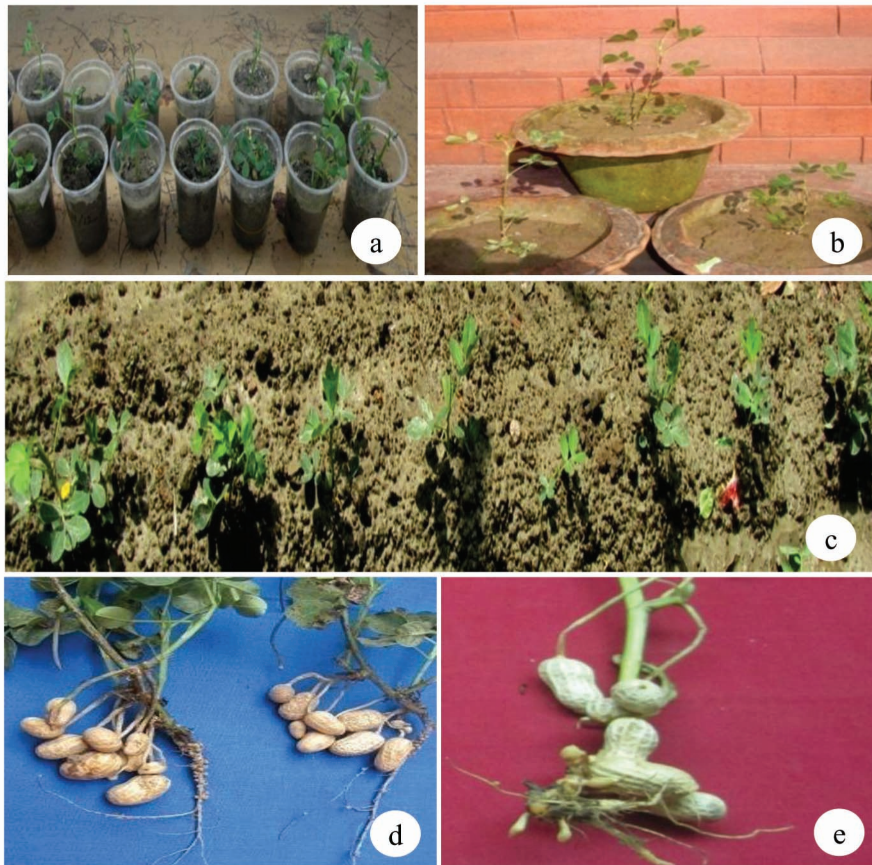

Fig. 29 (a - e). Acclimatization as well as formation of pods from *in vitro* regenerated plantlets of BARI Chinabadam-8 and Binachinabadam-4. (a) *In vitro* regenerated plantlets of Binachinabadam-4 transferred to soil in small pots; (b) Transplantation of BARI Chinabadam-8 in large earthen pots; (c) *In vitro* raised plantlets of Binahinabadam-4 growing in the field; (d) Plants of Binachinabadam-4 with mature pods; (e) Plants of BARI Chinabadam-8 with pods.

4.2 Genetic Transformation

In this phase of investigation, a series of experiments were carried out to establish a suitable protocol for *Agrobacterium*-mediated genetic transformation in peanut using screenable marker gene *GUS* (β – Glucoronidase) as well as selectable marker gene *npt*II (Neomycin phosphotransferase). Further biotic stress tolerant fungal disease resistant gene *AFP* was integrated in peanut plants following the protocol developed using marker genes. For this purpose, genetically engineered *Agrobacterium* LBA4404 containing two different binary vector plasmid namely; pBI121GUS-NPTII considered as construct I and pCAMBIA2300AFP considered as construct II were used for transformation experiments (section 2.2). Transformation experiments were conducted using various explants, namely, immature leaflet, de-embryonated cotyledon and single cotyledon attached decapitated embryo of two varieties of peanut, viz, Dhaka-1 and BARI Chinabadam-8. During the regeneration experiments (section 4.1.1.3.7 - 4.1.1.3.10), it was noticed that the regeneration capability of decapitated half embryo explants was found to be comparatively lower than that of other explants like immature leaflet, de-embryonated cotyledon and single cotyledon attached decapitated embryo. For this reason decapitated half embryo explants were not included for transformation experiments. Results of these experiments are presented below in different headings.

4.2.1 Genetic transformation with *Agrobacterium* LBA4404/pBI121GUS-NPTII (construct I) containing the marker gene in Dhaka-1

In this investigation, genetic transformation experiments were carried out using *Agrobacterium* LBA4404 with plasmid pBI121GUS-NPTII containing the marker gene *GUS* as screenable marker and *npt*II gene as selectable marker conferring kanamycin resistance. This construct of *Agrobacterium* contained the screenable marker gene *GUS*. Therefore different factors or parameters which influenced the transformation efficiency were optimized through GUS expression using Dhaka-1 variety. Generally transient assay of such marker genes were routinely performed as a preliminary step to optimize the conditions required for successful transfer of desired gene/s. Results obtained from the optimization of different parameters for *Agrobacterium*-mediated transformation are presented under the following heads.

4.2.1.1 Optimization of different parameters influencing transformation efficiency

From the previous reports on *Agrobacterium*-mediated genetic transformation it was evident that efficiency of transformation was influenced by several factors, such as optical density (OD) of *Agrobacterium* suspension, duration of incubation period, duration of co-cultivation period, influence of light and dark period during co-cultivation of explants, etc. During this study optimization of these parameters were carried out using Dhaka-1 by monitoring the transient expression of the *GUS* gene.

4.2.1.1.1 Influence of optical density (OD) of *Agrobacterium* suspension on transformation

One of the most important influencing factors of transformation is optical density of *Agrobacterium* suspension. Overnight grown *Agrobacterium* suspension (section 3.10.6) was used to infect the explants of Dhaka-1 for transformation. Optical density of overnight grown bacterial culture ranging from 0.6 – 1.2 was measured at 600 nm. Findings of this set of experiment have been shown in Table 22. In case of immature leaflet explants, the maximum percentage (80.0%) of the transformation efficiency of explants was observed at 1.0 optical density of *Agrobacterium* suspension visualized through GUS assay. The GUS expression in immature leaflet explants has been presented in Fig. 30a. In case of de-embryonated cotyledon explants the maximum transformation efficiency was observed at 0.8 optical density of *Agrobacterium* suspension (Table 22). The GUS expression in de-embryonated cotyledon explants has been presented in Fig. 30 (b - c). Single cotyledon attached decapitated embryo explants showed the best GUS activity (83.33%) when *Agrobacterium* suspension had an optical density of 0.6 (Fig. 30d, Table 22). It was also noticed that single cotyledon attached decapitated embryo explants exhibited identical expression of GUS gene when *Agrobacterium* suspension had an optical density of 0.8 (Fig. 30e, Table 22). It was also observed that bacterial over growth was occurred around the co-cultured explants when de-embryonated cotyledon and leaflet explants were co-cultured using *Agrobacterium* suspension with an optical density of 1.0 or more than 1.0 (Fig. 31a and 31b). Therefore, bacterial suspension with an optical density of 0.8 was used for all the transformation experiments to avoid bacterial overgrowth in the regeneration medium.

Table 22. Influence of optical density (measured at 600 nm) of overnight grown *Agrobacterium* (LBA4404 with pBI121GUS-NPTII) suspension on transformation of various explants of Dhaka-1 analyzed by transient GUS histochemical assay.

Explants	OD_{600}	No. of explants infected	No. of explants assayed for GUS	No. of explants +ve for GUS	% of GUS +ve explants
Immature leaflet	0.6	80	30	19	63.33
	0.8	80	30	22	73.33
	1.0	**80**	**30**	**24**	**80.0**
	1.2	80	30	22	73.33
De-embryonated cotyledon	0.6	80	30	22	73.33
	0.8	**80**	**30**	**26**	**86.66**
	1.0	80	30	22	73.33
	1.2	80	30	22	73.33
Single cotyledon attached decapitated embryo	**0.6**	**80**	**30**	**25**	**83.33**
	0.8	80	30	22	73.33
	1.0	80	30	20	66,.66
	1.2	80	30	22	73.33

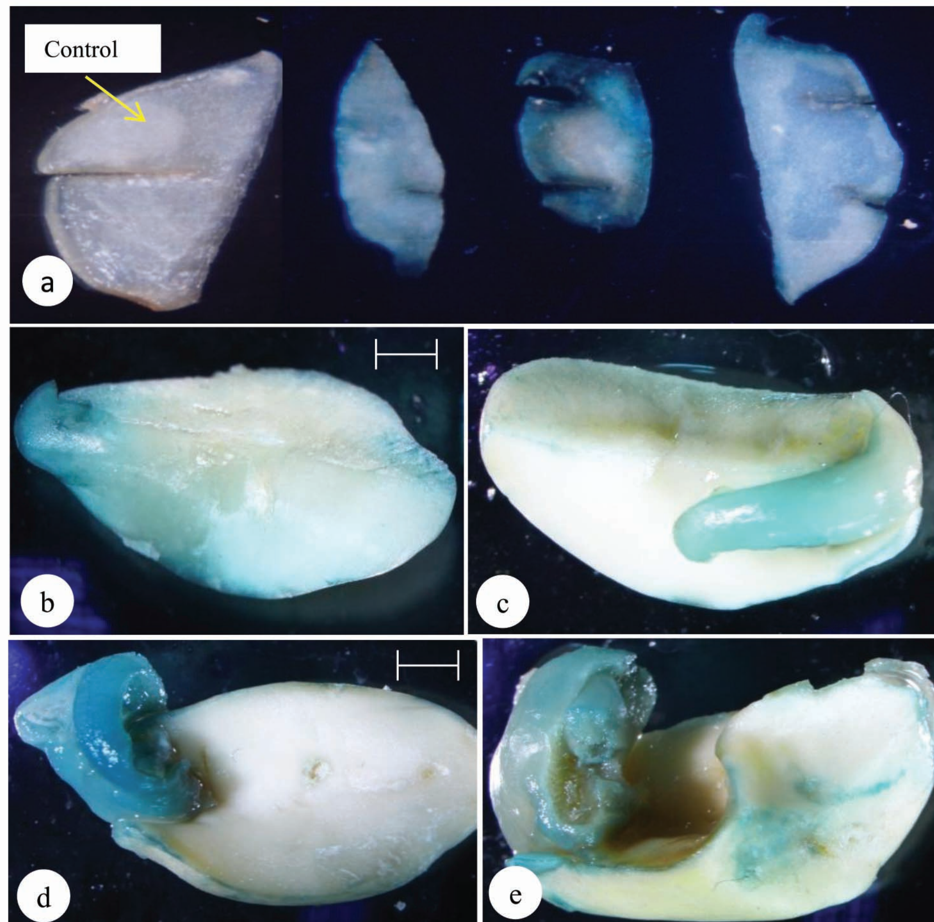

Fig. 30 (a – e): Stereomicroscopic view of histochemical localization of GUS activity of different explants at various optical density (OD) and incubation period of *Agrobacterium* suspension. (a) GUS expression in leaflet explants at optical density of 1.0 with 30 min incubation period, arrow indicates the control explant; (b) GUS positive expression in de-embryonated cotyledon explants at OD 0.8 with 10 min incubation period (Bar = 6.1 mm); (c) same as Fig. 30b but in case of OD 1.0; (d) GUS expression in single cotyledon attached decapitated half embryo explants at OD 0.6 with 10 min incubation period (Bar = 6.2 mm); (e) same as Fig. 30d but in case of OD 0.8.

4.2.1.1.2 Influence of incubation period of explants in bacterial suspension on transformation

Incubation period in *Agrobacterium* suspension was another important parameter where the explants were incubated for a definite period to allow the bacteria to infect the plant tissues. In this set of experiment, the effect of different incubation periods was investigated. For this purpose, different incubation periods, such as 15, 30 and 45 minutes were applied using bacterial suspension with a constant optical density of 0.8 in case of three types explants. Leaflet explants showed maximum GUS positive expression when the explants were incubated for a period of 45 minutes in *Agrobacterium* suspension. It was observed that bacterial overgrowth was visualized in culture media when de-embryonated cotyledon and single cotyledon attached embryo explants were incubated more than 15 minutes in *Agrobacterium* suspension. Therefore, in case of de-embryonated cotyledon and single cotyledon attached decapitated embryo explants were incubated for few seconds, 10 and 15 minutes with *Agrobacterium* suspension. De-embryonated cotyledon and single cotyledon attached embryo explants showed maximum GUS activity in 15 minutes of incubation with *Agrobacterium* suspension. In many cases the explants showed bacterial over-growth if the explants were incubated for longer period with *Agrobacterium* suspension. Therefore, the optimum incubation period was maintained at 30 minutes for leaflet explants and 10 minutes for de-embryonated cotyledon and single cotyledon attached decapitated embryo explants at 0.8 OD of the *Agrobacterium* suspension (Table 23).

4.2.1.1.3 Influence of co-cultivation period on transformation

Co-cultivation period of explants is another important factor which influences transformation efficiency. In this experiment the transformed explants were co-cultivated for different days such as 2, 3, 4 and 5 days obtain specific time period for sufficient transformation of explants. It was observed that the transformation efficiency was found to be slightly variable for different periods of co-cultivation of the explants (Table 24). In this experiments optical density of *Agrobacterium* suspension was maintained at 0.8. A period of 3 - 4 days co-cultivation period showed the optimum transformation efficiency determined by GUS assay. However, percentage of transformation could be increased with the increase of co-cultivation period but longer co-cultivation period (more than 4 days) produced bacterial over-growth on the co-culture medium (Fig. 31a and 31b). This

type of bacterial over-growth was not suitable for survival of co-cultured explants. Therefore, co-cultivation period of 3 - 4 days was found to be the most suitable when transformation experiment was performed under optimum condition (Table 24).

Table 23. Influence of different incubation periods of over night grown *Agrobacterium* (LBA4404 with pBI121GUS-NPTII) suspension on transformation of various explants of Dhaka-1 analyzed by transient GUS histochemical assay.

Explants	Incubation Period (mins)	No. of explants infected	No. of explants assayed for GUS	No. of explants +ve for GUS	% of GUS +ve explants
Immature leaflet	15	80	30	18	60.00
	30	**80**	**30**	**24**	**80.00**
	45	**80**	**30**	**26**	**86.67**
De-embryonated cotyledon	Few seconds	80	30	23	76.66
	10 min	**80**	**30**	**24**	**80.00**
	15 min	**80**	**30**	**27**	**90.00**
Single cotyledon attached embryo	Few seconds	80	30	20	66.66
	10 min	**80**	**30**	**24**	**80.00**
	15 min	**80**	**30**	**24**	**80.00**

Table 24. Influence of different co-cultivation periods on transformation analyzed by transient GUS histochemical assay at a constant OD (600 nm) and incubation period of Dhaka-1.

