Development of drought and salinity tolerant peanut (*Arachis hypogaea* L.) lines through *Agrobacterium*-mediated genetic transformation

Ph.D. THESIS
Submitted
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DEDICATED

TO

MY RESPECTED PARENTS

AND

BELOVED FAMILY

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– The Author

CERTIFICATE

This is to certify that the thesis entitled "**Development of drought and salinity tolerant peanut (*Arachis hypogaea* L.) lines through *Agrobacterium*-mediated genetic transformation"** submitted by Shamima Nasrin has been carried out under our supervision in the Plant Breeding and Biotechnology Laboratory of the Department of Botany, University of Dhaka. This is further to certify that it is an original work and suitable for submission for the award of Ph.D. in Botany.

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ABSTRACT

A series of experiments were carried out to establish a suitable protocol for *Agrobacterium*-mediated genetic transformation in two locally grown varieties of peanut (*Arachis hypogaea* L.), namely, Dhaka-1 and BARI Badam-8. As a prerequisite of the transformation protocol, an efficient *in vitro* regeneration system was established for these two peanut varieties. Three different types of explants, namely, immature cotyledonary leaflet, single cotyledon attached decapitated embryo (SCADE) and de-embryonated half cotyledon (DEHC) explants were used for *in vitro* regeneration. The best performance of multiple shoot development was achieved in case of Dhaka-1 (72.2%) and BARI Badam-8 (68%) varieties when the explants of SCADE and DEHC when initially cultured on MS medium supplemented with 88.8 µM BAP, followed by two subsequent cultures on lower concentrations of BAP (66.6 µM BAP and 13 µM BAP) containing medium. In most of the cases the regeneration of multiple shoots was obtained in 40 days. Best response towards the formation of roots from the excised *in vitro* regenerated shoots was observed on half strength of MS medium supplemented with 2.5 µM IBA or 1.0 µM IAA for the two varieties of peanut. 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 pBI121 harbouring *GUS* (*β-Glucoronidase)* and *nptII* (*neomycin phosphotransferase*) genes (construct I) was used. Transient GUS histochemical assay revealed that among the two explants used maximum transient GUS expression (88%) was observed from de-embryonated half cotyledon explants of variety BARI Badam-8. In this case the required optical density of *Agrobacterium* suspension was 0.8 at 600 nm with 10 minutes for the incubation of explants in that bacterial suspension. Transformed shoots were cultured on 150 - 200 mg/l kanamycin supplemented medium to select the putatively transformed shoots. During this experiment it was possible to develop a series of multiple shoots from the selected transformed explants using *Agrobacterium* strain having marker genes *(GUS and nptII).* Histochemical GUS assay of the putatively transformed shoots on optimum concentrations of kanamycin showed the integration of marker genes. However, gene specific bands were not amplified during PCR experiment.

During this study *Agrobacterium* strain containing EHA105/pCAMBIA 1301-PDH45 harbouring *PDH45* and *hptII* genes were used for the transfer of drought and salinity tolerant gene in the peanut plants. Single cotyledon attached decapitated embryo (SCADE) and de-embryonated half cotyledon (DEHC) explants of variety Dhaka-1and BARI Badam-8 were used for transformation experiments. Among the explants studied maximum transformation efficiency was observed in de-embryonated half cotyledon (DEHC) explants with bacterial suspension having an optical density of 0.5 at 600 nm with 25 min incubation period and 48 hrs of co-cultivation period. Transformed shoots were selected using 20 mg/l hygromycin. Stable integration of *PDH45* and *hptII* genes were confirmed through PCR analysis using the genomic DNA isolated from transformed shoots. In this case, transformation efficiency was found to be 1.08% in case of BARI Badam-8. A total 13 putatively transgenic plants (T_0) of BARI Badam-8 were confirmed through PCR analysis. These T_0 peanut plants survived through acclimatization and developed till maturation. Transgenic lines were maintained in the greenhouse. T_0 seeds were collected and raised following the Biosafety guidelines in the greenhouse for further investigation.

1. INTRODUCTION

1. INTRODUCTION

Peanut, or groundnut (*Arachis hypogaea* L.), is a major leguminous oilseed crop of the world. The dry seeds of this plant contain 44 - 56% oil and 22 - 30% protein (Savage and Keenam, 1994). According to El-Akhal *et al*. (2013) and Meena *et al*. (2016), peanut is an economically important oil seed crop, and its seeds are a rich source of dietary essential fatty acids including oleic and linoleic acids (Toomer *et al*. 2018). About 46 percent of the fat in peanut oil is monounsaturated (mostly oleic acid), 32 percent is polyunsaturated (mostly linoleic acid), while 17 percent is saturated (mostly palmitic acid) (Ozcan *et al*. 2010).

Peanuts belong to the family Leguminosae (Fabaceae). It may be pointed out here that Leguminosae (Fabaceae) is considered as the third largest family of higher plants and peanut (*Arachis hypogaea* L.) is one of the most valuable oil seed species of this family (Krishna *et al.* 2015). From the ancient times, leguminous seeds are one of the fundamental components of human nutrition. Legumes have traditionally been regarded as foods that are good for human health. According to recent analytical studies, eating peanuts and tree nuts has been linked to improved weight management, decreased risk of some types of cancer, and decreased cardiovascular disease (Jones *et al*. 2014; Gonzalez *et al*. 2006). Furthermore, peanuts are a good source of dietary fiber, calcium, iron, p-coumaric acid, resveratrol, vitamin A, vitamin B complex, thiamine, riboflavin, niacin, folates, and vitamin E. (Pandey *et al*. 2012; Janila *et al*. 2013; Krishna *et al*. 2015). Vitamin E, a significant antioxidant component, is present in peanut oils (Jonnala *et al*. 2006). It has a complete vitamin content of 130 to 1300 'Lg/mL without tocotrienols and contains α-tocopherol (48 to 373 µg/ml), β-tocopherol (0 to 140 µg/mL), γ-tocopherol (88 to 389 µg/mL), and 15-tocopherol (0 to 20 µg/mL) (Pattee *et al.* 2002). In addition to vitamin E, peanuts also contain considerable levels of vitamins A, B, C, D, and K. Apart from vitamins, its oil also includes sterols, primarily the anticancer ß-sitosterol (0.09 percent to 0.3 percent) (Awad *et al*. 2000). As a dietary supplement of biologically active polyphenols, flavonoids, and isoflavones may be present in peanuts (Janila *et al*. 2013).

The nutraceutical qualities of peanut seed are enhanced. Arginine, a semi-essential aminoalkanoic acid, is found in abundance in peanut seeds and other peanut-based foods

(Tapiero *et al.* 2002). Arginine content in peanuts ranges from 8.6 to 23.5 µg/g seeds (Aninbon *et al*. 2017; Young *et al*. 1972). Consuming arginine prevents problems with the digestive system and has anti-aging properties. It is also involved in gametogenesis and muscle activity (Duggan *et al*. 2002). Additionally, phytosterols found in significant amounts in peanut seeds have positive benefits on health. Given that peanuts contain 80% ß-sitosterol, 10% campesterol, and 5% stigmasterol as phytosterols (Jonnala *et al*. 2006), blood LDL cholesterol levels in blood vessels are consequently reduced (Ostlund *et al*. 2002; Varady *et al*. 2007).

Peanut seeds have 570 kcal of energy and a great source of numerous B vitamins, vitamin E, and a number of dietary minerals, including manganese (95 percent DV), magnesium (52 percent DV), phosphorous (48 percent DV), and dietary fiber (USDA, 2014). As a very good source of monounsaturated fats, peanuts are highlighted in the heart-healthy diet. Additionally, resveratrol, a phenolic antioxidant found in peanuts that lowers the risk of cancer and cardiovascular disease.

Peanut kernels may be consumed directly raw, roasted, cooked or processed or indirectly as confectionary, vegetable oil. For upscale snacks, Virginia-type peanuts are farmed mostly in Virginia and the North geographic region and account for more or less 15% of the US peanut industry. They also have the largest kernel size. Another type of peanuts, which are frequently used in peanut sweets and snacks because of their smaller kernel size and higher oil content than other species of peanuts, are also known as Spanish peanuts. About 4% of the US peanut market is made up of Spanish type peanuts, which are grown in Texas and Oklahoma. Valencia varieties are usually roasted and sold in the shell, have three or more small kernels per pod, and are quite sweet in flavor. In the United States, peanuts can be consumed raw, boiled, or cooked. They are also widely used to make a variety of prepackaged meals (peanut butter, candies, confections, and snack goods), and in developing nations, these are a primary source of protein. During World Wars I and II, peanuts and peanut butter were play an important role in the American military's rations, and the US Army made the peanut butter and jelly sandwich popular as a snack during combat II (National Peanut Board, 2017). Over 60% of worldwide production of peanut seeds crushed to extract oil for industrial and edible uses and remaining 40% of the world's production of peanuts are consumed in food and other uses. Due to great frying medium, peanut oil has a high smoking point (Singh and

Diwakar 1993). In North and South America, as well as Europe, about 70 percent of production is used to make food, whereas in Asia, 35 percent of the assembly is used to make food. Animal feed can be made from peanut byproducts, including oil pressings, seeds, green material, and haulms (meals containing oil cake). Shells may be used as animal feed, fuel or in fiber board producing and as filler in feed and chemical trade (Birthal *et al*. 2010).

Haulms of peanuts provide wholesome food for animals. They have higher concentrations of protein (8-15%), lipids (1-3%), minerals (9-17%), and carbohydrates (38-45%) than cereal fodder. When given to cattle, peanut haulm has a crude protein digestibility of 88 percent and a nutritional digestion of about 53 percent. Haulms can release up to 2337 cal per kilogram of dry matter, however salmon and mahogany red rose are the most abundant (Wanja *et al*. 2011)

Legumes also include a wide range of non-nutrient substances known as bioactive substances with anti-oxidant, hypoglycemic, hypolipidemic, and anticarcinogenic effects. Incorporating legumes into new food products and formulations is becoming more and more popular, and this growing trend and research activity will assist to increase the intake of legumes and thus improving the human diet across the globe. Legumes have a significant role in our sustainable future due to the benefits, they provide in terms of nutrition and health, as well as the vital economic and environmental issues. Legumes are safe to eat, reasonably priced, and widely available. Because of these properties they are found in the diets of millions of people around the world (Martin-Cabrejas *et al*. 2019).
Scientific name of peanut (*Arachis hypogaea* L.) has derived from the Greek words *Arachis,* which means "legume," and *hypogaea,* which means "below ground," and alludes to the development of pods in the soil (Pattee *et al.* 1995). According to their morphological characteristics and growth patterns, the four botanical types of cultivated peanuts comprising *hypogaea*, *hirsuta*, *vulgaris,* and *fastigiata*. The very first two of these botanical types correspond to the subspecies *hypogaea* and the rest two to *fastigiata* (Krapovickas *et al*. 1994). Each botanical type has unique morphological traits, notably in spp.*Hypogaea* lacks floral axes on the main stem and growing along the ground, whereas all botanical types in spp. *Fastigiata* have fertile main stems and an upright plant habit.

It is thought that peanut originated in South America between Bolivia and Argentina, where it was first undergoing domestication (Bertioli *et al*. 2011; FAO, 2011). Another evidence points to the Peruvian Incas as the highly advanced ancient agricultural civilization that farmed it all along the coast. The adventurers of Spain brought peanuts to Europe from wherever they were traded to completely diverse parts of the world, including Africa and Asia (Hammons *et al*. 2016). As mentioned earlier, this is a significant oilseed crop grown mostly in a number of humid, subtropical, and hot temperate regions of the world. It's grownup within the warm climates of Africa, Asia, Australia and North and South America. It grows in regions where temperatures range from 25°C to 30°C between the latitudes of 40°N and 40°S. (Weiss, 2000). Currently, the top prominent exporters of peanuts are China, India, USA and Argentina.