Co-cultivation period (days)	No. of explants assayed for GUS expression	No. of GUS +ve explants	% of GUS +ve explants
2	25	19	76
3	**25**	**21**	**84**
4	**25**	**21**	**84**
5	**25**	**22**	**88**

4.2.1.1.4 Effect of cocultivation under dark and light conditions on transformation

An experiment was carried out to observe the effect of dark (within the incubator) and light (growth room condition) conditions on transformation efficiency during co-cultivation of explants. The result of the experiment was also analyzed by transient GUS histochemical assay and immature leaflet, de-embryonated cotyledon and single cotyledon attached decapitated embryo explants were used. The two treatments (dark and light) during co-cultivation did not show any significant differences on the transformation efficiency. Results of this experiment have been presented in (Table 25). It was observed that the immature leaflet and de-embryonated cotyledon explants showed similar GUS positive response both under light and dark conditions. However, single cotyledon attached decapitated embryo explants showed slightly better response when the explants were co-cultivated under dark condition.

Table 25. Effect of darkness and light (growth room condition) in co-culture period towards transformation at optical density 0.8, 30 mins of incubation period and 3 days of co-cultivation (analyzed by transient GUS histochemical assay).

condition	Explants	No. of explants assayed for GUS expression	No. of GUS +ve explants	% of GUS +ve explants
Dark condition	IL	20	14	70
	DEC	**20**	**16**	**80**
	SCAE	20	14	70
Light (growth room condition)	IL	20	14	70
	DEC	**20**	**16**	**80**
	SCAE	20	13	65

IL= Immature leaflet; DEC= De-embryonated cotyledon; SCAE= Single cotyledon attached decapitated embryo.

4.2.1.2 Regeneration from co-cultured explants

Transformation experiments were performed with immature leaflet, de-embryonated cotyledon and single cotyledon attached decapitated embryo explants of Dhaka-1. During these experiments a few selected explants infected with the *Agrobacterium* were subjected to GUS histochemical assay to monitor the transformation efficiency of the

explants. The remaining co-cultivated explants were washed with sterile distilled water containing 300 mg/l ticarcillin for the elimination of *Agrobacterium* attached on the surface of the explants. Following this step the explants were transferred to MS medium containing 22.2 μM BAP (for leaflet explants) and MSB with various BAP supplemented media for de-embryonated cotyledon and single cotyledon attached decapitated embryo explants (Section 4.1.1.3.14 and 4.1.1.3.16) for the regeneration of shoots. In case of peanut, selection pressure was not applied immediately after co-cultivation, instead the explants were allowed to regenerate (minimum for 15 days) in regeneration medium without the selection pressure only containing 100 mg/l ticarcillin. It was observed that if selective agent (kanamycin) was applied immediately after co-cultivation the *Agrobacterium* infected explants did not show further regeneration and in most of the cases the explants failed to survive. When the multiple shoots elongated up to 0.2 - 0.5 cm in length the explants with the newly developed shoots were transferred to the medium with selective agents.

4.2.1.3 Determination of optimum kanamycin concentration for selection of transformed shoots

Agrobacterium strain LBA4404 harbouring pBI121GUS-NPTII contained *npt*II gene conferring resistance to kanamycin. Therefore, kanamycin was used as selective agent during the transformation experiments with that *Agrobacterium.* To determine the optimum selection level using kanamycin, shoots derived from non-infected explants (those served as negative control) were subjected to selection pressure. From this experiment it was revealed that with the increase of kanamycin concentration the percentage of survived shoots were found to decrease. For this purpose, the regenerated shoots where transferred to suitable regeneration medium containing various concentration of kanamycin. The concentration of kanamycin was increased gradually from 50 mg/l to 250 mg/l in the following manner 50, 100,150, 200 and 250 mg/l kanamycin. Non transformed (control) shoots were transferred to various kanamycin contained selection medium. In case of peanut, shoots did not show albinism in presence of 50 mg/l kanamycin after 30 days of culture (Fig. 32a). Control shoots began to show the effect of selection pressure in 100 mg/l kanamycin after 12 - 25 days of culture (Fig. 32b). Most of the cases leaves were found to become albino after 8 - 16 days and then the bleaching extended to the stems. All the non infected explants (negative control) failed to survive in presence of 250 mg/l kanamycin within 30 days (Fig. 32c.).

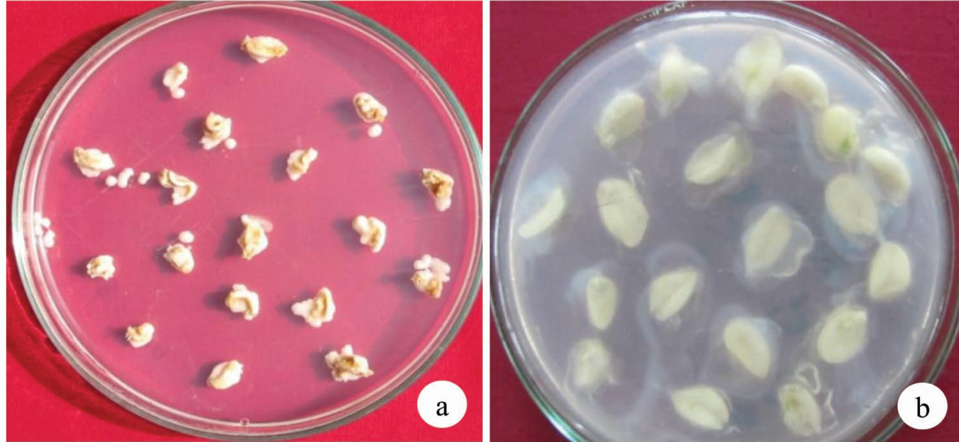

Fig. 31 (a - b): Overgrowth of *Agrobacterium* in co-culture media. (a) Overgrowth observed in co-culture media around leaflet explants at optical density of 1.2 for *Agrobacterium* suspension and 45 minutes incubation period; (b) Same as Fig. 31a but in case of de-embryonated cotyledon explants in 20 mins incubation period.

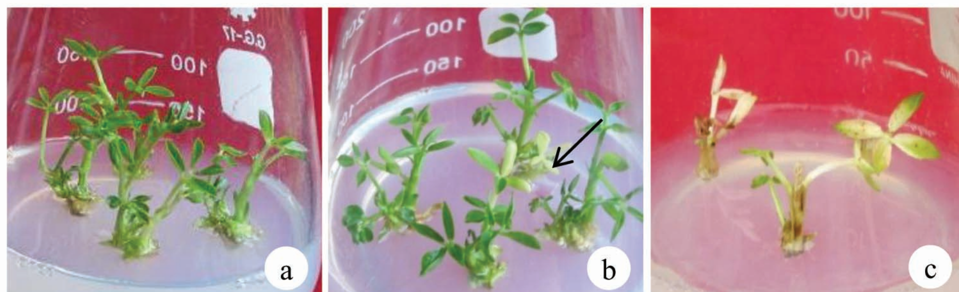

Fig. 32 (a - c): Effect of kanamycin in non-transformed (control) shoots. (a) No albinism observed in the presence of 50 mg/l kanamycin supplemented media after 30 days of culture; (b) Shoots started to show albinism (arrow) in the presence of 100 mg/l kanamycin supplemented media; (c) All control shoots failed to survive in 250 mg/l kanamycin supplemented media.

For this reason 100 mg/l kanamycin was considered as first selection pressure and 250 mg/l kanamycin was considered as highest selection pressure in the present study when the *Agrobacterium* LBA4404 containing plasmid pBI121GUS-NPTII. Effect of kanamycin towards the control shoots have been shown in Fig. 32 (a - c).

4.2.1.4 Selection of transformed shoots of Dhaka-1 using kanamycin

For the development of transformed plantlets it is important to select the transformants from the regenerating shoots. As successfully transformed shoots have the capability to express the gene conferring kanamycin resistance, they were supposed to survive in the selection medium. On the contrary, shoots regenerated from uninfected explants became albino and could not survive in the selection medium since they did not possess the kanamycin resistant gene. It has been mentioned before that 100 mg/l kanamycin concentration was taken as the first selection pressure then the concentration of kanamycin was increased gradually like 150 mg/l, 200 mg/l, 250 mg/l. Shoots those remained green and healthy and survived on the medium containing optimum selection pressure (250 mg/l) for 30 days were selected as putatively transformed shoots. Various stages of regeneration and selection of transformed shoots in the present study have been shown in Fig. 33 (a - i). The results of transformation experiment are shown in Table 26.

It was observed that, none of the explants showed any regeneration response if the infected explants were inoculated directly in first selection pressure containing medium (100 mg/l) after co-culture. Among the three explants studied de-embryonated cotyledon and single cotyledon attached decapitated embryo explants showed better transformation efficiency then leaflet explants. In case of de-embryonated cotyledon explants, out of approximately 590 infected explants, 16 shoots of Dhaka-1 were recovered on final selection medium. Therefore the frequency of recovery of putatively transformed shoots from de-embryonated cotyledon explants was about 2.7%. In single cotyledon attached decapitated embryo explants the frequency of recovery of putatively transformed shoots was about 1.6% (Table 26).

From the Table 26 it is observed that no shoots were survived from immature leaflet explants in media containing higher selection pressure. Some shoots were recovered on 150 mg/l kanamycin supplemented medium but they were not survived when they were subjected to higher selection pressure of 250 mg/l kanamycin. The response of shoot regeneration from infected leaf explants was very slow; it took 110 - 120 days or more for

multiple shoot formation. Moreover in many cases bacterial growth was observed in leaflet explants after 3 - 4 sub-culture which hampered the regeneration from infected explants.

Table 26. Effect of kanamycin on selection of infected shoots of Dhaka-1 from immature leaflet, de-embryonated cotyledon and single cotyledon attached decapitated embryo explants with construct pBI121GUS-NPTII.

Explants	No. of explants infected	No. of survived shoots in regeneration media with kanamycin (mg/l)				% of survived shoots
		100	150	200	250	
IL	1208	520	120	-	-	No shoots survived
DEC	590	1050	510	38	16	2.7
SCAE	420	220	110	26	7	1.6

IL= Immature leaflet; DEC= De-embryonated cotyledon; SCAE= Single cotyledon attached decapitated embryo

4.2.1.5 *In vitro* root formation from the putatively transformed shoots of peanut and transplantation of plantlets to soil

During the transformation experiments the shoots that survived in the kanamycin selection medium were transferred to half strength of MS medium containing 2.5 µM IBA with 50 mg/l ticarcillin for the induction of roots. During this study altogether 60 shoots of Dhaka-1 were inoculated in root induction medium which was survived in selection pressure contained medium. It was observed that the shoots that survived in selection pressure containing medium showed slow and lower response towards root formation (Table 27). In this variety 36.66% shoots showed response towards induction of roots (Fig. 34a, b). It was noticed that 90% plantlets were found to survive on soil (Fig. 34c, e).

Table 27. Formation of roots and transplantation of plantlets in soil from the *in vitro* regenerated shoots survived in selection pressure.

Variety	No. of transformed shoots inoculated for root induction	Days required for root initiation	No of shoots showed rooting response	% of shoots forming roots	% of survival rate of transplanted plantlets
Dhaka-1	60	20 - 45	22	36.66	90

Fig. 33 (a - i): Regeneration and selection of putative transformants. (a) Infected immature leaflet explants in filter paper to reduce the *Agrobacterium* suspension from the surface of explants ; (b) Shoots regenerated from infected de-embryonated cotyledon explants on selection free regeneration medium; (c) Multiple shoot regenerated from infected de-embryonated cotyledon explants without selection pressure; (d) Shoot formation from infected leaflet explants after co-cultivation; (e) Development of multiple shoots from infected leaflet explants on 100 mg/l kanamycin supplemented; (f) Leaflet explants derived non-transformed shoots died when cultured on 150 mg/l kanamycin; (g) Same as Fig. 33c but in de-embryonated cotyledon explants on 200 mg/l kanamycin; (h - i) Putative transformed shoots survived on 250 mg/l kanamycin supplemented media.

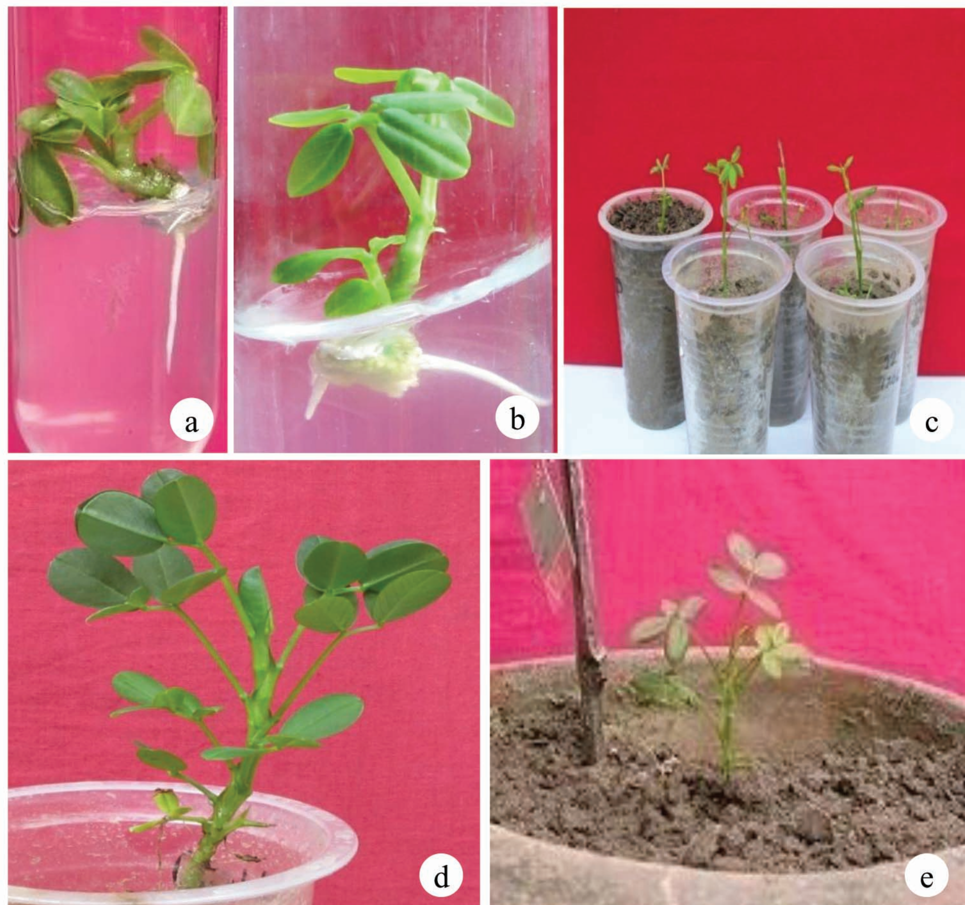

Fig. 34 (a - e): Formation of roots from base of the regenerated shoots and transplantation of putatively transformed plantlets of Dhaka-1 in soil. (a, b) Formation of roots from the base of the putatively transformed shoots of Dhaka-1; (c) Transplantation of putatively transformed plantlets in small plastic pots containing soil; (d) Fully developed putatively transformed plantlet in small plastic pot; (e) Same as Fig. 34d but showing a fully developed transformed plant of Dhaka-1 in earthen pot containing soil.

4.2.1.6 Analysis of transgene integration

Insertion of the transgenes within the transformed plantlets was confirmed by amplification of *GUS* and *npt*II genes present within the genomic DNA of the transformed plantlets of peanut. For this purpose total genomic DNA was isolated from the leaf of putatively transformants as well as control peanut shoots. This isolated DNA was subjected to Polymerase Chain Reaction (PCR) for the amplification of *GUS* and *npt*II genes.

At the same time plasmid DNA from genetically engineered *Agrobacterium* construct pBI121GUS-NPTII was isolated to use as positive control during PCR analysis. Specific primers were used for the amplification of *GUS* and *npt*II genes (Section 2.3.7). Among the 34 rooted plantlets only 5 showed GUS positive expression through GUS assay in Dhaka-1 (Fig. 35a - c). PCR was perform using these GUS positive plantlets. Two GUS positive plantlets of Dhaka-1 showed PCR positive result considering the *GUS* and *nptII* gene (Fig. 36a, b). Table 28 shows the results of transformation efficiency of peanut using de-embryonated cotyledon explants. The results obtained following PCR analysis have been shown in Fig. 36 (a - b). Results of PCR analysis indicated that presence of transgene within the genomic DNA of transformed plants.

Table 28. Transformation efficiency of Dhaka-1 using de-embryonated cotyledon explants.

Variety	No. of explants infected	No. of shoots survived in 250 mg/l kanamycin	No of rooted plantlets showed GUS+ assay	No. of PCR + ve plants	% of transformation efficiency
Dhaka-1	590	23	5	2	0.34%

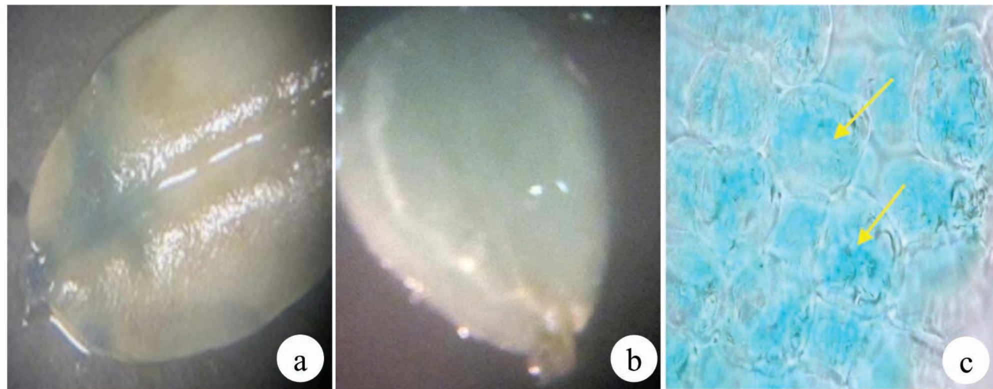

Fig. 35 (a - c): Histochemical localization of GUS in leaf part of transformed plants. (a, b) Histochemical GUS expression of *gus* gene (blue color) in transformed plants of Dhaka-1; (c) A part of transformed macerated leaf tissue of Dhaka-1 showing the presence of GUS positive blue color (arrow, ×100).

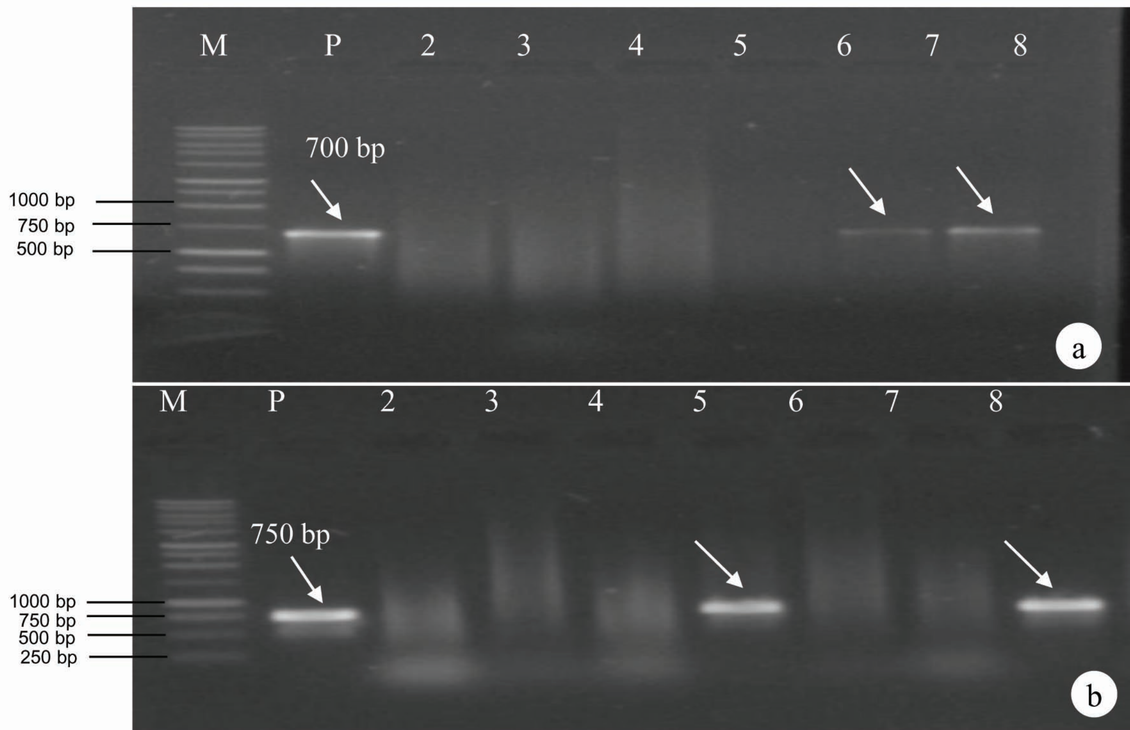

Fig. 36 (a - b): PCR amplification of *nptII* and *gus* gene in putatively transformed plants of Dhaka-1. (a) PCR amplification of *nptII* gene (Lane M - 1kb ladder, lane P - positive control (plasmid of LBA4404/pBI121), lane 2 - negative control, lanes 6 and 7 - amplified genomic DNA of transformed shoots, identical to the band amplified in positive control lane P; (b) PCR amplification of *gus* gene (Lane M - 1kb ladder, lane P - positive control (plasmid of LBA4404/pBI121), lane 2 - negative control, lanes 5 and 8 - amplified genomic DNA of transformed shoots, identical to the band amplified in positive control lane P.

4.2.2 Transformation of peanut with *Agrobacterium* LBA4404/pCAMBIA2300 enh35SAFP (construct II) containing the antifungal gene

This part of the transformation experiments *Agrobacterium*-mediated genetic transformation was carried out using LBA4404 containing the plasmid pCAMBIA2300enh35SAFP (construct II) to integrate fungal disease resistant gene in peanut varieties. This *Agrobacterium* construct containing *npt*II gene as selectable marker that conferring kanamycin resistance and anti fungal protein gene (*AFP)* as the gene of interest. In this case de-embryonated cotyledon and single cotyledon attached decapitated embryo explants of Dhaka-1 and BARI Chinabadam-8 varieties of peanut were used. For this purpose transformation experiments were carried out following the protocol developed in transformation experiments with marker gene described in the section 4.2.1.

4.2.2.1 Determination of optimum kanamycin concentration for selection

From the previous experiments (section 4.2.1.3) it was observed that non-transformed shoots were initially failed to survive on the media with 100 mg/l kanamycin. As a result 100 mg/l kanamycin concentration was used for the primary selection pressure for the selection of transformed shoots. In case of transformation experiments with construct pCAMBIA2300enh35SAFP similar selection pressure were used for the selection of transformed shoots. It was observed that all control shoots were failed to survive in 250 mg/l kanamycin within 30 days. But in most of the cases shoot buds did not show any sign of further growth when the multiple shoots were subjected to higher selection pressure (250 mg/l kanamycin) containing media. For this reason in the present study, 200 mg/l kanamycin concentration was considered for the higher selection pressure for the selection of transformed shoots when transformation experiments was carried out with construct pCAMBIA2300enh35SAFP.

4.2.2.2 Selection of transformed shoots of Dhaka-1 and BARI Chinabadam-8 using kanamycin as a selectable agent

For the selection of transformed shoots of Dhaka-1 and BARI Chinabadam-8 kanamycin was used as a selectable agent. During the study the regeneration of shoots from infected explants were carried out following the regeneration protocol has been described in section 4.1.1.3.14 and 4.1.1.3.16 for both the explants such as de-embryonated cotyledon and single cotyledon attached decapitated embryo. The effect of gradual increase in kanamycin selection pressure on the regenerating shoots from various infected explants and the recovery of putatively transformants is presented in Table 29. Various stages of regeneration and selection of putatively transformed shoots from de-embryonated cotyledon and single cotyledon attached decapitated embryo in the present study have been shown in Fig. 37 (a - i) and 38 (a - f).

In case of Dhaka-1, out of approximately 1120 infected de-embryonated cotyledon (DEC) explants, 54 shoots and in out of approximately 562 single cotyledon attached decapitated half embryo (SCAE) infected explants 12 shoots were recovered on final selection medium infected with construct II (Table 29). In BARI Chinabadam-8 among approximately 940 infected DEC explants, 10 shoots were survived on final selection medium infected with construct pCAMBIA2300enh35SAFP, but no shoots were survived on higher selection pressure from single cotyledon attached decapitated embryo explants of this variety (Table 29). Therefore the frequency of recovery of putatively transformed shoots was almost 4.82% in de-embryonated cotyledon explants and 1.96% in single cotyledon attached decapitated embryo explants of Dhaka-1. In case of BARI Chinabadam-8, 1.06% shoots survived in higher selection pressure from de-embryonated cotyledon explants and no shoots were survived in higher selection pressure from single cotyledon attached decapitated embryo explants (Table 29).

Table 29. Effect of kanamycin (Kn) on selection of infected shoots of Dhaka-1 and BARI Chinabadam-8 from de-embryonated cotyledon explants (DEC) and single cotyledon attached decapitated embryo (SCAE) with construct pCAMBIA2300 enh35SAFP (construct II).

Variety	Explants	No.of explants infected	No. of survived shoots in culture (SEM) with Kanamycin (mg/l)			% of survived shoots
			100	150	200	
Dhaka-1	DEC	1120	155	75	54	4.82
	SCAE	562	92	45	11	1.96
BARI Chinabadam-8	DEC	940	90	25	10	1.06
	SCAE	410	350	28	No shoot survived	No shoot survived

DEC = De-embryonated cotyledon explants, SCAE = Single cotyledon attached decapitated embryo.

4.2.2.3 Root formation and transplantation of putatively transformed plants

Shoots that survived in the kanamycin selection medium were separated and transferred to root induction medium containing half strength of MS with 2.5 μM IBA as well as 50 mg/l ticarcilin. It was observed that the shoots which survived in higher concentration of kanamycin (200 mg/l) showed lower response towards root induction. Altogether 72 shoots of Dhaka-1 survived through selection were subjected for root induction. It was observed that out of 72 shoots 25 of them showed positive responses towards induction of roots (Fig. 39 b). Among these 25 rooted plantlets of Dhaka-1, 19 plantlets were survived following their transplantation in soil (Fig. 39 c, d). In case of BARI Chinabadam-8, 42 shoots survived under selection were subjected for roots induction. Out of 42 shoots 9 of them showed response towards root induction (Table 30). But unfortunately no rooted plantlets of BARI Chinabadam-8 were survived following their acclimatization in soil.

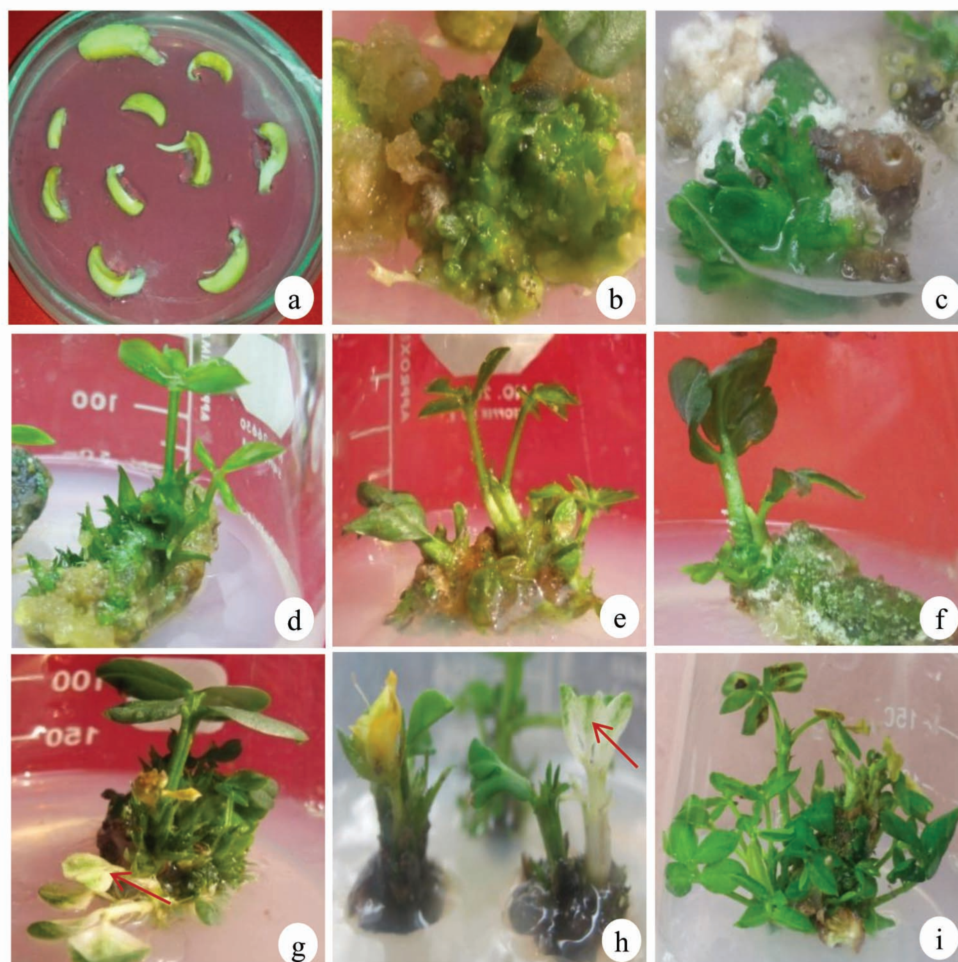

Fig. 37 (a - i): Shoot regeneration and selection of putatively transformed shoots from de-embryonated cotyledon explants using LBA4404/pCAMBIA 2300enh35SAFP. (a) Infected de-embryonated cotyledon explants on selection free regeneration medium; (b and c) Multiple shoots initiated from infected explants from selection free regeneration medium; (d) Elongated shoots developed on 100 mg/l kanamycin supplemented medium; (e and f) Shoots were survived on 150 mg/l kanamycin supplemented medium; (g and h) Green shoots along with albino shoots (arrow) on 200 mg/l Kanamycin supplemented medium; (e) Multiple shoots survived on 200 mg/l kanamycin supplemented media.