The genus *Arachis* is in the taxonomic group Papilionoidea, order Fables, tribe *Aeschynomeneae*, and subtribe *Stylosanthinae* of the pea Fabaceae family (Holbrook and Stalker, 2003). Almost 700 genera and 1800 species are included in this family (Polhill *et al.* 1981). The annual herbaceous peanut has an unpredictable growth habit and grows upright. There are nine sections in the genus, and some species are diploid while others, like *A. hypogaea*, have tetraploid (2n = 4x = 40) naturally (Brasileiro *et al*. 2014). According to the most recent data, A single recent hybridization event and spontaneous chromosomal duplication between diploid wild peanut species *A. duranensis* (AA) and *A. ipaensis* (BB) are considered to have produced the autogamous allotetraploid (2n = 4x = 40, AABB) (Moretzsohn *et al*. 2013). According to genetic study, the hybridization event most likely only happened once and resulted in the development of *A. monticola*, a wild variety of peanut that only grows in a few specific areas in Argentina's north-western parts and has evolved *into A. hypogaea* by artificial selection (Seijo *et al.* 2007; Kochert *et al.* 1996; Husted *et al*. 1936; Halward *et al.* 1992). *A. hypogaea* is significantly different from its wild relatives due to the artificial selection process used during domestication. Domesticated plants have a distinctive pod structure, have larger seeds, and are bushier and more compact. The first domestication may have occurred in southeastern parts of Bolivia or northwestern parts of Argentina, where today's most prevalent wild-like features in peanut landraces are grown (Krapovickas *et al*. 1994). From this core site of origin, diversity-creating farming spread and gave rise to subsidiary and tertiary centers in the Republic of Peru, Ecuador, Brazil, and other South American countries and nations.

Peanuts are one of the most common and widely grown legumes in the world. Currently, it is grown in about 116 different nations around the globe including China, India, USA, Nigeria, Sudan, Myanmar, Argentina, etc. In terms of quantity if production, peanut's position is thirteenth (13th) among the food crop and sixth (6th) among the oil seed within the world (Nigam *et al.* 2014). A total of 28.57 million hectares of land was under peanut cultivation whereas in India and China the land under peanut cultivation is 5.30 million and 4.63 million hectares respectively. About 45.95 million tons peanut pods were created annually that was equivalent to regarding 34.6 billion US dollars globally. Asia has the biggest share in peanut production that is 62.5% followed by Africa (26.1%) and North and South America (combinedly 11.3%). Highest amount of peanut is produced in China (17.33 million metric tons) followed by India (6.7 million metric tons) and also the US. (2.48 million Metric tons) (FAOSTAT 2020). Top ten peanuts producing countries of the world is presented in Fig. 1.

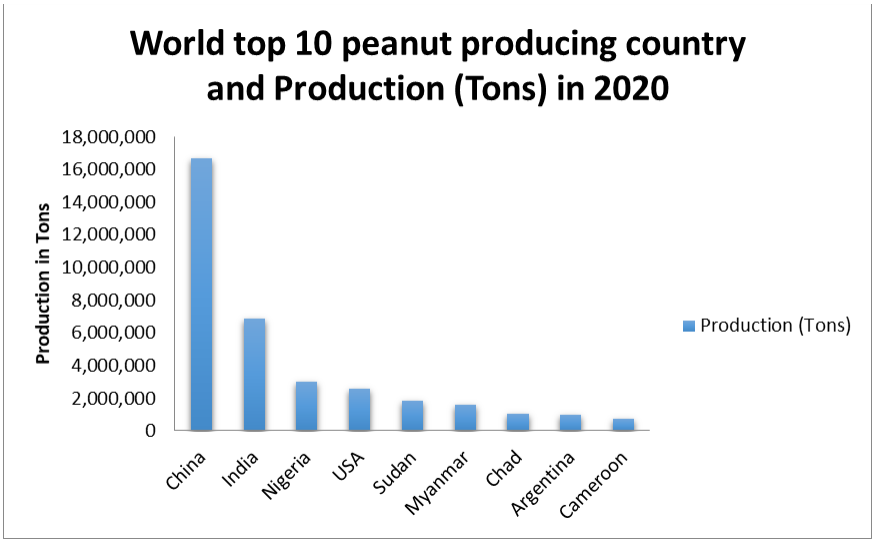


Fig. 1: World top 10 peanut producing countries and production (Tons) in 2020 (FAOSTAT 2020).

Together with other crops, peanut has significant role in food industries of Bangladesh. In the food industry, it is used as edible oil to produce cake, biscuits, and bakery goods. It is typically consumed as fried "badam," and oil cake is fed to livestock. To meet the daily requirements, shelled peanuts and peanut oil are frequently imported into Bangladesh (Deb *et al*. 2015). Bangladesh has excellent soil and climate conditions for cultivating peanuts. More specifically, sandy soils and riverbeds are used for cultivation. It is known that the vast "Char" areas of Bangladesh where no other crops but peanut can be

cultivated. In these “Char” areas peanut is cultivated mostly by marginal farmers (Nath *et al*. 2002).

In Bangladesh, oilseed crops were planted on 1196 thousand acres which comprises 6.23 percent of all arable land in 2017-18 season. Season of Rabi in 2017-18, top five peanut producing districts were Panchagarh, Faridpur, Noakhali, Bhola and Chattagram (BBS, 2018). However, production and area of peanut has shown an increasing pattern in the recent years. Among seven major oilseed crops cultivated in Bangladesh, mustard and rape alone occupies about 78% of the oilseed land followed by sesame, linseed, peanut, coconut and castor (BBS, 2018). Bangladesh ranks 38 as a peanut producing country in the world in terms of production (FAOSTAT, 2018). However, it is not currently being used for oil extraction in Bangladesh. The total quantity of peanut is used as roasted and in the bakery industries (Akter *et al*. 2012). In the late 2000s, Noakhali, Faridpur, Dinajpur, Dhaka and Chattagram were top five peanut producing districts which comprised 60% of peanut cultivation area and 58% of the country's peanuts production (Deb *et al*. 2015). Its area and production almost remained static over the period of 1900s and 2000s. An increasing trend was found in production although a slightly decreasing trend was observed in area. Climatic factors like drought and rainfall were mainly responsible for yield fluctuation over the years (Akter *et al*. 2012). However, the area and production of peanut has shown an increasing pattern in the recent years. Within the year 2017-18, 94 thousand acres of land was cultivated with the peanut. It had been cultivated in 13186 acres and production was 9401 MT in Kharif season 2016-17. In Rabi season 2017-18, 81311 acres of land was cultivated with a production of 57863 MT peanut (BBS, 2018).

Peanut is self-pollinated plant and grows to a height of 30 to 50 cm. Each leaflet measures one to seven cm in length and one to three cm in width. The leaves are opposite and pinnate, with four leaflets (two opposing pairs; no terminal leaflet). The leaves are nyctinasty, which means they require "sleep" movements and close at midnight, like a number of other legumes (Putnam *et al*. 1991).

In general, peanut pods are 25 to 50 mm long, two or more seeds are oblong shaped, cylindrical shape with smooth-edged and with a thin, netted, and spongy shell. Geocarpy is a rare phenomenon where peanut pods grow underground. The flowers are xanthous

orange with cherry veining and range in size from 0 to 1.5 cm (Krapovickas *et al*. 1994). They have a one-day lifespan and are produced in axillary clusters on stems above ground.

Peanuts are autogamous, however natural cross-impregnation caused by bee activity has been reported at amounts ranging from one to ten percent depending on the genotype, region and season (Nigam *et al*. 2014). The peanut's fruit might be a pod with one to three seeds that grows intermittently underground and is known as a "peg." It is a related, elongated ovarian structure that contains an ovule that has undergone fertilization and is formed at the base of the flower. When the peg comes into touch with the soil, the tip begins to develop into a distinctive pod and the extension of the peg is completely geotropic (Ntare *et al*. 2007). Peanut is actually a legume, despite having many similarities to nuts in name and appearance. **Fig. 2** shows the diagrammatic sketch of a typical peanut plant.

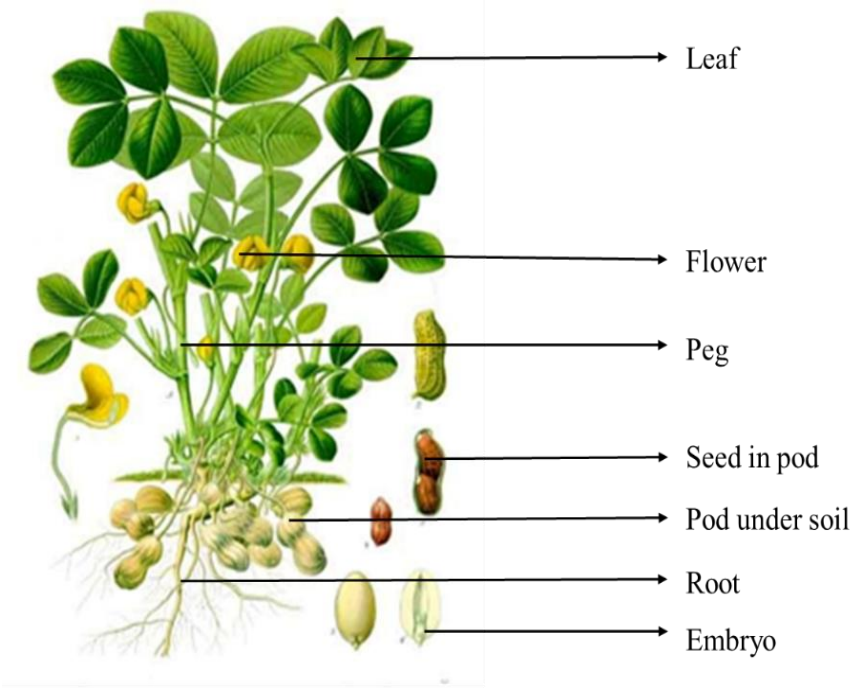


Fig. 2: A typical peanut plant (source: ttps://peanutbase.org/organism/*Arachis*/*hypogaea*)

The optimum temperature for growing peanuts ranges from 25°C to 35°C. Although a pH range of 5.5 to 7.0 is suitable but soil with a hydrogen ion concentration (pH) of 6.0 to 6.5 is optimum for peanut growth. The medium to late maturing large-seeded peanut cultivars requires 1000–1200 millimeters of rainfall, but the quick growing small-seeded species only require 300–500 millimeters (Ntare *et al*. 2008). As peanut has a very low salt tolerance, so saline soils don't seem to be the best choice (Weiss, 1983). Because of their

ability to fix nitrogen, peanuts are able to improve soil fertility while yet receiving little to no benefit from fertilizers that include nitrogen (Baughman *et al*. 2015). In case of peanut, light, temperature, and relative humidity all affect flowering; While temperatures between 22°C to 33°C and a soil moisture level of 40% are excellent for flowering, high temperatures and low relative humidity inhibit flowering. For optimum development, a light intensity greater than 45 percent of full daylight is required. Flowering is promoted by light received three days previous to the opening of flowers, and only one bloom opens at a time at each node at sunrise (Nigam, 2014). The nutritionary demand is totally different for peanut since the pegs develop within the soil. For efficient *Rhizobium* nodulation and organic process, the ideal pH range is 6.0-6.5). Maintain proper amounts of phosphorus, potassium, calcium, metals, and micronutrients by using a balanced fertility program based on soil testing is very important in peanut cultivation. During the development of the seed and pod, there should be enough Ca in the pegging zone. Peanut pods exhibit geocarpy, a rare characteristic of underground growth. Since most of the calcium in peanuts is immediately absorbed by the pods, fertilizers containing calcium are sprayed to the pod zone during the height of flowering to ensure that the calcium is accessible to the pods (Janila *et al*. 2013). Even when the pods are ready for harvest, some remain immature because peanut plants continue to produce blossoms while the pods are growing. To maximize output, the timing of harvest is an important consideration. There will be many unripe pods if it is too early. If it's too late, the pods may separate at the stalk and remain in the ground. The entire plant, along with the majority of the roots, are dug up from the ground for harvest. The elevated veins on the pods are interspersed with constricted spaces between the seeds.