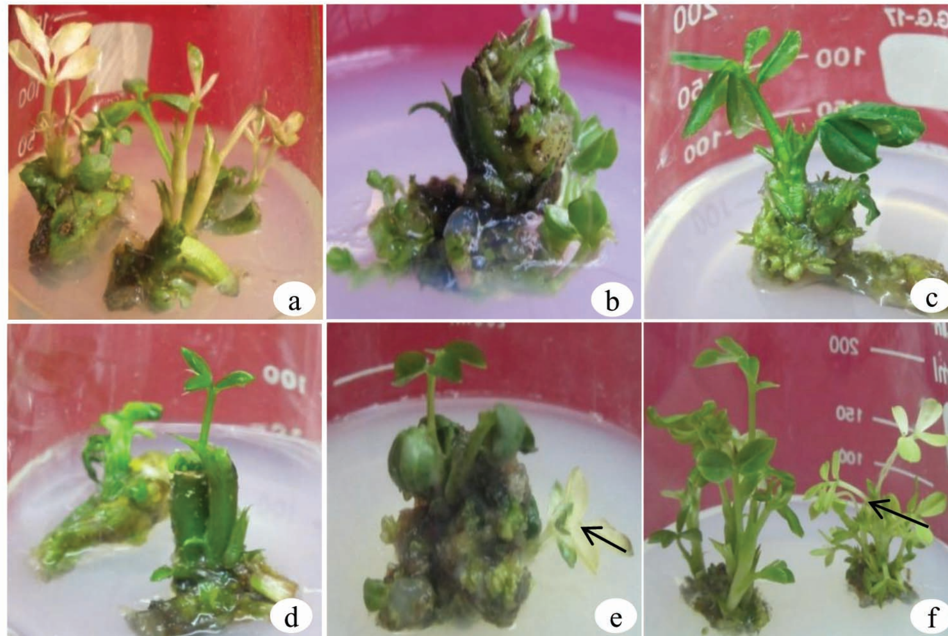

Fig. 38 (a - f): Shoot regeneration and selection of shoots from infected single cotyledon attached decapitated embryo explants. (a) Non transformed shoots failed to survive on kanamycin containing selection medium; (b - c) Initiation of multiple shoots from infected explants; (d) Shoots survived on 200 mg/l kanamycin supplemented medium; (e) Green shoots along with albino shoots (arrow) on 200 mg/l kanamycin supplemented medium; (f) Putatively transformed shoot survived on higher selection pressure of 200 mg/l kanamycin and non-transformed shoots became albino (arrow) on higher selection pressure.

Table 30. Response of survived shoots of Dhaka-1 and BARI Chinabadam-8 towards *in vitro* root formation.

Variety	No. of shoots (under selection) inoculated for root induction	Days required for root initiation	No. of shoots showed rooting response	% of rooting response	% of survived plantlets in soil
Dhaka-1	72	22 - 45	25	34.72	76
BARI Chinabadam-8	42	22 - 40	9	21.423	Plants failed to survive

4.2.2.4 Analysis of transgene integration and efficiency of transformation in two peanut varieties

Molecular analysis through PCR amplification was performed to confirm the presence of *AFP* and *npt*II genes in putative transformants. The genomic DNA from the putatively transformed plantlets along with their control shoots was isolated for the amplification of *AFP* and *npt*II gene through Polymerase Chain Reaction (PCR) (Section 2.3.7). Plasmid DNA of pCAMBIA2300enh35SAFP of *Agrobacterium* LBA4404 was used as positive control during PCR analysis. Among the 25 rooted plantlets, 17 plantlets showed the PCR positive amplification towards *AFP* and *npt*II gene in Dhaka-1. Out of 17 PCR positive plantlets of Dhaka-1, 10 plants were finally survived in soil. In case of BARI Chinabadam-8, out of 9 rooted plantlets 8 plantlets showed the PCR positive result towards *AFP* gene. But unfortunately no rooted plants were survived further in soil in case of BARI Chinabadam-8. The transformed plantlets of Dhaka-1 were successfully maintained in double layered insect proof net house for further progeny analysis (Fig. 40 a - d). The results of PCR analysis and transformation efficiency of two peanut varieties for the integration of *AFP* gene has been presented in Table 31.

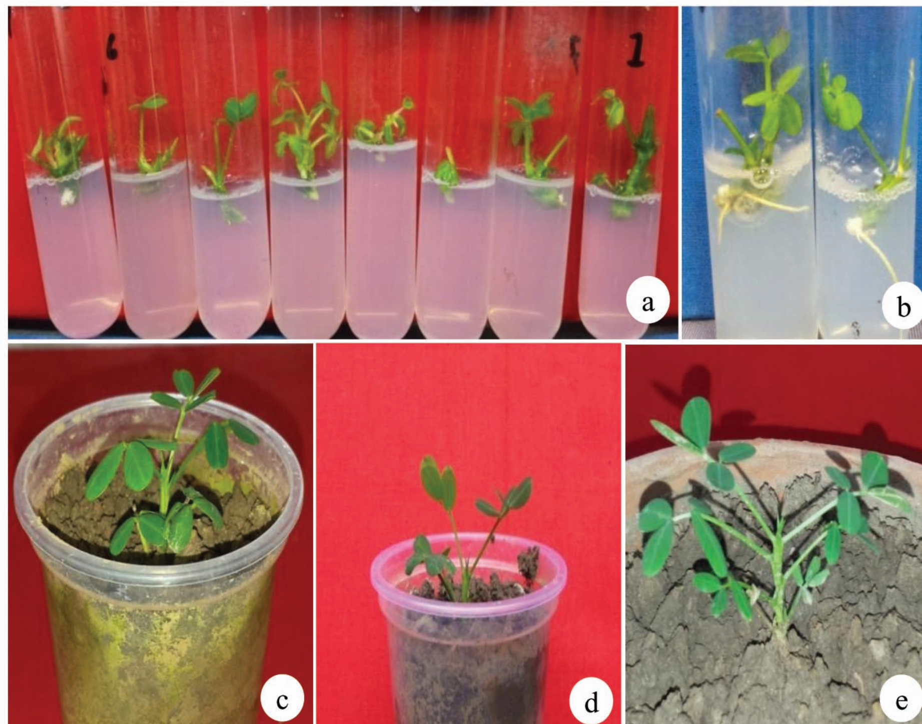

Fig. 39 (a - e): Formation of roots from base of the putatively transformed shoots and transplantation of transformed plants in soil. (a) The putatively transformed shoots were transferred on root induction medium (half strength of MS medium with 2.5 µM IBA); (b) Formation of roots from the cut end of the putatively transformed shoots of Dhaka-1; (c - d) Developed putatively transformed plantlets of Dhaka-1; (e) Showing a developed T_0 plantlets of Dhaka-1 in earthen pot growing in the net house.

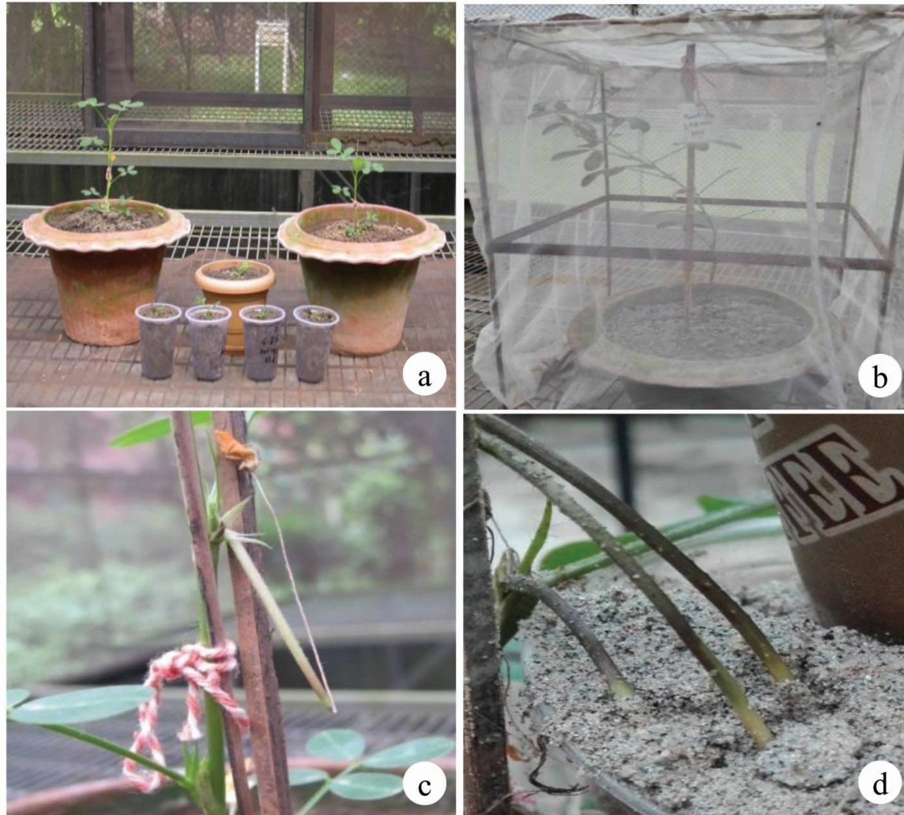

Fig. 40 (a - d): The T_0 plants in plastic and earthen pot maintained in double layered insect proof net house prepared for maintaining proper biosafety regulation. (a) Developed T_0 plantlets in plastic and earthen pot in net house; (b) Well developed T_0 plantlet were kept under mosquito net; (c) Formation of peg in T_0 plant; (d) Insertion of pegs in soil.

Table 31. Transformation efficiency of two peanut varieties using de-embryonated cotyledon explants.

Variety	No. of infected explants	No. of shoots survived in 200 mg/l kanamycin	No. of PCR + ve plants	% of transformation efficiency
Dhaka-1	1120	54	17	1.52
BARI Chinabadam-8	940	10	8	0.85

4.2.2.5 PCR analysis for the amplification of *AFP* and *npt*II genes

The genomic DNA isolated from leaves of 25 rooted plantlets of Dhaka-1 was subjected to PCR analysis for the amplification of *AFP* and *npt*II genes. 17 plantlets of Dhaka-1 were found showing corresponding band for both the genes (at 190 bp for *AFP* gene and 700 bp for *npt*II gene) after PCR amplification, identical to the band of positive control (Fig. 41a and 41b). In case of BARI Chinabadam-8, genomic DNA of 8 putatively transformed rooted plantlets was used for PCR analysis for the amplification of *AFP* and *npt*II genes. It was observed that in case of *AFP* gene amplification, all eight plants were found showing band, identical to the band of positive control (Fig. 42 a). On the other hand, five *npt*II gene corresponding bands were produced after PCR amplification, identical to the band of positive control Fig. 42 b).

4.2.2.6 PCR for the amplification of *picA* gene

This experiment was conducted to verify the probable presence of *Agrobacterium* cells in T_0 plants. *picA* is an *Agrobacterium tumefaciens* chromosomal locus, identified by Mu d11681 mutagenesis (Rong et al. 1991). PCR amplification was performed using *picA* gene specific primers. For the amplification of PCR, 5'-ATG CGC ATG AGG CTC GTC TTC GAG-3' was used as forward primer and 5'-GAC GCA ACG CAT CCT CGA TCA GCT-3' used as reverse primer. Among the seventeen transformed plants of Dhaka-1 and eight transformed plants of BARI Chinabadam-8, no bands were found to amplify after PCR amplification, identical to the band of positive control (Fig. 41c and 42c). Only band was found to amplify in lane of positive control. Thus it was concluded that the transformed plantlets did not have any *Agrobacterium* contamination.

4.2.2.7 Southern blot analysis of T_0 plants

Among the 17 PCR positive plantlets of Dhaka-1, 14 plantlets survived in soil. Southern blot analysis was carried out with ten rooted transgenic plants of Dhaka-1 and a control plant to determine the stable integration and copy number of transgene integrated into genome of peanut plants (Fig. 43). Genomic DNA digested with *Hind*III enzyme that cut only once within the T-DNA was used for hybridization with a DIG-labeled DNA *npt*II gene as probe. Southern hybridization results confirmed the integration of the transgenes into genome of transgenic peanut plants. These results showed that seven of the ten positive transformants possessed a single copy insert whereas three had two copies. These results also indicate that no transgene insertion was detected in non-transformed control plant DNA (Fig. 43)

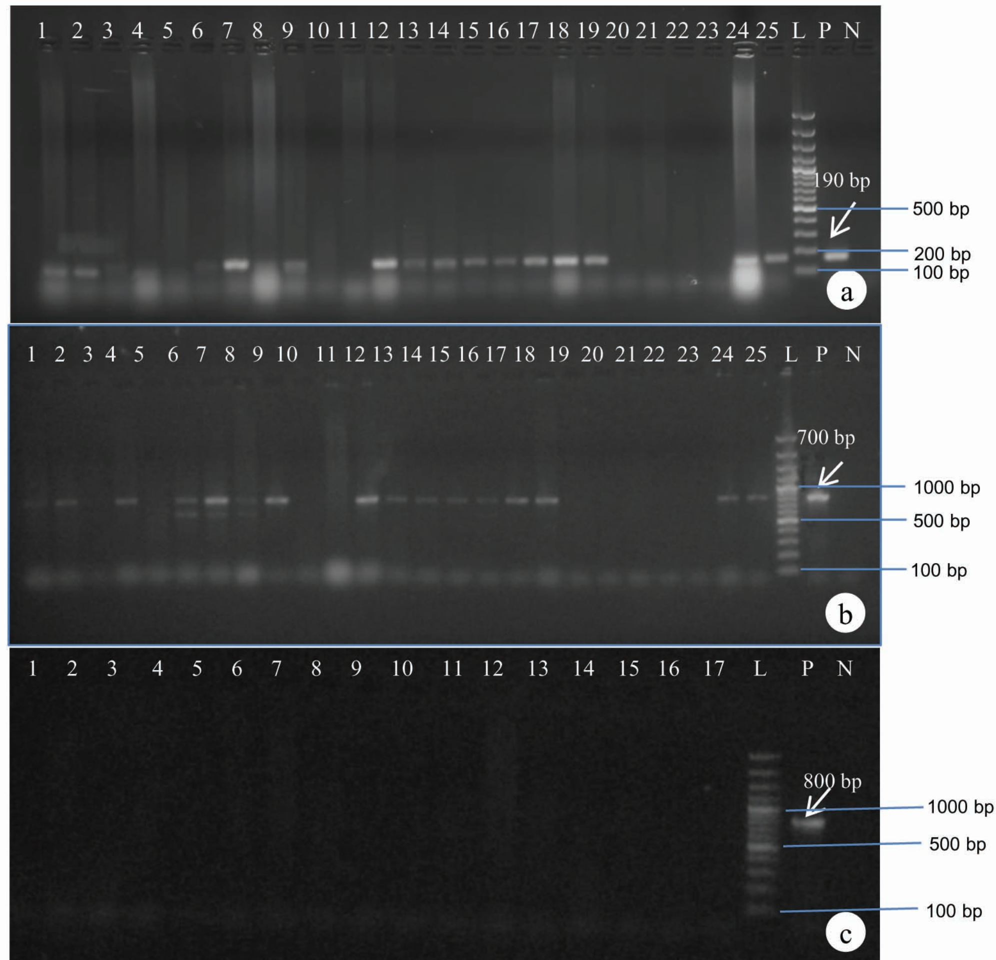

Fig. 41 (a-c). PCR amplification of the putative transformants of Dhaka-1.

(a) PCR amplification of *AFP* gene from transformed shoots (lane L-100 bp ladder; lane P-plasmid DNA of pCAMBIA2300-enh35S AFP as positive control; lane N - negative control; lanes 1-25 genomic DNA of transformed shoots; note that lane 1, 2, 4, 6, 7, 8, 9, 12, 13, 14, 15, 16, 17, 18, 19, 24 and 25 amplified corresponding band, identical to the band amplified in positive control lane P, no band was amplified in negative control lane N;

(b) PCR amplification of *nptII* gene from transformed shoots (lane L - 100 bp ladder; lane P - positive control (plasmid DNA same mentioned as Fig. 41a); lane - N negative control; lanes 1-25 genomic DNA of transformed shoot; note that lane 1, 2, 4, 6, 7, 8, 9, 12, 13, 14, 15, 16, 17, 18, 24 and 25 amplified corresponding band, identical to the band amplified in positive control lane P, no band was amplified in negative control lane N;

(c) PCR amplification of *picA* gene from transformants (lane L - 1kb ladder; lane P-plasmid DNA of pCAMBIA2300-enh35S AFP as positive control; lane-N negative control; lanes 1-17 genomic DNA of transformed shoot; note that no bands were amplified in genomic DNA lane 1-17 after PCR amplification, Only band was found to amplify in lane of positive control in lane P.

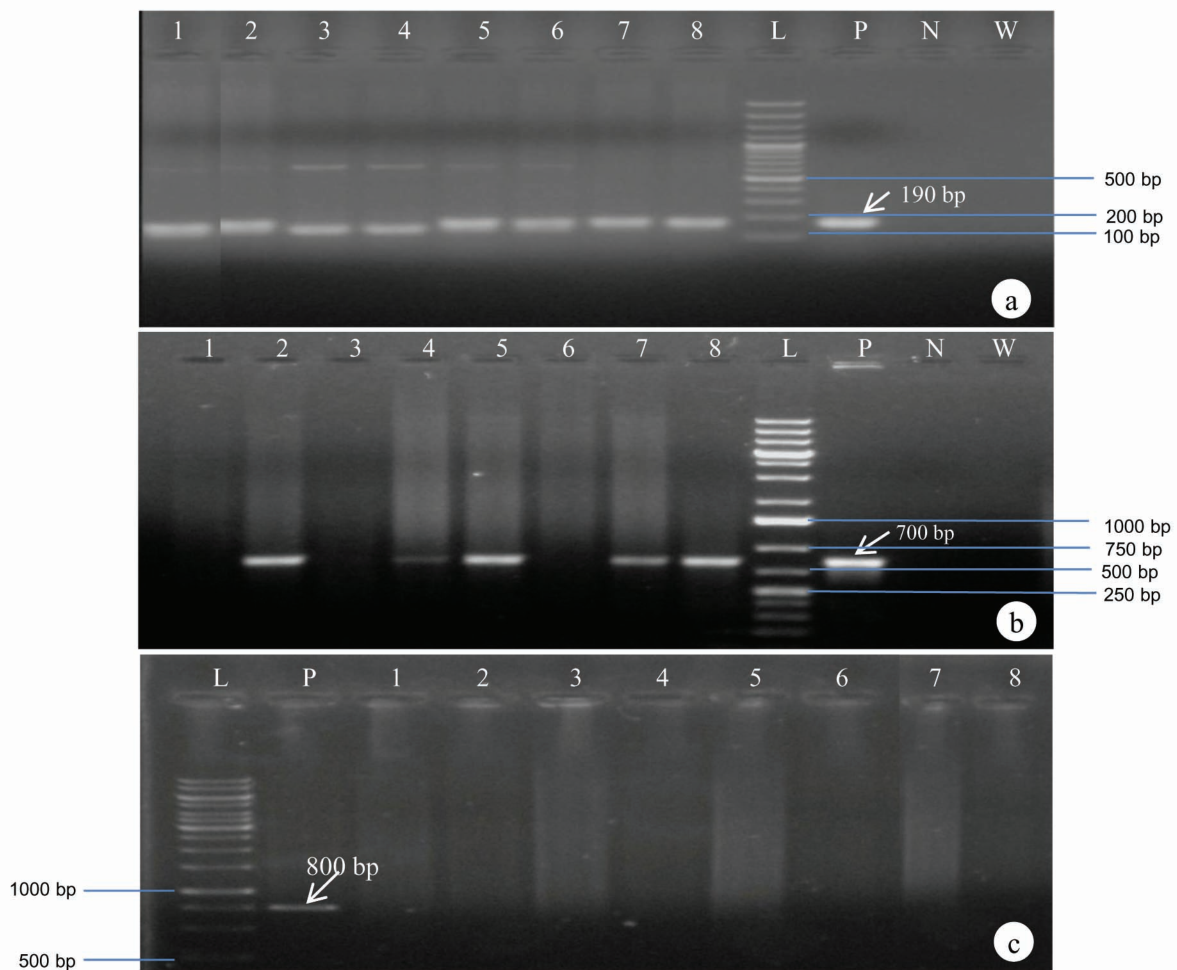

Fig. 42 (a-c). PCR amplification of the putative transformants of BARI Chinabadam-8; (a) PCR amplification of *AFP* gene from transformed shoots (lane L-100 bp ladder; lane P- positive control (plasmid DNA same mentioned as fig 41 a); lane N- negative control; lane W-water control; lanes 1-8 genomic DNA of transformed shoots of BARI Chinabadam-8; note that lane 1-8 amplified corresponding band, identical to the band amplified in positive control lane P, no band was amplified in negative control lane N and water control lane W;

(b) PCR amplification of *nptII* gene from transformed shoots (lane L- 1 kb ladder; lane P - plasmid DNA as positive control; lane - N negative control; W- water control; lanes 1-8 genomic DNA; note that lane 2, 4, 5, 7 and 8 amplified corresponding band, identical to the band amplified in positive control lane p, no band was amplified in negative control lane N and water control lane W;

(c) PCR amplification of *picA* gene from transformants (lane L-1kb ladder; lane P - plasmid DNA of pCAMBIA2300-enh35SAFP as positive control; lanes 1 - 8 genomic DNA of transformed shoot; note that no bands were amplified in genomic DNA lane 1-8 after PCR amplification, Only band was found to amplify in lane of positive control in lane P.

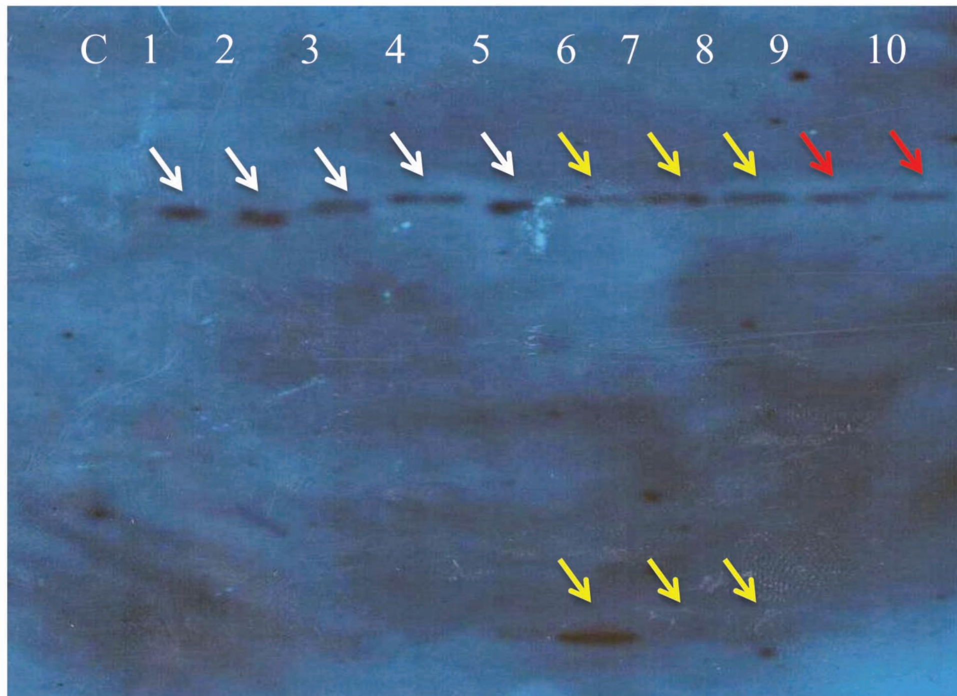

Fig. 43: Southern blot analysis of *Hind*III digested genomic DNA from T_0 transgenic plants. The blot was hybridized with a DIG-labeled *npt*II probe. Lane 1–10 transgenic plants, Lane c- non-transformed plant. Plants 1,2, 3, 4, 5, 9 an10 represent single copy integration.

5. DISCUSSION

5. DISCUSSION

Bangladesh is an agricultural country and its main economic strength based on agricultural sector. This sector contributes 19.10% to GDP (at current prices) and employs 50.28% of the labor force. Among the different crops, grain legumes play a significant role in rain fed agriculture of Bangladesh. Peanut *(Arachis hypogaea* L.) is a major grain legume which plays a vital role in the economy of Bangladesh. The productivity of peanut in Bangladesh is low and unstable due to various biotic constraints. Among the various biotic constraints, the fungal foliar diseases are prominently responsible for the lower production of peanut in Bangladesh as well as in many countries of the world. Due to these constraints, constraints the average peanut production is not up to the adequate and to obtain desired performance improvement of this crop is essential. Conventional breeding methods alone are not adequate for the improvement of peanut. Now a days plant genetic transformation technology is considered as one of the most useful biotechnological methods which exploring an alternative to conventional methods towards the improvement of this crops. This transformation technology is regarded as a pre-breeding method that can provide a solution to certain constraints that hampered crop production.

In the present investigation *Agrobacterium*-mediated genetic transformation technology was used to establish a protocol for the development of fungal disease resistant transgenic peanut line. Two different construct- pBI121GUS-NPTII containing β-glucoronidase (*GUS*) and neomycin phosphotransferase (*nptII*) genes (named as construct I) and pCAMBIA2300enh35SAFP containing antifungal protein gene (*AFP*) and neomycin phosphotransferase (*nptII*) genes (named as construct II) in a *Agrobacterium* strain LBA4404 were used to integrate the desired gene in peanut genome. Among these two construct, construct I was used for the optimization of suitable condition of transformation in peanut, whereas construct II was used for the integration of antifungal gene into peanut genome. The transformants developed through this study were characterized through molecular analysis, like PCR and Southern blot analysis to confirm the introgression of foreign genes.

As an integral part of *Agrobacterium*-mediated genetic transformation, experiments were carried out to establish an efficient *in vitro* regeneration protocol using three peanut

varieties, namely, Dhaka-1, BARI Chinabadam-8 and Binachinabadam-4 commonly cultivated in Bangladesh. Several experiments were carried out to optimize *in vitro* regeneration system using four different explants, namely, immature leaflet, the decapitated half embryo, de-embryonated cotyledon and single cotyledon attached decapitated embryo. Establishment of regenerated plants to soil was also carried out following the development of sufficient root system.

An efficient and reproducible *in vitro* regeneration system is required to be developed for the particular plant species to establish a transformation protocol (Gardner 1993). Earlier studies revealed that most of the legumes, including groundnut, are considered recalcitrant towards regeneration and genetic transformation (Fatokun et al. 2002, Tuteja et al. 2013). However, recent literature survey yielded a number of reports on the transformation and regeneration of different cultivars of peanut across the globe. It was also reported that the *Agrobacterium*-mediated transformation efficiency in peanut is genotype/cultivar dependent (Livingstone and Birch 1995, Chu et al. 2013). Since then several attempts were made to establish a suitable regeneration protocol for peanut. Due to recalcitrant nature success of transformation in leguminous crops is limited (Sainger et al. 2015). In spite of this fact, several attempts have been made in regenerating plantlets from a number of legumes including *Pisum sativum* L., *Cicer arientum* L., *Phaseolous vulgaris* L., *Vigna unguiculata* L., *V. radiata* L. Wilczek, *Glycine max* L. Merril, *Arachis hypogaea* L. and *Lens culinaris* Medik. (Little et al. 2000; Sarker et al. 2003; Kumar et al. 1995; Pniewski and Kapusta 2005; Shan et al 2005; Mohamed et al 2006; Hoque et al. 2007; Verma et al. 2009; Shan 2009; Aasim et al. 2011, 2013; Vadawale 2011). Although considerable success has been achieved in regeneration of legumes, very few efficient regeneration protocols are presently available to use them in transformation experiment. This seriously hindered the progress toward the implementation of gene transfer technology to improve leguminous crops.