Cultivation of peanut is more profitable, because of it's a decent soil conditioner. The nodule morphogenetic program and the mechanism of rhizobial invasion are shown to be under the control of the host plant (Gleason *et al*. 2006; Tirichine *et al.* 2006). Because of the symbiotic nitrogen fixation by nodule forming bacteria, peanut benefits very little or not the least bit from nitrogen fertilization (Baughman *et al*. 2015). Previous analysis of Reddy *et al.* found that an interaction among few factors such as the *Rhizobium* strain, host plant genome and environment determine the symbiotic nitrogen fixation (Reddy *et al*. 2012). Timberland and Uchida have been reported that one hectare of peanut crop cultivated land produced approximately 72-124 kg nitrogen in a year (Silva *et al*. 2000). It’s additionally an effective cover crop for land exposed to soil erosion.

Although peanut is a vital grain legume, its cultivation and production remain lower compared to alternative peanut producing regions in Bangladesh. Abiotic conditions including salinity, drought, and seed dormancy as well as biotic pressures like illnesses caused by fungus, viruses, nematodes, and insect pests are the main causes of yield loss in this crop, which in turn affects the quality and quantity of the crop (Akter *et al*. 2012). Numerous diseases, such as rust (*Puccinia arachidis* Speg.), early leaf spot (*Cercospora arachidicola*), late leaf spot (*Phaeoisariopsis personata*), and aflatoxin contamination by *Aspergillus flavus* and *Aspergillus parasiticus*, are major global barriers to the production of peanuts (Mishra *et al*. 2015).

Abiotic stress is a general phrase that could refer to a variety of conditions, including heat, cold, excessive light, drought, logging of waterways, wounding, exposure to ozone and UV-B radiation, osmotic shock, and salinity. Only 10% of cultivable land has been calculated to be in the "no stress" category, indicating that 90% of arable lands have one or more environmental pressures on the crops grown there (Dita *et al*. 2006). Abiotic stresses negatively impact on growth and productivity and causes a variety of physiological, morphological, biochemical, and molecular variations in crops (Fig. 3). Also, abiotic stress has the power to alter a wide range of proteins, whether they are soluble, structural, or exist both before and after folding in the plant cell (Qureshi *et al*. 2007). It has been suggested that they reduce average yields by more than 50% for most major crops worldwide (Mahajan *et al*. 2005).

Among them, drought and salinity are major factors that limit peanut production globally (Stansell *et al*. 1985). Simultaneously, soil salinity influences soil N contribution and total N (nitrogen) intake, which reduces production (van Hoorn *et al*. 2001). Numerous abiotic factors affecting legume crops may directly affect dependent relationships, hence restricting the growth of the bean. Additionally, crops experiencing abiotic stress are frequently more vulnerable to weeds, insects, and diseases, which increases severe losses (Reddy *et al*. 2004).

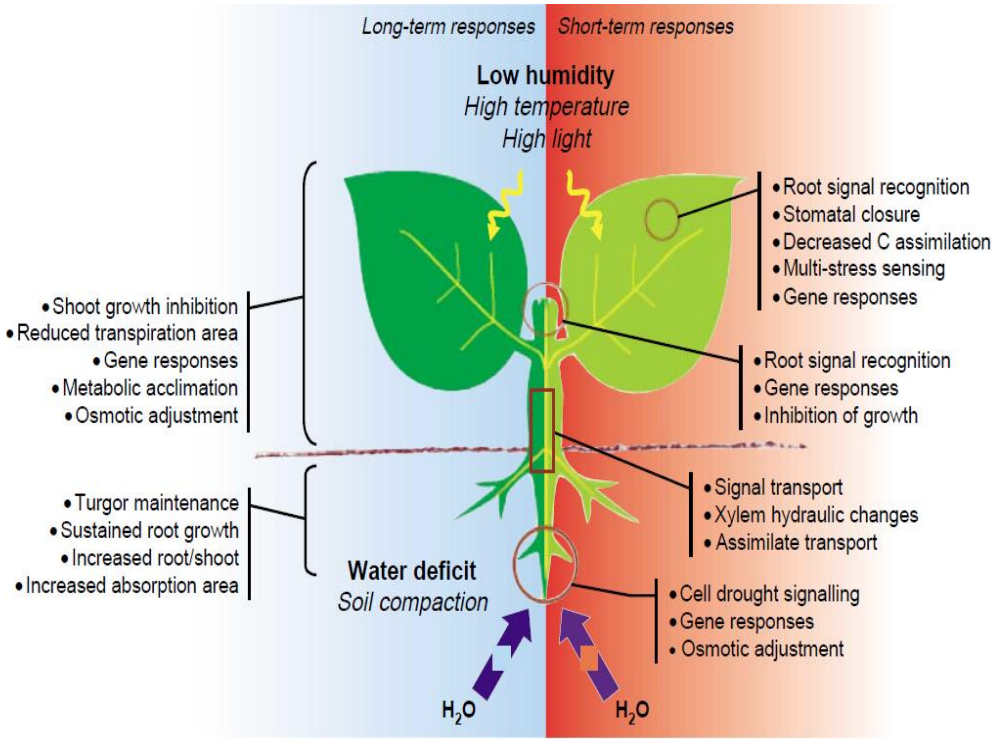


Fig. 3: Plant response during different abiotic stresses (source: de Oliveira *et al*, 2013b)

Drought is the major constraint that causes considerable losses to crop yields (Tuteja *et al*. 2009). As a result of hyper osmotic stress brought on by drought stress, which is characterized by decreased turgor pressure and water loss, membranes become disorganized, photosynthesis is inhibited, reactive oxygen species build up, and finally cells and plants die (Boudsocq *et al*. 2005). Arid and semi-arid regions make up more than half of the production area and account for 70% of the peanut-growing area. In these areas, peanuts are frequently subjected to drought pressures of various intensities and durations (Reddy *et al*. 2003). With an increase in the frequency and severity of droughts and higher temperatures, the impending climate change has intensified negative effects on the production of peanuts (Bhatnagar-Mathur *et al*. 2007; Akcay *et al*. 2010; Asif *et al*. 2011; Vadez *et al*. 2013). Drought results in an annual calculable loss in peanut production of around US$520 million. Furthermore, according to Cole *et al*. (1989), dryness is known to make peanuts more susceptible to aflatoxin contamination, rendering them unsafe for human consumption. Plants under drought stress lose moisture from their pods, which results in a decrease in the physiological activity of the seed and worsens the situation for fungal invasion. Drought stress is notorious for altering the nutritional integrity of peanut seed proteins in addition to affecting food quality (Kambiranda *et al*. 2011).

When rainfall is insufficient in rainy season that year Bangladesh endures a long dry period usually from November to May (Habiba *et al*. 2011). The northwest portion of the country is the most prone to drought because of the significant rainfall variability (Shahid *et al*. 2008). Due to the low soil moisture-retention capacity and infiltration rate, Pabna, Kushtia, Rajshahi, Naogaon, Chapai-Nawabganj, Bogura, Rangpur and Dinajpur (Fig. 4) more frequently experience drought. A region of 5.46 million hectares has experienced moderate-to-severe droughts during the dry season, and 33 percent of the overall land area is insufficient for sustainable farming. A terrible severe drought affects 0.45 million ha of land during the Rabi season, while 0.40 million ha and 0.34 million ha are afflicted during the pre-kharif and kharif seasons, respectively (Habiba *et al*. 2011). The production of several other oilseeds, as well as peanuts, is severely harmed by these droughts (Ahmed *et al*. 2006).

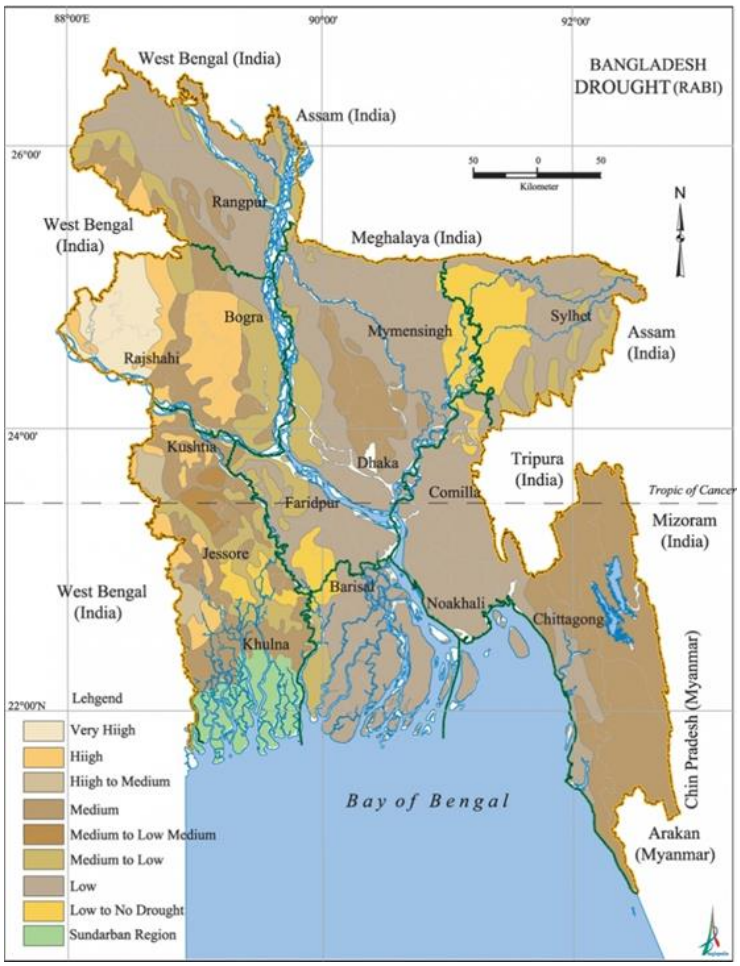


Fig. 4: Drought map of Bangladesh (source: Habiba *et al*. 2011).

The possible effects of climate change on coastal places around the world are caused by increased salinity from saltwater intrusion (Dasgupta *et al*. 2015). Approximately 600 million people live in low-elevation coastal regions around the globe, which will be impacted by growing salinization (Ciesin *et al*. 2010; Wheeler *et al.* 2011). While most studies have concentrated on the flooding and losses caused by heightened storm surges, increased salinity from saltwater intrusion may actually be the biggest threat to livelihoods and public health due to its effects on infrastructure, coastal ecosystems, agriculture, aquaculture, and the availability of fresh water for domestic and commercial use. High salinity's molecular pathways can cause physiological drought conditions and ion toxicity in addition to interfering with plant growth and development (Zhu *et al*. 2002). A changed K+/Na+ ratio is the outcome of ion-specific stressors brought on by salinity. Salinity causes the amounts of Na+ and Cl- in the cytosol to increase, which may ultimately be harmful to the cell. Higher sodium ion concentrations (over 100 mM) are harmful to cell metabolism and can limit the action of numerous vital enzymes, cell division, and growth. They can also cause membrane instability and osmotic imbalance, which can ultimately inhibit growth. Reactive oxygen species generation and photosynthesis can both be decreased by higher sodium ion concentrations (Chaves, *et al*. 2009). Additionally, excessive salinity can harm the cells of transpiring leaves, which inhibits growth (Fig. 5). The osmotic balance, the operation of stomata, and the activity of specific enzymes can all be affected by changes in K+ ions (caused by the effects of high salinity stress).