In case of peanut several attempts had been made in the past regarding the development of a suitable protocol for plant regeneration. The plants have been regenerated through tissue culture *via* organogenesis using a wide range of explants of peanut like immature leaflet, mature leaf, cotyledon, epicotyl and hypocotyls (Pittman and Dunbar 1992, Bhuiyan 1994, Chengalrayan et al. 1995, Kanyand et al. 1997, Sarker and Islam 1999, 2000, Sharma and Anjaiah 2000, Akasaka et al. 2000, Tiwari and Tuli 2008, Verma et al.

2009, Shan 2009, Vadawale 2011) and via somatic embryogenesis from immature zygotic embryos, other seedling explants and leaflets (Sellars et al. 1990, Baker and Wetzstein 1992, Little et al. 2000, Radhakrishnan et al. 2000, Pacheco 2007, Joshi et al. 2008, Philip Robinson et al. 2011).

During the present study *in vitro* plant regeneration was carried out through organogenesis using four different types of explants, namely, immature leaflet, decapitated half embryo, de-embryonated cotyledon and single cotyledon attached decapitated embryo from three peanut varieties cultivated in Bangladesh. In case of Dhaka-1 and BARI Chinabadam-8, only immature leaflet and decapitated half embryo explants were used whereas de-embryonated cotyledon and single cotyledon attached decapitated embryo explants were used in case of all three varieties of peanut. Leaflet explants were collected from aseptically grown germinated seeds. Cotton soaked with sterile distilled water was found to be most effective for *in vitro* seed germination with n. Leaflets from 7 days old germinated seeds of Dhaka-1 and 8 days old germinated seeds of BARI Chinabadam-8 were suitable for regeneration of shoots from leaflet explants. Half embryo, de-embryonated cotyledon and single cotyledon attached decapitated embryo explants were harvested from overnight socked seeds.

In case of immature leaflet and de-embryonated cotyledon explants regeneration of shoots was obtained via callus formation as well as without the intervention of callus. Sarker and Islam (1999) reported similar results from leaflet explants. Direct regeneration was observed in case of rest of the explants. Lacroix (2003) also showed direct shoot regeneration from embryo explants. Vadawale et al. (2011) used immature folded leaflets for regeneration of shoots from the 7 days old seedlings. Rey et al. (2000) used fully expanded leaflets from adult plants for regeneration via organogenesis and somatic organogenesis. Lacroix et al (2003) used overnight soaked seeds to collect cotyledon explants. However, Tiwari and Tuli (2008), Sharma and Anjaiah (2000) used similar explants from surface sterilized seeds soaked in sterile distilled water for 2 - 3 h.

The various factors on which *in vitro* callus induction and shoot regeneration depends include the composition of culture medium, proper concentration of growth regulators and the responses of explants as well as the genotype of the plant material. The results obtained from the study demonstrated that, MS and MSB medium with different concentrations and combinations of BAP alone and in combination with BAP and Kn was

effective in regenerating multiple shoots directly or via intervention of callus phase from different explants of peanut. This result is in agreement with that of the findings of Mroginski et al. (1981) Narasimhulu and Reddy (1983), Reddy and Reddy (1993). However, several reports are also available where modified MS medium has been found to be equally effective (Pittman and Dunbar 1992, Bhuiyan 1994, Venkatachelam et al. 1998, Little et al. 2000, Tiwari and Tuli 2008). This differential response on shoot regeneration on different media may be due to the use of different genotypes by the above researchers.

In the present investigation, it has been observed that the immature leaflet explants of Dhaka-1 and BARI Chinabadam-8 produced highest number of healthy multiple shoots on MS medium supplemented with 22.2 μM BAP. In this case the 73.33 - 80% explants responded towards shoot initiation. A series of experiments were also conducted to investigate the effect of different hormonal supplements such as BAP, Kn and NAA on *in vitro* shoot regeneration from the leaflet explants. In case of BAP and Kn supplemented MS medium 22.2 μM BAP and 2.3 μM Kn showed best response towards regeneration of multiple shoots, though the mean no of shoots/explant was low compare to the shoots regenerated on MS with 22.2 μM BAP. Previously Sarker and Islam (1999, 2000) and Sarker and Nahar (2003) observed best shoot regeneration using leaflet explants of DM-1, ACC-12, DG-2 and Dhaka-1 varieties on MS with 5.0 mg/l BAP and 0.5 mg/l Kn which corresponds to the concentrations of BAP and Kn used in the present investigation.

Experiments were also carried out to observe the multiple shoot regeneration ability of leaflet explants on MS containing various concentrations of BAP and NAA. It was observed that Dhaka-1 showed positive response towards regeneration of shoots on MS medium supplemented with 44.4 μM BAP and 2.6 μM NAA, whereas the BARI Chinabadam-8 showed best response on 22.2 μM BAP and 2.6 μM NAA containing MS medium. In this combination of BAP and NAA 38-40% explants showed responses towards initiation of shoots. Large amount of callus was produced in the various combinations of BAP and NAA. Vadawale et al. (2011) showed up to 70% direct shoot development on MS medium with 13.32 μM BAP and 2.68 μM NAA from immature leaflet explants. Joshi et al. (2008) used 2,4-D, TDZ and NAA for the production of somatic embryos from leaflet explants. Rey et al. (2000) obtained most suitable combination for regeneration from fully expanded leaf through organogenesis on MS

media with 10 mg/l NAA and 1mg/l BA. But in the present investigation it was noticed that leaflet explants in all combinations of BAP and NAA with MS medium did not show notable response towards shoot initiation. Highest mean no of shoots/explant (4.0) was observed on MS with 44.4 µM BAP and 2.6 µM NAA which was lower than the shoots produced on MS with BAP alone or in combination with Kn.

In the present study, regeneration experiments were also conducted using decapitated half embryo explants explant of Dhaka-1 and BARI Badam-8 varieties. The highest number of multiple shoots (5.0) was obtained on MS medium with 22.2 µM BAP and 2.3 µM Kn from decapitated half embryo explants of Dhaka-1. However, in case of BARI Chinabadam-8 the highest number of multiple shoots (4.2) was obtained when explants were cultured on MS supplemented with 11.11 µM BAP and 2.3 µM Kn. No remarkable variation was observed on regeneration and mean number of shoots on different hormonal combinations. Lacroix et al. (2003) got found efficient regeneration system from overnight socked embryo axes on a medium containing 1 mg/l BAP and 1 mg/l NAA from Bambara groundnut plant. However, Pacheco et al. (2007) showed distinct shoot regeneration pattern from embryo axes explants on MS with 4.4 µM BAP supplemented medium. But in the present study among the various concentrations of BAP alone was used for regeneration of shoots, best response obtained on 22.2 µM BAP supplemented media combination with comparatively low mean no of shoots/explants (4.0 - 4.2).

Among the four explants used for the regeneration of shoots, the highest multiple shoots were obtained within the shortest period from de-embryonated cotyledon explants. It was noticed that regeneration of shoots was not observed from de-embryonated cotyledon explants on MS with various hormonal combinations from leaflet and decapitated half embryo explants. MSB medium supplemented with various concentrations of BAP showed best response towards regeneration of shoots from de-embryonated cotyledon explants of three varieties of peanut. Dhaka-1 showed the best result where 80% explants responded towards multiple shoot formation from the de-embryonated cotyledon explants on MSB with various concentrations of BAP. For this purpose, MSB medium supplemented with 88.8 µM BAP followed by subculture on 66.6 µM BAP which was considered as shoot induction medium (SIM). For further development and elongation of shoots, explants were sub-cultured on MSB with 13 µM BAP which was designated as shoot elongation medium (SEM). In this concentration of BAP, Binachinabadam-4 also

showed positive response towards regeneration of shoots from the de-embryonated cotyledon explants. BARI Chinabadam-8 showed slow response compared to other two varieties. Tiwari et al. (2008) used similar media supplements and optimized a genotype independent protocol for peanut using de-embryonated cotyledon explants which performed superior in shoot bud formation and high regeneration which is an agreement with the present investigation.

The effect of MSB medium supplemented with BAP and 2, 4-D on shoot regeneration from de-embryonated cotyledon explants was also investigated. Moderate response towards shoot initiation efficiency of de-embryonated cotyledon explants was found on MSB with BAP and 2, 4-D supplemented media but mean number of shoots/explant was low compared to MSB with BAP supplemented medium in all the three varieties of peanut. According to Tiwari et al. (2015), three types of shoot initiation medium SM1 (MSB with 66.6 µM BAP), SM2 (MSB with 20 µM BAP + 10 µM 2, 4-D) and SM3 (initially MSB with 88.8 µM BAP (SIM1) and then sub-cultured on 66.6 µM BAP (SIM2) were used for the regeneration of de-embryonated cotyledon explants. Results demonstrated that highest regeneration frequency was found on SM1 medium (MSB with 66.6 µM BAP) and highest mean number of shoots/explant was recorded on SM2 (MSB with 20 µM BAP + 10 µM 2, 4-D). But in the present experiment, highest shoot regeneration frequency and mean number of shoots/explant was observed when initially cultured on SIM1 (88.8 µM BAP) and then sub-cultured on SIM2 (66.6 µm BAP) which was identical to SM3 of Tiwari et al. (2015).

Sharma and Anjaiah (2000) demonstrated high frequencies (90%) adventitious shoot bud production from de-embryonated cotyledon explants from mature peanut seeds on modified MS containing BAP and 2,4-D. But in the present study MSB medium supplemented with BAP and 2,4-D did not show optimum response towards shoot regeneration. Although adventitious shoot buds were formed at the proximal end of explants but most of the cases they were not elongated properly even after two to three subcultures. Use of cotyledon segments as explant for *in vitro* regeneration have been demonstrated in a number of legume species including *Vigna radiata* (Chandra et al. 1995), *Dalbergia sissoo* (Roxb.) (Chand and Singh 2004) and *Lens culinaris* (Medik.) (Khawar et al. 2004), *Cicer arietinum* L. (Tripathi et al. 2013).

It was noticed that regeneration of adventitious shoot bud from de-embryonated cotyledon explants, depends on the orientation of explants on the shoot induction medium. It is important that the proximal cut end should be embedded into the medium so that it remains in contact with the medium at least for the first two week of culture. Sharma and Anjaiah (2000), Beena et al. (2008), Tiwari et al. (2008) also reported identical observations in their studies with de-embryonated cotyledon explants of peanut. According to Tiwari et al. (2008), the orientation of de-embryonated cotyledon explants and auxin polarity on shoot induction medium played a significant role in efficient regeneration. Sharma and Anjaiah (2000) and Tiwari et al. (2015) also mentioned that proximal parts of the explants containing multiple adventitious shoot buds were excised and transferred on shoot elongation medium for the proper elongation of shoots. But in the present study it was observed that development and elongation of shoots was delayed when de-embryonated cotyledon explants were sub-cultured partially. Often it took 8-12 weeks for the elongation of shoots. Most of the cases, multiple shoots formed a compact structure from which individual shoots could not be separated. Venkatachalam et al. (2000) and Philip Robinson et al. (2011) also used cotyledons as explants to produce fertile plants *via* embryogenesis.

In case of single cotyledon attached decapitated embryo explants, it was observed that MSB medium with above mentioned concentrations of BAP was effective towards direct multiple shoot regeneration in all the three varieties of peanut, namely, Dhaka-1, BARI Chinabadam-8 and Binachinabadam-4. Beside this, other combinations like MSB with BAP and 2,4-D was tried for regeneration from this explant. In both the hormonal combinations, all varieties showed 92 - 96% response towards regeneration of shoots. Higher response towards formation of mean number of shoots/explant was observed on MSB and BAP supplemented medium. Cucco et al. (2000) showed regeneration of peanut from various explants like cotyledon with and without embryo, epicotyls in various concentration of 2,4-D. Banerjee et al. (2007), Verma et al. (2009) developed a protocol for multiple shoot regeneration without intervening callus stage on MS medium with 15.0 mg/l or 66.6 µM BAP and MS with 25.0 mg/l or 110.00 µM BAP from cotyledonary node explants of peanut. Anuradha et al. (2006) established regeneration system from cotyledonary node explants on MS with 4.0 mg/l BAP and 0.1 mg/l NAA. Rohini and Rao (2000) used single cotyledon attached decapitated embryo axis but for a non-tissue culture based approach for generating transgenic peanut plants.

Induction of healthy root, after successful regeneration of shoots, is an essential part for successful development of plantlets. In the present investigation various concentrations of auxin like 1.0, 2.5 and 5.0 µM of IBA, IAA and NAA were used for root induction. The results of the present study demonstrated that although roots were induced more or less in all the three combinations of IBA, IAA and NAA, 5.0 µM concentration of IBA was found most effective for root induction in regenerated shoots of Dhaka-1 and BARI Chinabadam-8, whereas both 2.5 µM and 5.0 µM IBA showed optimum response towards inducing healthy roots in Binachinabadam-4. Venkatachalam et al. (2000) reported best result for root formation from kanamycin resistant shoots on MS with 1.0 mg/l IBA which corresponds to 5.0 µM IBA used in the present investigation. Vadawale et al. (2011) reported that MS medium with 3.5 µM IBA was best for suitable root induction in peanut. On the other hand Verma et al. (2009) showed best rooting response in peanut on MS with 1.0 mg/l NAA and for some varieties of peanut best response was observed on 1.0 mg/l NAA and 0.5 mg/l IBA containing medium. Bhuiyan et al. (1992) reported that 0.2 mg/l IBA was found suitable for rooting of peanut. Similar result was reported by Nguyen and Le Tran (2012) who observed best rooting response in peanut using 0.3 mg/l of IBA.

In the present investigation, the effect of IAA on root induction was also studied. It was observed that among the different concentrations of IAA, best response was observed on half strength of MS medium supplemented with 2.5 µM IAA for BARI Chinabadam-8 and Binachinabadam-4, whereas Dhaka-1 showed best response on half strength of MS medium containing 1.0 µM IAA. Pacheco et al. (2007) showed the highest frequency of root formation from the shoots originated from mature somatic embryos of peanut on MS with 0.57µM and 2.85µM IAA. Kabir (2008) also observed best results using half MS with 0.2 mg/l IAA in both Dhaka-1 and BARI Chinabadam-6 varieties.

In case of various concentrations of NAA used for induction of roots, best result obtained on half strength of MS media with 2.5 µM NAA in all three varieties. However, it was observed that the root induction was relatively slower on NAA supplemented medium than IBA and IAA. Moreover, induction of roots as well as callus induction was also noticed at the cut end of the shoots. In the past Sarker and Nahar (2003), Venkatachelam, et al. (1998) were able to obtain best root induction on half strength of MS with 0.5 mg/l or 2.5 µM NAA supplemented media. Banerjee et al. (2007), Lacroix et al. (2003), Verma

et al. (2009) and Tiwari et al. (2008) reported that MS with 1.0 mg/l NAA showed best response for root induction in case of *in vitro* regenerated peanut shoots. Anuradha et al. (2006) got best root induction in peanut on MS+ 0.8 mg/l NAA. Sharma and Anjaiah (2000) observed best rooting response in *in vitro* regenerated peanut shoots on Modified MS containing 5 µM NAA. However, Eapen and George (1994), Sarker and Islam (1999) obtained best root induction in peanut on half strength of MS medium supplemented with 1.07 µM or 0.2 mg/l NAA. McKently et al. (1989, 1991) reported that 0.54 µM NAA were suitable for root induction in peanut.

After proper hardening, *in vitro* regenerated shoots with well developed roots of Dhaka-1, BARI Chinabadam-8 and Binachinabadam-4 were successfully transplanted into soil. Almost 100% transplanted plantlets were survived in soil. The survived plantlets grew well in large clay pots and afterwards they were transferred in experimental field.

The second phase of the investigation dealt with the genetic transformation of two peanut varieties, namely, Dhaka-1 and BARI Chinabadam-8. Among the different approaches, *Agrobacterium*-mediated genetic transformation has been considered as the most common and successful method used in various leguminous crop plants such as soybean (Meurer et al. 1998, Zhang et al. 2016, Li et al. 2017), chickpea (Kar et al. 1996, Indurker et al. 2010, Mehrotra et al. 2011, Tripathi et al. 2013), peanut (Sharma et al. 1994; Eapen and George 1994; Venkatachalam et al. 2000; Sharma and Anjaiah 2000; Sarker et al. 2000; Rohini and Rao 2001; Sarker and Nahar 2003; Bhatnagar et al. 2010; Tiwari and Tuli 2012; Mehta et al. 2013; Chen et al. 2015; Chu et al. 2013; Keshavareddy et al. 2013; Manjulatha et al. 2014; Pandurangaiah et al. 2014; Tiwari et al. 2008, 2011, 2015). Reports are also available for using several other methods of genetic transformation of plants including microprojectile bombardment, electroporation, sonication, chemical method of transformation, etc. (Singsit et al. 1997, Magbanua et al. 2000, Livingstone et al. 2005, Athmaram et al. 2006, Niu et al. 2009). All these methods are plant specific and cost intensive. Generally, *Agrobacterium*-mediated genetic transformation has been considered as the most convenient and cost effective than other techniques. It is the most popular method for genetic transformation because of high co-expression of introduced genes, potentially low copy number and preferential integration into active transcription regions (Sharma and Anjaiah 2000, Dodo et al. 2008, Tiwari et al. 2008, 2011).

In the present transformation study, genetically engineered *Agrobacterium* strain LBA4404 containing two different binary vector plasmid pBI121GUS-NPTII containing *GUS* (β – Glucoronidase) and *nptII* (Neomycin phosphotransferase) genes (considered as construct I) and LBA4404 containing plasmid pCAMBIA2300enh35SAFP containing *nptII* (Neomycin phosphotransferase) and *AFP* (antifungal protein) gene (considered as construct II) were used as transformation vectors to transform two local peanut varieties. Construct I was mainly used for the optimization of a suitable transformation protocol which includes various parameters required for *Agrobacterium*-mediated genetic transformation including the optical density of *Agrobacterium* suspension, suitable incubation and co-cultivation periods required for effective transformation. Construct II was used for the integration of antifungal gene into peanut genome with an aim to develop fungal disease resistant peanut lines. Attempts were also taken for selection and regeneration of putatively transformed plantlets. Transformation with LBA4404 containing plasmid pBI121GUS-NPTII was reported earlier by Sarker et al. (2000), Sarker and Naher (2003), Venkatachalam et al. (2000), Tiwari and Tuli, (2012) from various explants like immature leaflet, cotyledonary node, de-embryonated cotyledon, etc.

It is well known fact that the transformation and regeneration are influenced by various physical and chemical factors such as choice of genotypes, explants, co-cultivation time, virulence inducing agents, hormonal combinations and selectable markers (Manjulatha et al. 2014). An ideal transformation protocol should be qualified as the genotype independent and rapid, with minimal presence of chimerism in the regenerated transgenic plants, with high frequency of transformation. Factors like explant type, polarity and orientation of explants, hormonal combinations, addition of various antioxidants, time of infection, co-cultivation period, temperature, pre-culture, bacterial strains, nature of the genes and promoters used, use of sonication, selective agent, selection pressure and time of application, binary vectors with enhanced virulence, phenolic compounds are reported to increase transformation efficiency in peanut as well as many recalcitrant plant species (Sharma and Anjaiah 2000, Frame et al. 2002, Olhoft et al. 2003, Qiushen et al. 2005, Anuradha et al. 2006, Bhatnagar et al. 2010, Dutt et al. 2011, Tiwari and Tuli 2012, Tripathi et al. 2013, Rustagi et al 2015, Tiwari et al. 2015).

In the present study three explants, namely, immature leaflet, de-embryonated cotyledon and single cotyledon attached decapitated embryo explants of both the varieties were used for the evaluation of various factors influencing transformation efficiency of peanut. Factors that influence successful transformation efficiency, such as *Agrobacterium* strain-variety (host) compatibility and responsiveness of explants toward *Agrobacterium* infection were evaluated. Several other factors of transformation like optical density (O.D) of *Agrobacterium* suspensions, incubation duration of explants in to bacterial suspension, co-cultivation period, co-cultivation condition (dark/night), etc. were also optimized.

During optimization of the above mentioned factors it was found that maximum transformation efficiency was observed with bacterial suspension having an optical density of 1.0 at 600 nm in case of immature leaflet explants. In case of de-embryonated cotyledon explants best transformation efficiency was observed having an optical density of 0.8 and the single cotyledon attached decapitated embryo showed best GUS positive result having an optical density of 0.6. Based on the results of histochemical GUS assays it can be reported that 30 minutes of incubation period followed by 3 days of co-cultivation were found to be most effective towards transformation using immature leaflet explants. Eapen and George (1994) reported to obtain regeneration after 3 to 4 weeks from transformed leaflet explants of peanut which was incubated for 5 min in the bacterial suspension followed by co-cultivation for three days. Sarker et al. (2000) applied 60 mins of incubation period for transformation of peanut (variety DM-1) and co-cultured for 72 hours using immature leaflet explants and got the transformed peanut plants. Tiwari et al. (2008), Tiwari and Tuli (2012), Tiwari et al. (2015) reported that late log phase (OD600 1.0 to 1.6) of bacterial growth was optimum for the transformation in peanut. They infected the cotyledon explants with the A*grobacterium* suspension having an optical density of 1.4 - 1.6 with 20 minutes incubation period followed by 5 days of co-cultivation period to get transgenic peanut. In chickpea, Krishnamurthy et al. (2000) incubated mature embryo explants for 20 minutes and then co-cultured the explants for 3 days to obtain transgenic plants whereas Tripathi et al. 2013 obtained notable transformation efficiency when the infected explants were incubated in 2 days of co-cultivation. In the present study, de-embryonated cotyledon and single cotyledon attached decapitated embryo explants showed best result towards transformation through GUS expression when *Agrobacterium* suspension had an optical density of 0.6 - 0.8 followed

by 10 minutes of incubation period with 3 - 4 days of co-cultivation with all the *Agrobacterium* constructs. Anuradha et al. (2008) used the fresh culture of *Agrobacterium* in OD_{600} of 0.8 followed by 5 - 10 minutes incubation period with 4 days of co-cultivation of embryo axis and cotyledonary node explants of peanut. These conditions were almost identical to present study whereas Venkatachalam et al. (2000) employed the 2 days co-cultivation period for cotyledon explants having an OD of 0.6 with 10 min incubation period with continuous shaking. Vasudevan et al. (2007) also applied same parameters in case of cotyledon explants of peanut as like as Venkatachalam et al. (2000) but they used OD 1.0 instead of OD 0.6. The previous reports on peanut with de-embryonated cotyledon explants demonstrated that 72 hours co-cultivation with few seconds of incubation period was optimum for transformation (Sharma and Anjaiah 2000; Iqbal et al. 2012)

In the present study it was found that, longer incubation in the A*grobacterium* suspension (more than 30 minutes in case of construct I and more than 10 minutes for construct II) leads to the over-growth of bacteria in regeneration culture medium thus hampering the proper growth of infected explants. Akter (2006) observed over growth of *Agrobacterium* on the co-cultivation medium when they incubated the explants in bacterial suspension more than 35 minutes in case of Dhaka-1 and BARI Chinabadam-6.

Experiments were carried out to find the effect of dark (in incubator) and light conditions (growth room condition) during co-cultivation period after infection with *Agrobacterium*. No significant differences was observed towards the transformation efficiency between the dark and light (growth room condition) condition during co-cultivation. Both of the conditions showed almost similar results observed through GUS histochemical assay in case of immature leaflet, de-embryonated cotyledon and single cotyledon attached decapitated embryo explants of Dhaka-1. Tiwari and Tuli (2012), Tiwari et al. (2008) and Chen et al. (2015) obtained good transformation efficiency using dark conditions for the incubation of explants whereas Venkatachalam et al. (2000), Anuradha et al.(2006), Vasudevan et al. (2007), Iqbal et al. (2011) co-cultivated the explants under light condition instead of dark.

Both the constructs (pBI121GUS-NPTII and pCAMBIA2300enh 35SAFP) used in this investigation contained *npt*II gene within its T-DNA region and this gene confers kanamycin resistance to the transformed cells. Therefore, for the selection of transformed

tissue kanamycin was applied to the regeneration medium for the selection of transformants. To determine the level of selection agents (kanamycin) in medium, different concentrations of kanamycin were tested. In many reports kanamycin was applied immediately after co-cultivation for the selection of transgenic shoots/cells (Srinivasan et al. 1991, Venkatachalam et al. 2000, Vasudevan et al. 2007, Chopra et al. 2011). However, in the present study it was found that addition of kanamycin immediately after co-culture greatly hampered the growth of the explants. It was observed that even in the presence of lower concentration of kanamycin, co-cultivated explants failed to regenerate. Following the reports of Sharma and Anjaiah (2000); Iqbal et al. (2012), Anuradha et al. (2008), Beena at al. (2008), Bhatnagar-Mathur (2006) selection agent was added when the shoot buds were started showing their first signs of emergence. Therefore, a pre-culture period and a delayed selection with kanamycin were followed in obtaining regeneration from explants with high rate of transformation efficiency.

In the present study, it was observed that all control shoots died in the selection medium with 250 mg/l kanamycin. Non-transformed shoots were started to become albino in presence of 100 mg/l kanamycin. To establish the initial selection pressure 100 mg/l kanamycin was applied and this concentration of kanamycin increased gradually up to 250 mg/l to optimize the proper selection pressure. Shoots that survived on the selection pressure of 250 mg/l for 20 - 25 days were considered as putative transformed shoots. Sharma and Anjaiah (2000) reported that 200 μg/ml kanamycin as optimum for the selection of transformed shoots raised from cotyledon explants. However, Sarker and Nahar (2003), Akhter (2006) reported that 300 mg/l kanamycin was optimum to identify the non-transformed peanut explants and they applied higher selection pressure to select the transformed shoots of peanut. Eapen and George (1994) observed an average of 6.7% of shoot regeneration from leaf tissues on the selection medium containing 50 mg/l kanamycin. Anuradha et al. (2006) cultured the completely green shoots on shoot elongation medium with 175 mg/l kanamycin for two selections of two-week duration each. Beena et al. (2008) selected the transformed shoots on same process but where the kanamycin concentration was 125 mg/l. They suggested that 90% elimination of non-transformed shoots was observed when selection pressure was 125 mg/l kanamycin. Variation in selection pressure may be due to the use of different varieties of peanut. During the study 100 mg/l ticarcillin was used in all these culture media to check bacterial over growth. However, Venkatachalam et al. (2000), Anuradha et al. (2006),

Beena et al. (2008) used 250-300 mg/l cefotaxime to inhibit the growth of *Agrobacterium.*

Among the three explants used for transformation experiments with marker construct I, the higher number of shoots survived from de-embryonated cotyledon explants in the final selection pressure in both Dhaka-1(0.84%) and BARI Chinabadam-8 (0.71%). Shoots regenerated from single cotyledon attached decapitated embryo explants also survived in higher kanamycin contained medium. But none of the shoots regenerated from leaflet explants survived on the medium containing higher selection pressure. Finally transformed plants were obtained from de-embryonated cotyledon explants of Dhaka-1. According to Sharma and Anjaiah (2000), Tiwari and Tuli (2012), cotyledon explant was highly susceptible to *A. tumefaciens* and produced multiple transformed shoots per explants. For regeneration and transformation cotyledon explants showed excellent performance in several other crop species including *Brassica. napus* (Moloney et al. 1989). It may be mentioned here that the transformation efficiency in legumes is very low 0.03 - 5.1% (Yan et al. 2000, Senthil et al. 2004). Peanut also showed low transformation efficiencies ranging between 0.3 and 9.0% (Cheng et al., 1994, 1996; Eapen and George, 1994, Rohini and Rao, 2000). On the other hand, Sharma and Anjaiah (2000) obtained higher percentage (55%) of transgenic peanut plants using 200 mg/l kanamycin selection. Apart of these results, Tiwari and Tuli (2012) recorded up to 81% transformation efficiency from de-embryonated cotyledon explants using hygromycin selection. Venkatachalam *et al.* (2000), Anuradha et al. (2006) also reported 47% and 45% transformation efficiency with kanamycin selection from various explants of peanut like cotyledon, cotyledonary node, respectively.