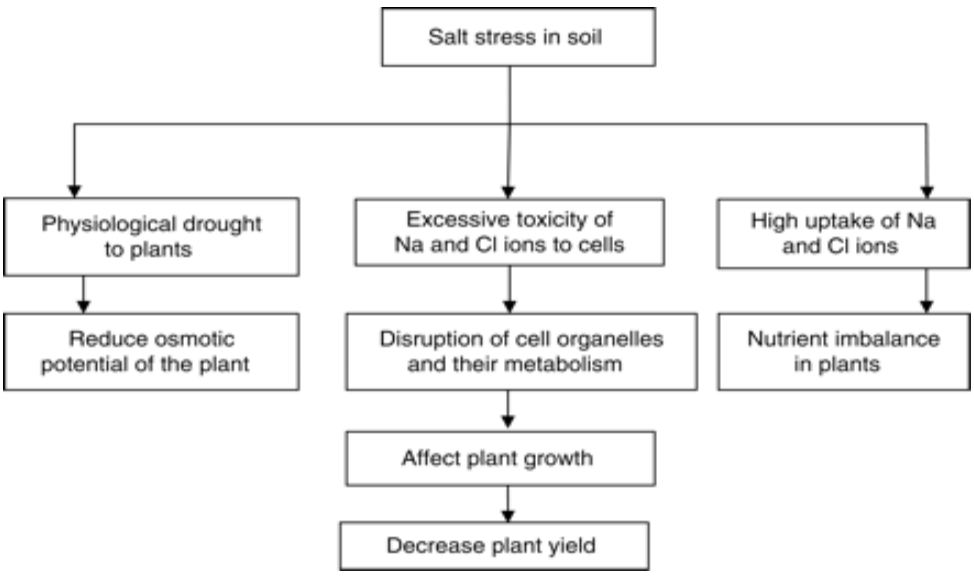


Fig. 5: Mechanism of causing adverse effect in plants by salt stress (source: de Oliveira, *et al.* 2013a).

Salinity of the soil is a global issue and Bangladesh is not an exclusion. The salinity of the soil is rising as a result of long-term irrigation use in agricultural systems, which has a negative impact on crop productivity. One of the main natural dangers limiting crop yield in Bangladesh is salt. Twenty percent of Bangladesh's land area is coastal, and of that, around 53 percent is affected by various salinity levels (Hossain *et al*. 2012). In Bangladesh, the coastal region contains more than 30% of the country's arable land. Diverse levels of salinity have an impact on around 1.0 million hectares of agricultural soils (Karim *et al*. 1990). Approximately 1.02 million ha (almost 70% of the cultivated lands) are impacted by varied degrees of soil salinity, and approximately 0.282, 0.297, 0.191, 0.450, and 0.087 million hectares of lands are affected by very slight, slight, moderate strong, and very strong salinity respectively (Haque *et al*. 2006). From the aforesaid discussion, it's quite obvious that peanut cultivation in Bangladesh faces severe difficulties because of salinity and drought.

Under these circumstances, development of salinity and drought tolerant peanut varieties is critical for the betterment of its production. From the ancient time, conventional breeding is one the approach to develop new peanut variety. Conventional breeding methods including hybridization techniques and selection are being carried out to develop improved varieties of many crops with high stable yield, better quality, wide adaptability towards biotic and abiotic stresses. Many attempts were made through conventional breeding to transfer fascinating traits in peanut cultivars within the past. Due to the lack of desired quantitative trait loci (QTLs) and levels of polymorphism in cultivated varieties, success in establishing abiotic stress tolerance in peanut by conventional breeding methods is limited (Rao *et al*. 2017). Furthermore, crossing in peanut is tedious because of small size of flowers. Genetic variation towards the improvement of any crop can also be obtained through introduction and gene mutation. Usually, conventional breeding takes more than seven to eight years because it involves crossing and selecting for specific features. In addition, inter-specific crosses of essential genes face obstacles (Daunay *et al*. 1991). But all attempts in making genetic variability in peanut through introduction, selection, hybridization and mutation did not yield the desired results. Since conventional breeding has several limitations, it is imperative that we have to look for some other methods to induce genetic variability in peanut.

Genetic variability of a species can be enriched through several other techniques besides conventional breeding. Somatic hybridization, tissue culture, and genetic engineering are a few of these methods for introducing somaclonal variation. According to Scowcroft *et al.* 1987, tissue culture technique can give rise to genetic variations at an unexpectedly high rate which can serve as a novel source of genetic variability in many plant species. Despite the fact that leguminous tissue *in vitro* is notoriously difficult to regenerate, numerous cases of effective peanut regeneration have been documented (McKently *et al*. 1991; Baker *et al*. 1992; Cheng *et al*. 1992; Ozias-Akinset *et al*. 1992; Pestana *et al*. 1999; Sarker *et al*. 2000). In spite of being important for achieving genetic diversity, tissue culture alone makes a little contribution to the development of stress tolerance and superior agronomic qualities in peanut (Mroginski *et al*. 1981; Narasimhulu *et al.* 1983; Atreya *et al*. 1984; Bhuiyan *et al*. 1992).

However, a replacement for traditional breeding programs employing recombinant DNA technology is genetic engineering, a tissue culture-based approach (Gardner *et al*. 1993). With success, genetic transformation is being utilized to introduce specific genetic information from any creature, regardless of its source (similar or distant plant, animal, even bacterial species). This approach thus ushers in a new era for the modification of elite varieties with desired features (tolerance to diseases and resistance to abiotic and biotic stresses), which is not attainable through breeding or tissue culture alone. Under these circumstances, genetic transformation can be successfully applied in crop improvement programs.

Since 1987, a large number of potentially beneficial transgenic plants have been created, including sugarcane, sugar beet, cotton, maize, potato, tobacco, rapeseed, raspberry, soybean, pea, tomato, rice, Hawaiian papaya, squash (Zucchini), etc. (Fisk *et al*. 1993). Biotech crop areas astonishingly rose from 1.7 million hectares in 1996 to 189.8 million in 2017, a growth of more than 100 times. 189.8 million hectares (469 million acres) were planted by up to 17 million farmers in 24 countries in 2017, an increase of 3% or 4.7 million hectares (11.6 million acres) from 2016. The top ten nations growing genetically modified crops are the United States (40 percent of the world's total areas, up 1% in 2016), Brazil (26 percent), Argentina (12 percent), Canada (7 percent), India (6 percent), Paraguay (2 percent), Pakistan (2 percent), China (1 percent), South Africa (1

percent), and Bolivia (1 percent). In 2017, a total of 14 new nations planted crops across about 3.7 million hectares (ISAAA, 2017).

Transformation of higher plants has been accomplished by completely different strategies (Grasser *et al*. 1989) for instance, direct strategies, like particle gun bombardment, microinjection, electroporation, *in planta* transformation methodology, chemical methodology and indirect methodology like, *Agrobacterium*-mediated genetic transformation methodology are used for transformation. The most economical methodology used for the transfer of desired foreign genes is thru the soil microorganism *Agrobacterium tumefaciens* in dicotyledonous species (Chandra *et al*. 2003; Rao *et al*. 2009; Grant *et al*. 1995).

Agrobacterium-mediated transformation, biolistic for DNA delivery, electroporation, and/or treatment with synthetic resin glycol (PEG) will be the main transformation events in grain legumes. Many different types of legumes have reportedly undergone stable transformation using the common indirect transformation technique with *Agrobacterium tumefaciens* or *A. rhizogenes*, as well as direct sequence transfer techniques like particle bombardment (Gulati *et al*. 2002; Masood *et al.* 1996; Oktem *et al*. 1999), electroporation of protoplasts (Christou *et al*. 1994; Atkins *et al.* 1979, *In planta* transformation technique (Sajib *et al*. 2008).

Crown gall development occurs naturally on a wide variety of plant species, including the majority of dicotyledonous and a few monocotyledonous species by the common soil bacterium *Agrobacterium tumefaciens* (Van Larebeke *et al*. 1974). Formation of gall is evoked by the microorganism which transfer of hormone-producing genes into plants. Crown gall development occurs naturally on a wide variety of plant species, including the majority of dicotyledonous and a few monocotyledonous species by the common soil bacterium *Agrobacterium tumefaciens* (Van Larebeke *et al.* 1974). Transfer of hormone-producing genes from the microorganism cell into the plant causes the gall to be elicited. A tumor-inducing (Ti) inclusion is a circular desoxyribonucleic acid molecule discovered among microorganism cells that carries onco-genes. Only a portion of the Ti inclusion known as the transfer desoxyribonucleic acid (T-DNA) is transferred to the plant during the infection process (Binns and Thomashow, 1988). The genetic modification of plants has been accelerated by using this *A. tumefaciens* sequence transfer approach, which has

undergone thorough study. The desired genes that are to be put into the plant are inserted into the T deoxyribonucleic acid region of those disarmed plasmids, which are absent from the artificially created *A. tumefaciens* Ti plasmids. Such *A. tumefaciens* cells can transmit the T-DNA sequence carrying the genes of interest into the plant cell where they stably integrate into the plant genome, but they are unable to produce a gall in an associated infected plant (Bevan *et al*. 1984; Klee *et al*. 1989). Specific reporter sequences, such as the GUS-A (β-glucuronidase) sequence and the GFP sequence, are integrated with the T-DNA in these transformation experiments along with the gene of interest. However, most monocotyledonous plants don't seem to be natural hosts of *A. tumefaciens. Agrobacterium's* role in the evolution of monocot transformation was problematic and unreliable (Sood *et al*. 2011). Several laboratories have studied plant transformation methods that do not include tissue regeneration or tissue culture and many plant species are rumored to be with success remodeled through *Agrobacterium* mediate in planta approaches. During this technique the entire plant will remodeled directly, *in vitro* regeneration isn't required, low cost, time saving technique, no somaclonal variation is anticipated (Bent *et al*. 2000).

The recalcitrant nature of legumes in culture has yielded less success in gene transformation (Nisbet and Webb 1990). However, in recent years, some transformation systems have been developed especially for legumes. These protocols are currently being improved for some of the sub-tropical legume crops (Sharma and Ortiz 2000). There are just a few transgenic legume plants that can be produced using *A. tumefaciens* as a gene vector: pea (Schroeder *et al*. 1993), lentil (Sarker *et al*. 2003), soybean (Zhang *et al*. 2016), alfalfa (Deak *et al*. 1986), mungbean, (Jaiwal *et al.* 2001) and chick pea (Tripathi *et al*. 2013).