The integration of antifungal gene via genetic transformation techniques to combat fungal diseases has been shown to be an effective strategy for rapid development of resistant plant to pathogens. Recently, several antifungal genes have been identified which are involved in plant defense against fungal infection. Antifungal protein (AFP) is a defensin gene. Defensins are small positively charged, antimicrobial peptides found to display antimicrobial activity not only against plant and insect pathogens, but also against human fungal pathogens including *Candida* and *Aspergillus* sp and they are employed as novel leads in antifungal therapeutics (Thevissen et al. 2007). In the present experiments, defensin gene containing construct pCAMBIA2300enh35SAFP in *Agrobacterium*

tumefaciens strain LBA4404 were used which considered as construct II. In case of construct II, it was observed that higher concentration of kanamycin (250 mg/l) hampered the development of shoot buds. Therefore, concentration of kanamycin was optimized to select the putatively transformed shoots. It was noticed that 200 mg/l kanamycin was found to be suitable for the selection of transformants. In this concentration, the non-transformed shoots from de-embryonated cotyledon and single cotyledon attached decapitated embryo explants, became albino and finally died whereas the putatively transformed shoots remain green and healthy. The percentage of survived shoots on selection pressure containing medium was 4.82% in de-embryonated cotyledon explants and 1.97% in single cotyledon attached decapitated embryo explants in Dhaka-1. Based on the results of survived shoots on selection medium of two varieties, it was observed that the transformation efficiency was higher for kanamycin selection in Dhaka-1 (1.01%) than BARI Chinabadam-8 (0.52%). Anuradha et al. (2006) shown that they had generated and characterized transgenic tobacco and peanut plants constitutively expressing the mustard defensin. Transgenic peanut plants showed enhanced resistance against the pathogens, *Pheaoisariopsis personata* and *Cercospora arachidicola*, which jointly cause serious late leaf spot disease.

Cheng et al. (1994, 1996) reported that among the different *Agrobacterium* strains (AGLO, LBA4404, C58 and EHA105) the most efficient one was LBA4404 followed by AGLO, EHA105 and C58. This could be due to differences in the functioning of different *vir* genes, which are important factors that influence the infection process by different *Agrobacterium* strains (Lacorte et al. 1991). The rate of infectivity is influenced by the combination and number of virulent genes involved in transcription (Gustavo et al., 1998). Yadav et al. (2014) also reported that among the different *Agrobacterium* strains e.g. LBA4404, EHA105 and GV3101, LBA4404 infected plants showed highest GUS activity in. Rohini and Rao (2000) reported the use of *Agrobacterium* strain LBA4404 in groundnut. Similar results were reported in *Vigna mungo* where transformation frequencies were superior with bacterial stain LBA4404 than those infected by EHA105 (Karthikeyan et al, 1996). High transformation frequency using *Agrobacterium* strain LBA4404 has been reported in pigeon pea (Rao et al. 2008), in pea (Orczyk and Orczyk 2000), in papaya (Azad et al. 2013) and in mungbean (Yadav et al. 2012).

Shoots that survived in higher concentration of selection medium were transferred to root induction medium. It was observed that no root induction was initiated when putatively transformed shoots were cultured on root induction medium containing kanamycin. For this reason, only ticarcillin was used in root induction medium to control the overgrowth of *Agrobacterium*. These findings were in agreement with the results of Anuradha et al. (2006). Sharma and Anjaiah (2000) also cultured the elongated shoots on root induction medium without any antibiotic.

Detection of integrated gene/s in the targeted host plant is an integral part of developing transgenic plants. Most trusted way to detect the integration of transgene is through polymerase chain reaction (PCR) analysis. In the present investigation, integration of transgene in the transformed plantlets was confirmed through the application of specific molecular techniques like polymerase chain reaction (PCR) analysis. The DNA isolated from both transformed and non-transformed shoots was subjected to PCR for the amplification of *nptII* and *GUS* gene. PCR amplified DNA was analyzed through agarose gel electrophoresis. 700 bp amplified bands corresponding to *nptII*, 750 bp for GUS and 190 bp for *AFP* were observed in each of the transformed plantlets were identical to the positive control. This result indicated that the respective target genes were inserted in the genomic DNA of transformed plantlets. In the present study, experiments were also conducted to nullify the probable presence of *Agrobacterium* cells in T_0 plants. *picA* is an *Agrobacterium tumefaciens* chromosomal locus, identified by Mu d11681 mutagenesis (Rong et al. 1990). It was observed that among the transformed plants of both the varieties, no bands were found to amplify after PCR amplification, but positive bands were observed in positive control. Thus it may be concluded that the transformed plantlets did not have any *Agrobacterium* contamination. Tiwari and Tuli (2012) reported the use of bacterial virG gene specific primers to exclude any false positives due to persistent *Agrobacterium* cells in T_0 plants.

Southern blot hybridization is used as a reliable method for further confirmation of transgene integration and to monitor the copy number of introduced genes in transgenic plant (Dai et al. 2001, Kumar et al. 2005, Rai et al. 2007, Maruthasalam et al. 2007). Transgenes expression in transgenic plants could depend much on number of introduced genes. Generally, a number of insertion sites of an introduced gene could lead to silencing of transgenes in plants. Therefore, it is required to know copy number of transgenes that

are introduced into genome of plants. Considering its importance of the copy number of transgene, Southern hybridization was used all through in our study. In the present study, Southern hybridization of ten PCR positive T_0 plants were carried out using DIG-labeled DNA probe (DIG non-radioactive nucleic acid labelling and detection system), corresponding to intact full length genes of kanamycin for hybridization. It is a simple adaptation of enzymatic labelling and offers a non-radioactive approach for the safe and efficient labeling of probes for hybridization reactions (Kruchen and Rueger, 2003). It was observed in the present study that out of 10 transgenic plants seven transgenic plants contained single copy of interrogated gene; while another three transgenic plants contained two copies of introduced gene. This result on the integration of multiple copies in the groundnut genome was reported by Prasad et al. (2013) using a non-radioactive DIG based system. Sharma and Anjaiah (2000) used probes with non-radioactive labeling AlkPhos direct system. Islam et al. (2015) used DIG non-radioactive nucleic acid labelling and detection system in Southern hybridization for the confirmation of putatively transgenic plants of rice. Anuradha et al. (2008), Tiwari and Tuli (2012), Venkatachalam et al. (2000) used radiolabelled probe to confirm the transgene in peanut through Southern hybridization.

From the foregoing discussion, it may be concluded that during the present investigation, it has been possible to develop high frequency regeneration system for three varieties of peanut growing in Bangladesh. It has also been possible to develop transgenic fungal disease resistant peanut plantlets. Those transgenic plants have been confirmed through selective agent (antibiotic) as well as polymerase chain reaction (PCR). Finally, Southern blot hybridization was carried out to check the stable integration of the transgene. Although there are some reports on the development of some transgenic peanut plants (Beena et al. 2008, Anuradha et al. 2008, Tiwari et al. 2008, Yang et al. 2011, Iqbal et al. 2012, Prasad et al. 2013, Manjulatha et al. 2014) however, no follow up reports on the establishment of advanced transgenic lines are available. To the best of our knowledge, this is the first report on the successful development of transgenic peanut plants using *Agrobacterium*-mediated genetic transformation in Bangladesh. Through the present research, it has been possible to raise 25 plantlets from both the varieties contained the antifungal protein *AFP* gene (using the construct pCAMBIA2300enh35SAFP). Among them, 10 transformed plantlets of Dhaka-1 were survived in pot and the presences of *AFP* gene in those 10 potted plants were confirmed by Southern blot analysis. The T_0 peanut

plants were grown in double layered insect proof net house prepared following proper bio-safety regulation. The seeds of T_0 peanut plants were successfully germinated to raise T_1 plants. The promising transgenic events identified in this research need further testing for developing advanced transgenic lines. Moreover, before consideration for releasing these transgenic lines further confirmation using proper bioassay for screening of transgenic plants towards fungal resistance under the laboratory, biosafety net house and field conditions. Food safety assessment as well as Environmental Risk Assessment (ERA) will also be needed to perform as the integral part of biosafety regulations of the country.

Peanut as a grain legume crop is considered as recalcitrant towards *in vitro* plant regeneration as well as *Agrobacterium*-mediated genetic transformation. However, the research results on both *in vitro* plant regeneration and genetic transformation may be considered as a milestone with the ultimate aim of developing fungus resistant transgenic peanut breeding lines. It is well established fact that fungal disease is the main biotic constraints for peanut production not only in Bangladesh but also in many other countries in the world. So, the developed transgenic peanut breeding lines will facilitate the farmers of Bangladesh for peanut cultivation without using toxic fungicide to save their crops as well as increasing the productivity.

6. REFERENCES

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7. APPENDIX

7. APPENDIX – A

ABBREVIATIONS

The following abbreviations have been used throughout the text:

%	:	Percentage
+ve	:	Positive
µ	:	Micron
µg	:	Microgram
µl	:	Micro liter
µM	:	Micromole
1 N	:	1 Normal
2, 4-D	:	2, 4-dichlorophenoxy acetic acid
A.	:	*Agrobacterium*
AFP	:	Anti fungal protein gene
B_5	:	B_5 basal medium
BAP	:	6-benzylaminopurine
BARI	:	Bangladesh Agriculture Research Institute
BBS	:	Bangladesh Bureau of Statistics
BINA	:	Bangladesh Institute of Nuclear Agriculture
bp	:	base pair
C	:	Centigrade / Celsius
$CaCl_2$	:	Calcium chloride
CaMV	:	Cauliflower Mosaic Virus
cDNA	:	Complementary DNA
CH	:	Casein hydrolysate
cm	:	Centimeter (s)
CTAB	:	Cyle tetramethyl ammonium bromide
DEC	:	De-embryonated cotyledon
dNTP	:	Deoxy nucleoside tri-phosphate
e. g.	:	Example gratia, for example
et. al.	:	et alil and others
etc	:	et cetra, and the rest
FAO	:	Food and Agriculture Organization
FAOSTAT	:	FAO Corporate Statistical Database
Fig/s	:	Figure / Figures

FW	:	Fresh weight
g	:	gram (s)
GM	:	Genetically modified
GUS	:	ß-glucoronidase
Ha (s)	:	Hectare
$HCCL_3$	:	Chloroform
HCL	:	Hydrochloric acid
$HgCl_2$	:	Mercuric chloride
Hr (s)	:	Hour (s)
i. e.	:	id est = which to say in other words
IAA	:	Indole- 3 – acetic acid
IBA	:	Indole- 3 – butyric acid
ICARDA	:	International Center for Agriculture Research in Dry Areas
Kan	:	Kanamycin
Kb	:	Kilo base pair
Kcal	:	Kilocalorie
Kn	:	Kinetin (6- furfurylaminopurine)
KNO_3	:	Potassium nitrate
l	:	Litre
LB	:	Liquid Broth
lb / sq. inch	:	Pound per square inch
m	:	Meter (s)
M	:	Molar
mg / l	:	Milligram per liter
mg	:	Milligram
min (s)	:	Minute (s)
ml (s)	:	Milliliter (s)
mm	:	Milimeter
mM	:	Millimolar
MS	:	Murashige and Skoog Medium 1962
MT	:	Metric tonne
MW	:	Molecular weight
Na_2 – EDTA	:	Sodium salt or ferric ethylene diamine tetra acetate
NAA	:	α- naphthalene acetic acid
NaOH	:	Sodium hydroxide
NH_4NO_3	:	Ammonium nitrate

nm	:	Nanometer
No.	:	Number
NOS	:	Nopaline synthase
*npt*II	:	Neomycine phosphotransferasae II
OD	:	Optical density
PCR	:	Polymerase Chain Reaction
pH	:	Negative logarithm of Hydrogen
PPT	:	Phosphinothricin
rpm	:	Rotation per minute.
RT-PCR	:	Reverse transcription polymerase chain reaction
SCAE	:	Single Cotyledon Attached Full Embryo
sec.	:	Second
SEM	:	Shoot Elongation Medium
SIM1	:	Shoot Induction Medium 1
SIM2	:	Shoot Induction Medium 2
Sp. / Spp.	:	Species
t	:	Ton
T- DNA	:	Transfer DNA
T_0, T_1 :	:	Transgenic lines (First generation inbreed progeny)
US	:	United States
US$	:	United States dollar
USDA	:	United States Department of Agriculture.
UV	:	Ultraviolet Wavelength
v / v	:	Volume by volume
Var. (s)	:	Variety (s)
Vir	:	Virulence region
Viz	:	Namely
w / v	:	Weight by volume
Wt.	:	Weight
X – gluc	:	5-bromo-4-chloro-3-indolyl glucoronide
YEP	:	Yeast Extract Peptone
YMB	:	yeast extract Mannitol Broth

APPENDIX - B

Murashige and Skoog (MS) Medium 1962

Components	Concentration
Macronutrients	**(mg/l)**
KNO_3	1900.00
NH_4NO_3	1650.00
KH_2PO_4	170.00
$CaCl_2.2H_2O$	440.00
$MgSO_4.7H_2O$	370.00
Micronutrients	
$FeSO_4.7H_2O$	27.80
Na_2-FeEDTA	37.30
$MgSO_4.4H_2O$	22.30
H_3BO_3	6.20
$ZnSO_4.4H_2O$	8.60
KI	0.83
$Na_2MoO_4.\ 2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Vitamins	
Glycine	2.00
Nicotinic acid	0.50
Pyridoxine-HCl	0.50
Thiamine-HCl	0.10
Inositol	100.00
Sucrose	30,000.00

pH adjusted to 5.8 before autoclaving.

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APPENDIX- C

MSB_5 medium

Macro and Micronutrients of MS medium and B_5Vitamins

(Gamborg *et al*.1968)

Components	Concentration
Macronutrients	**(mg/l)**
KNO_3	1900.00
NH_4NO_3	1650.00
KH_2PO_4	170.00
$CaCl_2.2H_2O$	440.00
$MgSO_4.7H_2O$	370.00
Micronutrients	
$FeSO_4.7H_2O$	27.80
Na_2-FeEDTA	37.30
$MgSO_4.4H_2O$	22.30
H_3BO_3	6.20
$ZnSO_4.4H_2O$	8.60
KI	0.83
$Na_2MoO_4. 2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Vitamins	
Nicotinic acid	1.00
Pyrodoxine-HCl	1.00
Thiamine-HCl	10.00
Inositol	100.00
Sucrose	30,000.00

PH adjusted to 5.8 before autoclave