Peanut has been thought of as a number of *A. tumefaciens* (Dong *et al*. 1990). The transformation and regeneration protocols for peanut area unit currently well established. Researchers are actively working to create transgenic peanut plants that can produce high-quality peanuts resistant to various diseases, insect pests, and abiotic stressors (Sharma *et al*. 2000; Rohini *et al*. 2001). Lacorte *et al*. (1991) demonstrated for the first time a successful integration of desired genes in peanut. However, the regeneration was unsuccessful from the remodeled tissues. The regulation of transformation potency of Peanut was rumored by Mansur *et al*. (1993). Although, Kumar *et al*. (1994) was able to

rework mature cataphyll and leaflet explants however did not regenerate plants from those tissues. Franklin and Jaya Kumar (1994) additionally got remodeled callus from hypocotyl tissues however they additionally did not regenerate plant from those tissues (Sharma *et al*. 1994) was thriving to regenerate shoots from the remodeled tissues of peanuts. Since then, several thriving genetic transformation protocols are rumored in peanut via *Agrobacterium*-mediated genetic transformation technique supported tissue culture (Li *et al*. 1997; Sharma *et al*. 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; Singh *et al*. 2014; Tiwari *et al*. 2015, Chen *et al*. 2015). By protecting embryo axes with *A. tumefaciens*, a non-tissue culture method called in planta transformation also produced modified peanut plants (McKently *et al*. 1995; Rohini *et al*. 2000; Manjulatha *et al.* 2014, Pandurangaiah *et al*. 2014). Remodeled peanut plants have additionally been developed victimization physical strategies like particle gun bombardment (Ozias-Akins *et al*. 1993; Asif *et al*. 2011). By using the electroporation approach, Padua *et al*. (2000) created transgenic plants from embryogenic explants of peanut leaflets. However, *Agrobacterium*-mediated genetic transformation is relatively a lot of wide accepted in peanut than different techniques and this method is tissue culture based mostly and takes less time to develop primary supposed transformants (Garladinne *et al*. 2016).

Plant genetic engineering has made it possible to create transgenic plants, which has the potential to cut down on agricultural losses. Increased crop productivity results from genetic engineering that confers resistance to several abiotic stress conditions (Mittler *et al*. (2004; Gill *et al*. (2014). Additionally, the function of DNA/RNA helicases in the tolerance to salt stress has come to light (Vashisht *et al*. 2006; Tuteja *et al*. 2013).

It is essential to use an integrative genomic and breeding strategy to identify organic process programs that increase grain quality and yield stability under unfavorable environmental conditions caused by abiotic challenges (Saeed *et al*. 2011).

In response to the stresses, plants experience a range of alterations in their transcriptome, proteome, and metabolome (Saeed *et al*. 2012). “Helicases” are characterized as major and effective players within the alleviation of multiple abiotic stresses (Pascuan *et al*. 2016). Salinity, dehydration, wounding and vasoconstrictive induce the expression of 1 of the potential stress responsive helicases, suggesting it to be a general issue concerned in

abiotic stress adaptation (Sanan-Mishra *et al*. 2005). According to Tuteja *et al*. (2004, 2012), ribonucleic acid helicases unfold the secondary structures in ribonucleic acid and are involved in transcription, cell organ biogenesis, and translation initiation. Helicases unwind duplex nucleic acids and are involved in a variety of significant processes, such as replication, repair, recombination, and transcription (Tuteja *et al*. 1996). DNA helicases are found to perform as molecular motor proteins in varied cellular mechanisms and as essential parts for nearly all desoxyribonucleic acid metabolic activities together with pre-mRNA splice in plants (Pham *et al*. 2000). The *PDH45* may be a member of the DEAD-box macromolecule family and contains all the celebrated canonical helicase motifs (Tuteja *et al*. 2012). Desoxyribonucleic acid helicase 45 (*PDH45*) was isolated from pea and shows structural similarities to eIF-4A. *PDH45* also has ATP-dependent DNA-dependent ATPase, ribonucleic acid helicase, and ATP-binding functions (Pham *et al.* 2000). Desoxyribonucleic acid helicases are constantly present, although little is known about their biological functions in plants and many other eukaryotic systems. In order to gain a deeper understanding of desoxyribonucleic acid transactions in plants, it may be helpful to do a close molecular analysis of desoxyribonucleic acid helicase. According to Pham *et al*. (2000) the major plant desoxyribonucleic acid helicase sequence, which encodes the biochemically active helicase macromolecule, is based on biological study. In bacteria, the helicase was overexpressed and refined into a 45.5 kDa macromolecule. *PDH45*, which stands for "pea desoxyribonucleic acid helicase forty-five (45) kDa in size," is the name given to this helicase. Many organs of the pea express the *PDH45* sequence. The cytoplasm and nucleus are where the accelerator is located. It increases the activity of topo-I, a topoisomerase found in peas (Pham *et al*. 2000). According to research, *PDH45* was upregulated in response to abiotic stressors (Sanan-Mishra *et al.* 2005). Its ability to increase stress tolerance in crop plants is demonstrated by the hyperbolic tolerance to salt it provides to tobacco plants. Overexpression of *PDH45* causes salinity and drought tolerance while not moving the yield of the plant.

As Bangladesh is losing its cultivated space due to salinity and drought the quantity of total productive space is decreasing day by day. During this regard, the event of salinity and drought tolerant native peanut cultivars may be an answer to the present stress-related cultivation and yield loss. As one of Bangladesh's main oil-producing crops, peanut production has decreased owing to biotic and abiotic conditions, and conventional breeding is time-consuming and has several drawbacks. As a result, modern

biotechnology has been utilized to boost peanut productivity. Considering Bangladesh's current condition, where 30% of the country's net cultivable land is located along the shore. The significance of peanuts in our agricultural scheme has already been discussed. In the Char regions, where poor marginal farmers have long resided, peanut farming is one of their primary sources of income. This crop may also be grown in Bangladesh's wide coastal regions, where it may provide a reliable source of income for the poor farmers. Unfortunately, at present there is no variety of peanut which can tolerate salt and drought stress.

As salinity and drought is a problem for better peanut production in Bangladesh. Due to climate change this problem is going to continue in Bangladesh. Peanut is a marginal crop, but it can be grown with little input. If the abiotic stress tolerance genes/characters be incorporated in local varieties of peanut, stress tolerance peanut can be cultivated in drought and salinity prone areas of Bangladesh. As a result, it is possible to increase peanut yield and include marginal lands in peanut farming.

The *PDH45* gene is used as a stress tolerance gene because overexpression of *PDH45* in plants provides more tolerant to drought and salinity. This gene can be used to give local peanut cultivars a trait that allows them to withstand abiotic stress.

Reviewing all these facts, the current investigation was conducted with the aim to develop an appropriate protocol for *Agrobacterium*-mediated genetic transformation of native peanut variety particularly, BARI Badam-8 to integrate the *PDH45* gene. Before that, an appropriate and reproducible *in vitro* regeneration system was developed to facilitated genetic transformation in peanut.

Therefore, the objectives of the present investigation are mentioned below:

1. Establishment of an efficient and reproducible regeneration system for the two important varieties of peanut, namely Dhaka-1 and BARI Badam-8 cultivated in Bangladesh.
2. Effect of salt stress on seed germination of local peanut varieties and evaluation of their growth performance at seedlings stage.
3. Development of an efficient *Agrobacterium*-mediated genetic transformation protocol using marker gene/s like *GUS* (β-glucuronidase) and *nptII* (neomycin phospho-transferase).
4. Development of a suitable *Agrobacterium*-mediated genetic transformation protocol for BARI Badam-8 peanut variety using drought and salinity tolerant *PDH45* gene.
5. Evaluation of putatively transformed peanut plants with the help of molecular techniques.

2. MARETRIALS

2. MATERIALS

2.1 Plant materials

Following two locally grown varieties of peanut (*Arachis hypogaea* L.) were used for the present investigation:

(i) Dhaka-1

(ii) BARI Badam-8

2.1.1 Source

Seeds of Dhaka-1 and BARI Badam-8 were collected from Oil Seed Division of Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur. Dhaka-1 variety was developed by Oilseed Research Centre, BARI in 1976 and BARI Badam-8 variety was developed by Oilseed Research Centre, BARI in 2008. The materials were maintained in the Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Dhaka. Fig. 6. represents the pods and seeds of peanut varieties used in the present investigation.

2.1.2 Description of the peanut varieties

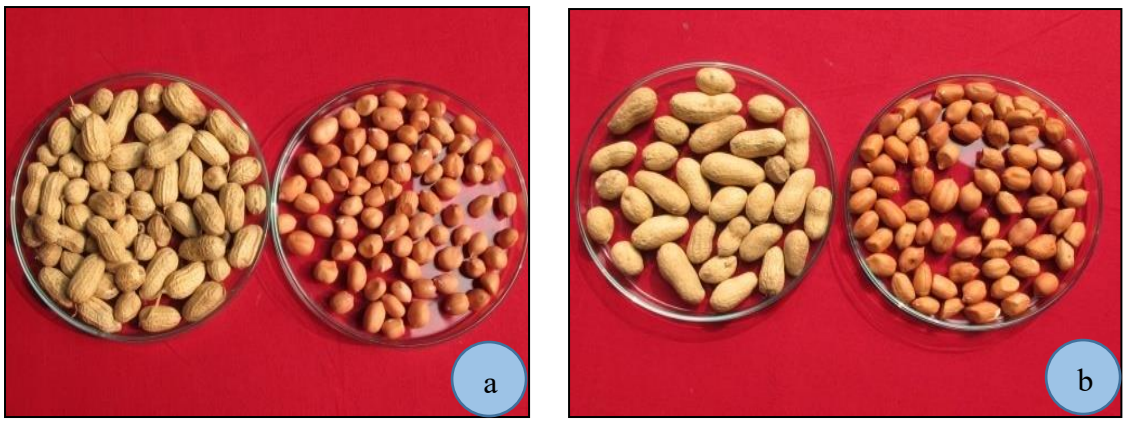
Important characteristics of these varieties used in the present investigation as are described below:

(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 Badam-8: This variety; 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 pods, seeds are large in size, non-dormant, shell of the nuts is smooth and soft, testa reddish in colour; life cycle is 140-150 days in 'Rabi' season and 125-140 days in Kharif' season, 100 seeds-weight is about 55-60 g, shelling percentage is 65 - 70%; highly susceptible to Cercospora; average yield is 2.5 tons per hectare.

2.1.3 Explants

Three different types of explants, namely, immature cotyledonary leaflet (CL), single cotyledon attached decapitated embryo (SCADE) and de-embryonated half cotyledon (DEHC), were used in the present investigation. Various types of explants have been presented in Fig. 7. Immature cotyledonary 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 as well as for *Agrobacterium*-mediated genetic transformation.



Dhaka-1 **BARI Badam-8**

Fig. 6 (a-b): Pods and seeds of two peanut varieties (Dhaka-1 and BARI Badam-8) used in the present study. (a) Pods and seeds of Dhaka-1; (b) Pods and seeds of BARI Badam-8.

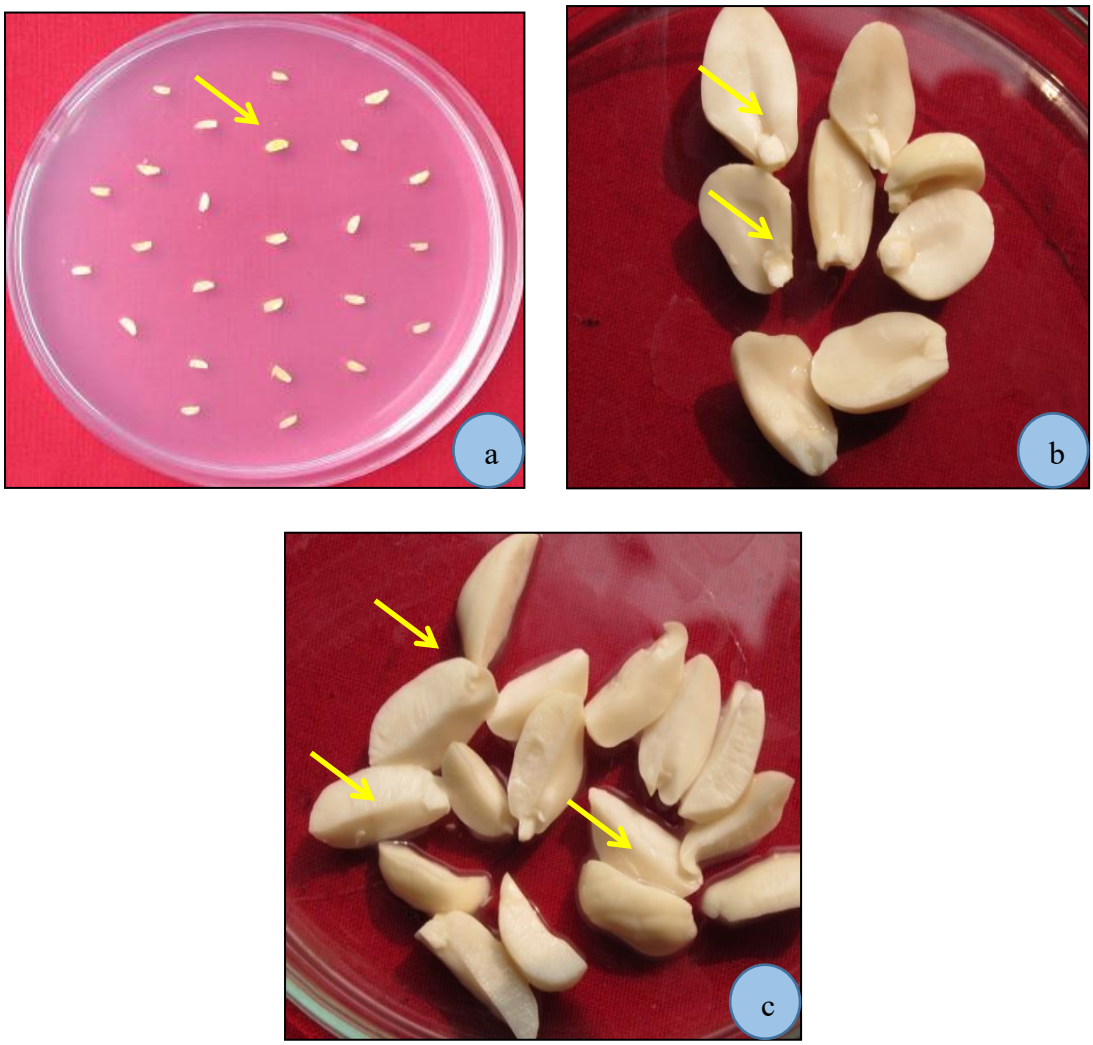


Fig. 7 (a-c): Different types of explants used in the present study. (a) Cotyledonary leaflet; (b) Single cotyledon attached (arrow) decapitated embryo (c) De-embryonated half cotyledon (arrow)

2.2 *Agrobacterium* strain and vector plasmids:

2.2.1 Strain I: LBA4404 (pBI121)

Agrobacterium tumefaciens strain LBA4404 contains plasmid pBI121 of 14 KDa (binary vector). This binary vector contains following genes within the right border (RB) and left border (LB) region of the gene construct (Fig. 8a):

(i) The *udiA* 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.

(ii) The *nptII* gene (Herrera-Estrella *et al*. 1983) encoding neomycin phosphotransferase II conferring kanamycin resistance, driven by 35S promoter and NOS terminator.

(iii) The bacterium also contains plasmid pAL4404 which is a disarmed Ti plasmid (132 KDa) containing the virulence genes.

2.2.2 Strain II: EHA105 (pCAMBIA1301PDH45)

Genetically engineered *Agrobacterium tumefaciens* strain was used for transformation experiments which is mentioned below with its relevant characteristics. Genetically engineered *Agrobacterium tumefaciens* strain EHA105 containing plasmid pCAMBIA1301 was used for transformation experiments. This construct was kindly provided by Dr. Narendra Tuteja, Plant Transformation Group, ICGEB. This vector contains following genes within the right border (RB) and left border (LB) region of the construct (Fig. 8b):

(i) The *uidA* 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.

(ii) The *hptII* gene (Waldron *et al.* 1985; Vanden Elzen *et al*. 1985) encoding hygromycin phosphotransferase conferring hygromycin resistance, driven by CaMV 35S promoter and NOS terminator.

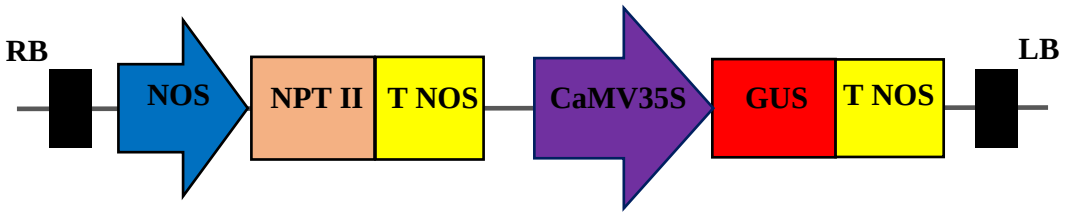


Fig. 8: Diagrammatic representation of gene construct of *Agrobacterium tumifaciens;* (a) T-DNA region between left (LB) and right (RB) border of *Agrobacterium tumifaciens* strain LBA4404 containing pBI121GUS-NPTII construct;

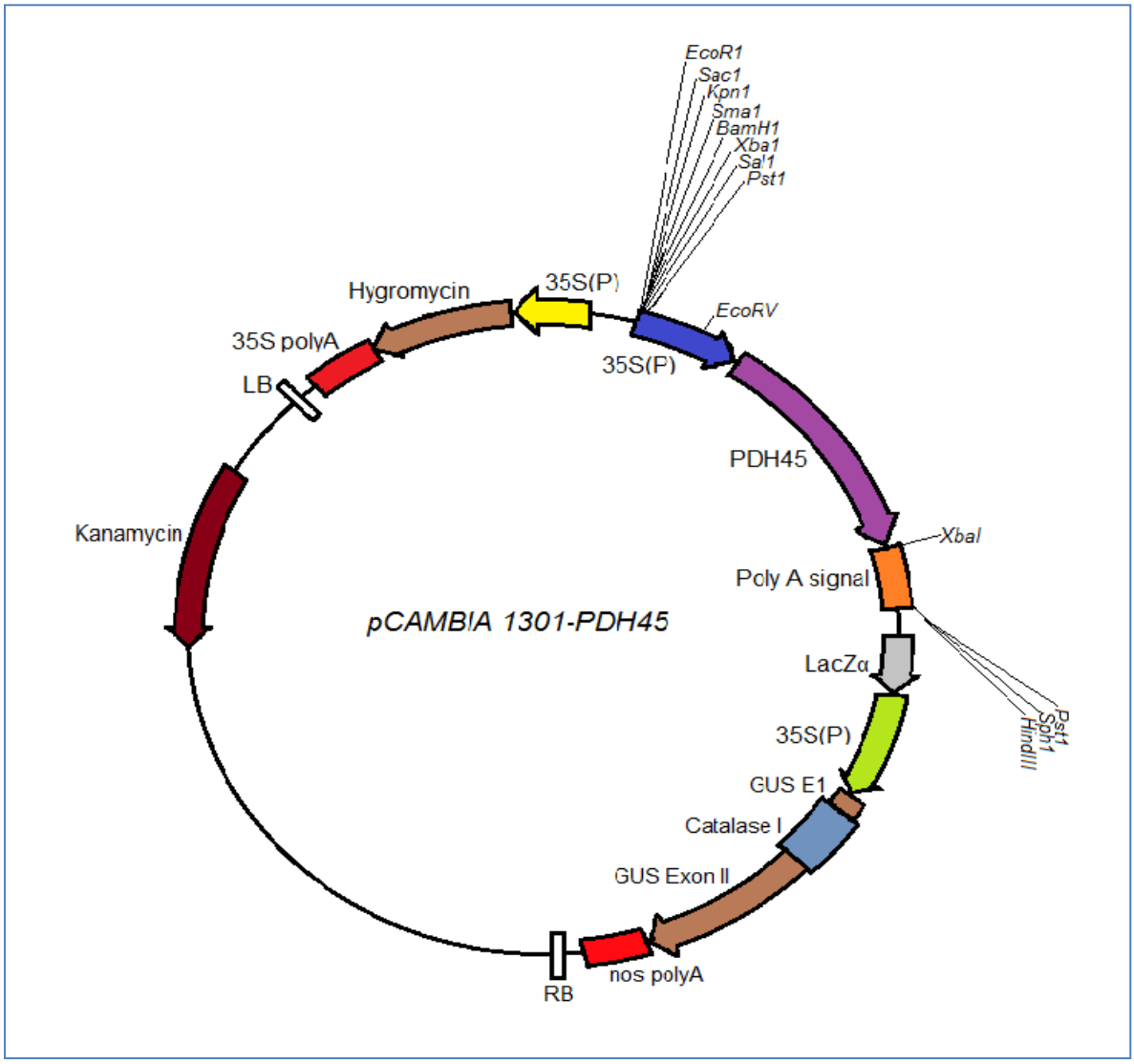


Fig. 8 (b): Diagrammatic representation of the plasmid pCAMBIA1301PDH45 of *Agrobacterium tumefaciens* strain EHA105.

2.3 Chemicals used for various purposes

2.3.1 Component of culture medium

Substance	Molecular weight	Source
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	Source	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

2.3.3 Plant hormones and additives

Substance	Molecular weight (MW)	Source	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
GA3	346.37	DUCHEFA, Netherlands	1[N] NaOH
Acetosyringone	196.2	ROTH, Germany	DMSO

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	Source
Gene Ruler^{TM} 100 bp DNA ladder	0.5 mg/ml	MBI Fermentas
Gene Ruler^{TM} 1 kbp DNA ladder	0.5 mg/ml	MBI Fermentas

2.3.6 Solvent, sterilizers and others

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

2.3.7 Primers

Primer	Sequence (5'-3')	Product	Source
GUS	CCT GTA GAA ACC CCA ACC CG TGG CTG TGA CGC ACA GTT CA	750bp	Macrogen, South Korea
SFKan-F SFKan -R	GAA AAA CTC ATC GAG CAT CA TTG TCC TTT TAA CAG CGA TC	700bp	Macrogen, South Korea
iii) Primer *PDH45* gene		1250bp	Invitrogen USA
PDH45-F 5'-ATGGCGACAACTTCTGTGG-3'			
PDH45-R 5'-GAGTCTAGATTATATAAGATCACCAATATC-3'			
iv) Primer *hptII* gene		750bp	Invitrogen USA
hptII-F 5'- CGAAGAATCTCGTGCTTTCAGC-3'			
hptII-R 5'- AGCATATACGCCCGGAGTCG-3'			

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 + RNAse A

10 mM Tris-HCl, pH 8.0

1 mM EDTA

10 µg/ml RNAse A.

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. l00 mM Tris-HCl (pH-8.3 at room temperature).

iii. 0.1% Gelatin.

b) 25 mM MgCl in water for PCR.

c) l0 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

During the present investigation, experiments were conducted to develop an efficient protocol for *Agrobacterum* mediated genetic transformation in peanut (*Arachis hypogaea* L.). The transformation protocol was established using marker gene constructs, further the transformation experiments were conducted with an aim to integrate drought and salinity tolerant gene in local varieties of peanut. Moreover, *in vitro* regeneration system was developed for the peanut varieties using different explants to facilitated *Agrobacterum* mediated genetic transformation. The different procedures used in the present study have been described under the following heads:

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

Different stock solutions were prepared for the preparation of medium used in different steps of plant regeneration and selection of putatively transformants. The stock solutions of various constituents of the medium were prepared for ready use during the preparation of various medium.

3.1.1 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.

3.1.1.1 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.

3.1.1.2 Stock solution B (Micro nutrients) for MS medium

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

(i) 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.

(ii) 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.

3.1.1.3 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.

3.1.1.4 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 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) Gibberellic 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 hormone 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.

3.2.1 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) Cefotaxime (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.

3.2.2 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 1 litre 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 solutions 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.

3.2.3 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.

3.2.4 Media for *in vitro* 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 (Gibberellic acid) were used during the present experiment.

3.2.5 Media for *in vitro* 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.

3.2.6 Preparation of *Agrobacterium* culture medium

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

3.2.6.1 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.

3.2.6.2 YEP medium was prepared in the following manner

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

All of these ingredients of the medium were added in 250 ml distilled water and mixed properly. Then the final volume was made up to 500 ml by adding distilled water. The pH was adjusted to 7.0 - 7.2.

For the preparation of bacterial maintenance medium, 7.5 g agar was added to the medium before autoclaving. Filter sterilized antibiotic kanamycin (100 mg/l), streptomycin (50 mg/l) and rifampicin (25 mg/l) were added 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. The plates were stored at 4 ˚C for further use.

3.2.7 Preparation of bacterial suspension media

1.0 litre medium for suspending *Agrobacterium tumefaciens* for transformation was prepared using 4.405 g MS powder and 90 g sugar without any hormonal supplement. pH of the medium was adjusted to 5.8 and then autoclaved.

3.2.8 Preparation of Co-culture media

Co-culture medium for co-cultivation (infection of peanut explants together with *Agrobacterium*), was prepared using MSB5 powder containing 100 µg/l acetosyringone (ROTH, Germany) without any hormonal supplement **.**

3.2.9 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 aluminum 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.

3.2.10 Preparation of selection medium for putatively transformed plantlets

For the selection of transformed tissues/plantlets antibiotic, namely, hygromycin was used, as the *Agrobacterium* strains used in the present investigation contained *hptII* (hygromycin resistance) 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 air flow cabinet. Medium was then poured into suitable culture vial and allowed to solidify. The antibiotic such as carbenicillin was also used to control the overgrowth of bacteria.

3.2.11 Seed germination

Surface sterilized seeds were soaked overnight in sterilized distilled water to be used as a source of de-embryonated half cotyledon (DEHC) and single cotyledon attached embryo (SCADE) 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.

3.3 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, Microcentrifuge 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.

3.3.1 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

(i) 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.

(ii) Explants culture (Inoculation)

Immature leaflet (IL), de-embryonated half cotyledon (DEHC) and single cotyledon attached decapitated embryo (SCADE) 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 half 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/MSB media supplemented with

different concentrations and combinations of BAP, Kn, 2,4-D, NAA, for *in vitro* regeneration of shoots.

(iii) 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.

(iv) 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.

(v) 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.

3.3.2 Transformation

3.3.2.1 Preparation of *Agrobacterium* culture for transformation

Glycerol stocks (-86ºC) of *Agrobacterium tumefaciens* EHA105/pCAMBIA1301-PDH45 were used to prepare bacterial plates (YEP plates) by streak method. The plates were incubated at 28ºC for 48 hours in a dark chamber and then the bacterial growth was observed. After achieving suitable growth, another fresh YEP plate was streaked with bacterial cells from the plates. The plate was incubated in the same conditions as mentioned above and then used for transformation.

3.3.2.2 Suspension of bacterial cells in the suspension medium

After observing proper growth in step 3.11.1, bacterial cells were suspended in 25 ml of bacterial suspension medium (described in 3.5.3). Then optical density (O.D.) of this suspension was determined at 600 nm with the help of a spectrophotometer. Bacterial suspension of O.D. 0.5 was preferred for transformation. This bacterial suspension was prepared before 30 minutes of the infection process. 200 µg/l acetosyringone was added to the suspension before infection.

3.3.2.3 Infection of explants with bacterial cells

The explants were dipped in the bacterial suspension and incubated at 28 ºC for 20 minutes in the shaking incubator at 180 rpm.

3.3.2.4 Co-culture

The explants were co-cultured on regeneration medium after infection and incubation. The explants were soaked in filter papers for a short period of time to remove excess bacterial suspension prior to the transfer of all explants to co-cultivation media. All the explants were maintained on co-culture medium for 48 hours at 25±2 ºC in the dark.

3.3.2.5 Culture of *Agrobacterium*-infected explants

Co-cultured explants were washed with distilled water for 3-4 times until no opaque suspension was seen. They were then washed for 10 minutes with distilled water containing 300 mg/l carbenicillin. After that, the explants were gently soaked with a sterile filter paper and transferred to regeneration medium with 300 mg/l carbenicillin. After 10-15 days, all the regenerated shoots were sub-cultured on selection medium containing 20 mg/l hygromycin and 300 mg/l carbenicillin. Sub-culture of regenerated shoots was done regularly at an interval of 15-18 days on selection medium. Shoots survived on regeneration medium were transferred to root induction medium for root formation.

3.4 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.

3.4.1 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.

3.4.2 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.

3.5 Plant genomic DNA isolation from putatively transformed plantlets

3.5.1 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.

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

The following chemicals were used for plant genomic DNA isolation.

(i) 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.

(ii) 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.

(iv) 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.

(v) β-Mercaptoethanol

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

(vi) Ribonuclease A stock solution

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

(vii) 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.

(viii) 70% Ethanol (100 ml)

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

(ix) 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 4º C.

(x) 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.

3.5.3 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.

3.5.4 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.

3.5.5 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.

3.5.5.1 Measurement of DNA concentration and quality by Agarose Gel Electrophoresis

3.5.5.2 Preparation of stock solutions used for Gel Electrophoresis

3.5.5.3 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.

3.5.5.4 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 cyanole 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.

3.5.5.5 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.

3.6 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 electrophoresis was continued until DNA fragments were separated well.

3.7 Polymerase Chain Reaction

3.7.1 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 Master cycler gradient).

3.7.2 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 *PDH45* gene

Step	Temperature	Time	No. of Cycle
Initial denaturation	95 °C	5 min	1 (first)
Denaturation	95 °C	1min	35 Cycles
Annealing	54 °C	30 sec	
Elongation	72 °C	1.30 min	
Final elongation	72 °C	5-10 min	1 (last)

The PCR condition for *hptll* gene

Step	Temperature	Time	No. of Cycle
Initial denaturation	95 °C	5 min	1 (first)
Denaturation	95 °C	1min	35 Cycles
Annealing	56 °C	30 sec	
Elongation	72 °C	1.30 min	
Final elongation	72 °C	5-10 min	1 (last)

he PCR condition for *GUS* and *nptII* gene

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

3.7.3 Analysis of PCR product

3.7.3.1 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).

3.7.3.2 Transplantation and acclimatization of plantlets

The transgenic shoots were rooted in root induction medium as well as transplanted into plastic pots having sterilized soil. The roots were caringly washed to remove agar attached with the roots. Then the plastic pots were covered with transparent porous polythene bags. Inner side of these bags was moistened with water to prevent desiccation. The plants were acclimatized in the culture room for 1 to 2 weeks and subsequently maintained in net house for biosafety regelation until flower and pod formation.

4. RESULTS

4. RESULTS

The objective of the present study was to establish an effective *in vitro* regeneration system for two varieties of peanut (*Arachis hypogaea* L.), namely, Dhaka-1 and BARI Badam-8, as well as to develop a reliable protocol for *Agrobacterium*-mediated genetic transformation of BARI Badam-8 variety with the ultimate goal of transferring drought and salinity tolerance genes into this plant. Further the aim of this study was to develop peanut lines that could be utilized in the future breeding program in developing drought and salinity tolerance in peanut.

The entire study was divided into three phages. In the initial phase, the effect of various concentrations of salt (NaCl) on germination of seeds as well as development of seedlings of peanut varieties was determined. Through this part of the study the performance of peanut varieties was examined against salt stress. In the second phase, an *in vitro* regeneration protocol was optimized through experiments using three different types explants, such as cotyledonary leaflet, single cotyledon attached decapitated embryo (SCADE) and de-embryonated half cotyledon (DEHC) explants. In the third phase, a series of experiments were conducted to develop a protocol for *Agrobacterium*-mediated genetic transformation using two different genetically engineered gene constructs. For this investigation the *Agrobacterium* strain LBA4404 harboring plasmid pBI121GUS-NPTII (considered as construct I) also the strain EHA105 harboring plasmid pCAMBIA1301PDH45 (considered as construct II) were utilized.

Construct I (pBI121GUS-NPTII) was mainly used for the development of transformation protocol since it contained two marker genes namely, *GUS* (β-glucuronidase) as screenable marker and *nptII* (*neomycin phosphotransferase*) as selectable marker gene. On the other hand, strain EHA105 with plasmid pCAMBIA1301PDH45 (construct II) contained two genes conferring drought and salinity tolerant gene *PDH45* (pea DNA helicase, considered as a gene of interest) and *hptII* gene as selectable marker for the transformation of peanut. Construct pCAMBIA1301PDH45 was used for the integration of drought and salinity tolerant gene into peanut genome with an aim to develop drought and salinity tolerant peanut lines. Additional experiments were conducted using construct I to optimize the various parameters required for *Agrobacterium*-mediated genetic transformation, such as the required optical density of the bacterial suspension, the

incubation period, the co-cultivation period, as well as the optimization of the concentration of the selectable agent, etc. Experiments were also performed to regenerate plantlets from *Agrobacterium* infected explants as well as for the selection of transformed plantlets. Moreover, molecular characterization of putatively transformed plantlets was carried out to examine the integration of transgenes. The results of the present study obtained from different experiments are presented in the following sections.

4.1 Evaluation of salt tolerance

The ability of plants to grow in soil with variable concentration of salt varies from species to species and is influenced by the salt content of the root zone. The most effective criterion for choosing salt tolerance are seedling and germination traits. The most effective criterion for choosing salt tolerance are seedling and germination traits. In this present investigation salt tolerance ability of the local varieties of peanut was examined using various concentrations of salt solutions.

4.1.1 Effect on seed Germination Rate under Salt Stress Conditions

The establishment of plants growing in saline soils depends on their ability to tolerate salt during germination, which is a pivotal step in the life history of each individual plant. In the present study, the seeds of two local peanut varieties, such as Dhaka-1 and BARI Badam-8 were allowed to germinate in cotton beds containing 0, 50, 100, 200 and 300 mM of NaCl solution. The days required for germination and the length of radicle after 7 days of their incubation were observed.

4.1.1.1 Effect of various NaCl concentrations on seed germination in Dhaka-1 Surface sterilized seeds of Dhaka-1 variety were allowed to germinate in sterile cotton beds containing 0, 50, 100, 200 and 300 mM of NaCl solution to observe the effects of salinity on seed germination. In this case, salinity had a major impact on germination properties. Table 1 exhibits the findings of this experiment. These observations demonstrated that the germination rate was gradually declined with rising saline levels. Besides that, the number of days needed to germinate increased gradually as the saline level rose. In case of radicle length, the effect was also significant, as the length of radicle was decreased gradually with increasing levels of salinity Fig. 9 (a- b).

Table 1. Effect of various concentrations of NaCl on germination of seeds in case of Dhaka-1.

NaCl concentration (mM)	No. of seeds inoculated	No. of seeds germinated	% of germination	Days required for germination	Mean length of radicle after 7 days (cm)
0 (control)	20	19	95	2 – 3	3.0
50	20	18	90	3 – 4	2.2
100	20	16	80	3– 5	1.5
200	20	10	50	4– 6	0.5
300	20	0	0	-	-

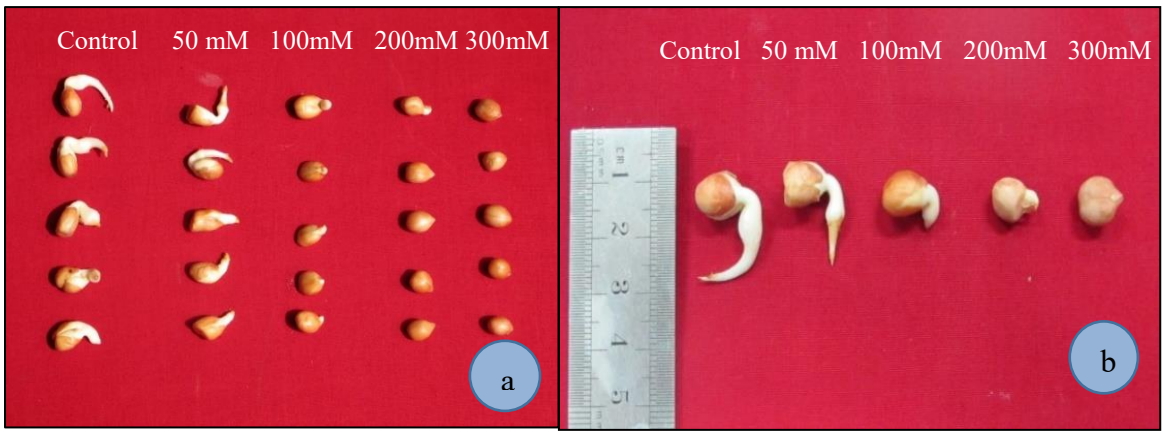


Fig. 9 (a-b): (a) Germination of seeds of Dhaka-1 on different concentrations of NaCl solutions and without any salt stress (control); (b) Gradual decrease of radicle length of seeds of Dhaka-1 on different concentrations of NaCl solutions.

4.1.1.2 Effect of various concentrations of NaCl on seed germination of BARI Badam-8

Surface sterilized seeds were allowed to germinate in sterile cotton beds same way as mentioned above, containing 0, 50, 100, 200 and 300 mM of NaCl solution to observe the effect of salinity on seed germination. The effects were almost identical in case to Dhaka-1 variety. The results of the experiment are shown on Table-2. From the results it was observed that, in this case also, the seed germination rate, length of radicle was decreased by the increase of salt concentration. Fig. 10 (a-b) are presented the results of this experiment. It was observed that more days were required for the germination of seeds when kept in increased concentration of NaCl.

Table 2. Effect of different concentrations of NaCl on seed germination of BARI Badam-8.

NaCl concentration (mM)	No. of seeds inoculated	No. of seeds germinated	% of germination	Days required for germination	Mean length of radicle after 7 days (cm)
0 (control)	20	20	100	2 – 3	3.5
50	20	18	90	3 – 5	2.0
100	20	14	70	4 – 6	0.5
200	20	0	0	0	0
300	20	0	0	0	0

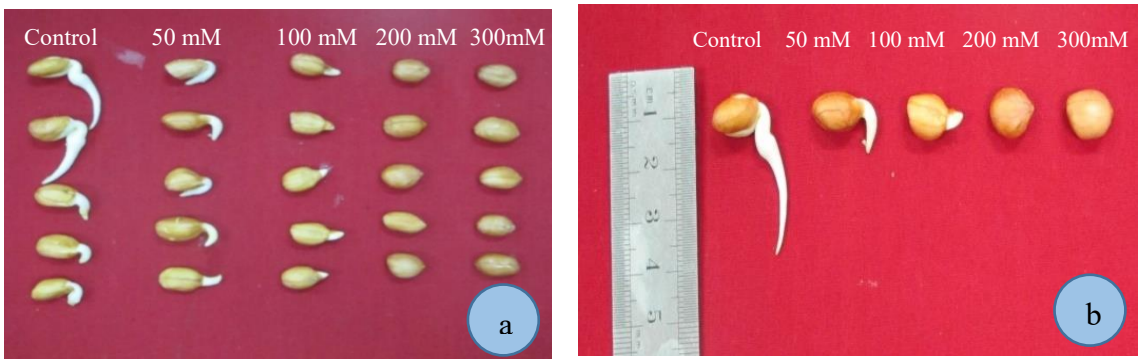


Fig. 10 (a): Germination of seeds of BARI Badam-8 on different concentrations of NaCl solutions and without any salt stress (control); (b) Gradual decrease of radicle length of seeds of BARI Badam-8 on different concentrations of NaCl solutions.

4.1.2 Effect of salt stress in growth and development of seedlings

8-day-old seedlings were cultured on Murashige and Skoog (1962) media supplemented with 50, 100, 200, or 300 mM NaCl and MS medium alone (experimental control) under continuous white light at 25 2°C at a 16-h photoperiod in order to observe the effects of salt stress on seedlings. The impacts of salt stress on seedlings were recorded for all experiments.

4.1.2.1 Salt stress effect in growth and development of seedlings of Dhaka-1 and BARI Badam-8 varieties in different NaCl concentrations

8-day-old seedlings were transferred to MS media supplemented with 50, 100, 200 and 300 mM NaCl, as well as to MS medium alone (experimental control) to examine the effects of salt stress. The number of seedlings that tolerated the stresses was recorded. Additionally, the plantlets that obtained from each treatment were used to measure the length of roots and shoots individually. The effects of various NaCl concentrations on seedling growth and development are depicted in Figs. 11 and 12, and the results due to these observations are presented in Table 3.

Table 3. Effects of various concentrations of NaCl on growth and development of seedlings of Dhaka-1 and BARI Badam-8 varieties.

Concentration of NaCl solution (mM)	Dhaka-1				BARI Badam-8			
	Root length (cm)	No. of roots	Shoot length (cm)	No. of leaves	Root length (cm)	No. of roots	Shoot length (cm)	No. of leaves
0	6.4	55	6.0	28	6.2	50	5.2	26
50	4.5	40	5.5	16	4.2	35	4.8	16
100	4.0	35	2.5	12	3.5	1	2.5	10
200	0.8	1	0.6	-	1.5	1	2.5	8
300	0.4	1	-	-	0.4	-	-	-

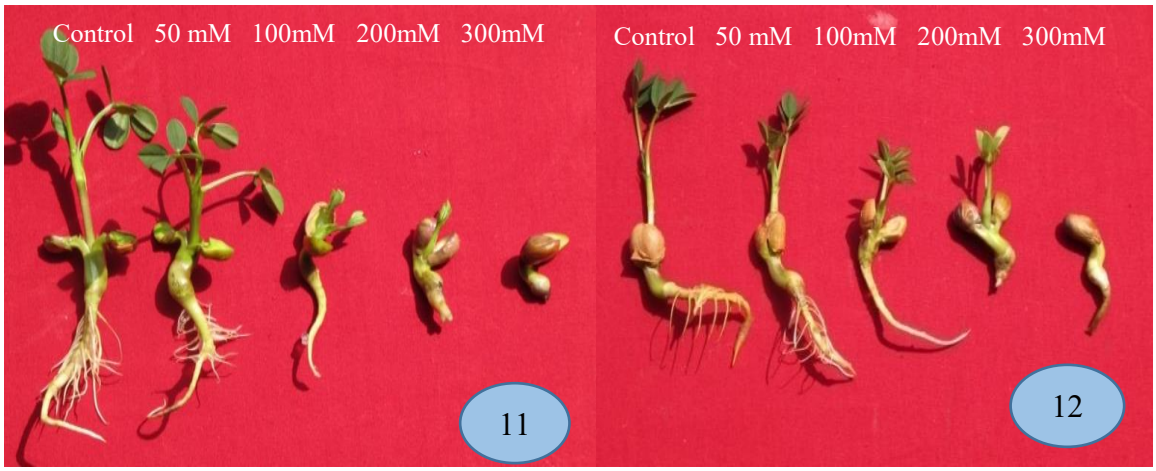


Fig. 11 and 12: Seedlings cultured on MS medium supplemented with different concentrations of NaCl solution and without salt supplementation (control) in case of Dhaka-1(Fig. 11) and BARI Badam-8 (Fig. 12) varieties.

4.2 *In vitro* regeneration

A successful plant genetic transformation requires a compatible *in vitro* regeneration system. The age of the tissue or organ employed as the initial explant has a significant impact on the effectiveness of *in vitro* regeneration. Explants from juvenile plant tissues, especially those from seedlings are very sensitive. In order to develop an *in vitro* regeneration system that was compatible with transformation, the cotyledonary leaflet, single cotyledon attached decapitated embryo (SCADE), and de-embryonated half cotyledon (DEHC) explants were used in this investigation.

Autoclaved cotton soaked with sterile distilled water was used for seed germination (Table 4) and their consequent development for seedlings. Germinated seedlings were used as source of leaflet explants. Apart from the leaflet explants, the other two explants (decapitated embryo (SCADE) and de-embryonated half cotyledon (DEHC) were collected from over-night soaked or four hours-soaked seeds before use. The number of shoots per explant, the number of responsive explants, and the number of days needed for the initiation of shoots from explants were recorded for each of the experiment. *In vitro* raised excised shoots were used for *in vitro* root development. After suitable hardening, rooted plantlets were acclimated in soil and allowed to develop under field environments.

4.1.1 *In vitro* regeneration of shoots

For *in vitro* regeneration of shoots on MS media supplemented with various quantities and combinations of auxins and cytokinins were used. For this purpose, different types of explants from two varieties of peanut, namely Dhaka-1 and BARI Badam-8, were utilized. The results of different experiments for this part of investigation are described in the following sections.

4.1.1.1 Sterilization of seeds surface used for *in vitro* germination

0.1 per cent $HgCl_2$ solution and 70% alcohol were used to surface sterilize seeds while maintaining an aseptic environment to prevent unintended contamination of explants. Healthy seeds were dipped into 70% alcohol for one minute and then sterilized with 0.1% HgCl2 solution for 8 - 10 minutes. The seeds were then four to five times rinsed in sterile distilled water to get rid of any $HgCl_2$ residue. From aseptically germinated seeds, leaflet explants for in vitro regeneration were obtained. Autoclaved cotton soaked with sterile distilled water was used for seed germination and their consequent development for

seedling. Germinated leaflet explants were obtained from seedlings. Under these circumstances, seed germination took 2 to 4 days. (Table 4). Leaflet explants were collected from 6 - 8 days old seedlings (Fig. 13). Single cotyledon attached decapitated embryo (SCADE) explants and de-embryonated half cotyledon (DEHC) explants were prepared from the overnight soaked or four hours-soaked seeds before use. These were then used for *in vitro* regeneration and transformation experiments for Dhaka-1 and BARI Badam-8 varieties.

Table 4. 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	48	96	2 – 3
Bari Badam-8	50	47	94	3 – 4

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

It was found that the age of immature cotyledonary leaflet explant had a significant impact on the *in vitro* regeneration of shoots. In case of regeneration of shoots from leaflet explants, it was found that in most of the cases shoot regeneration was seriously influenced by the age of leaflet. Both the varieties of peanut seeds took 2 - 4 days for germination (Table 4). It was found that leaflet explants collected from early germinating seeds (4 - 5 days) was not suitable for initiation of shoots (Fig. 13). 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. 14a). But in case of BARI Badam-8, it took 8 days to collect the most responsive leaflet explants from the germinated seeds (Fig. 14a). 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. 14 (a- c).

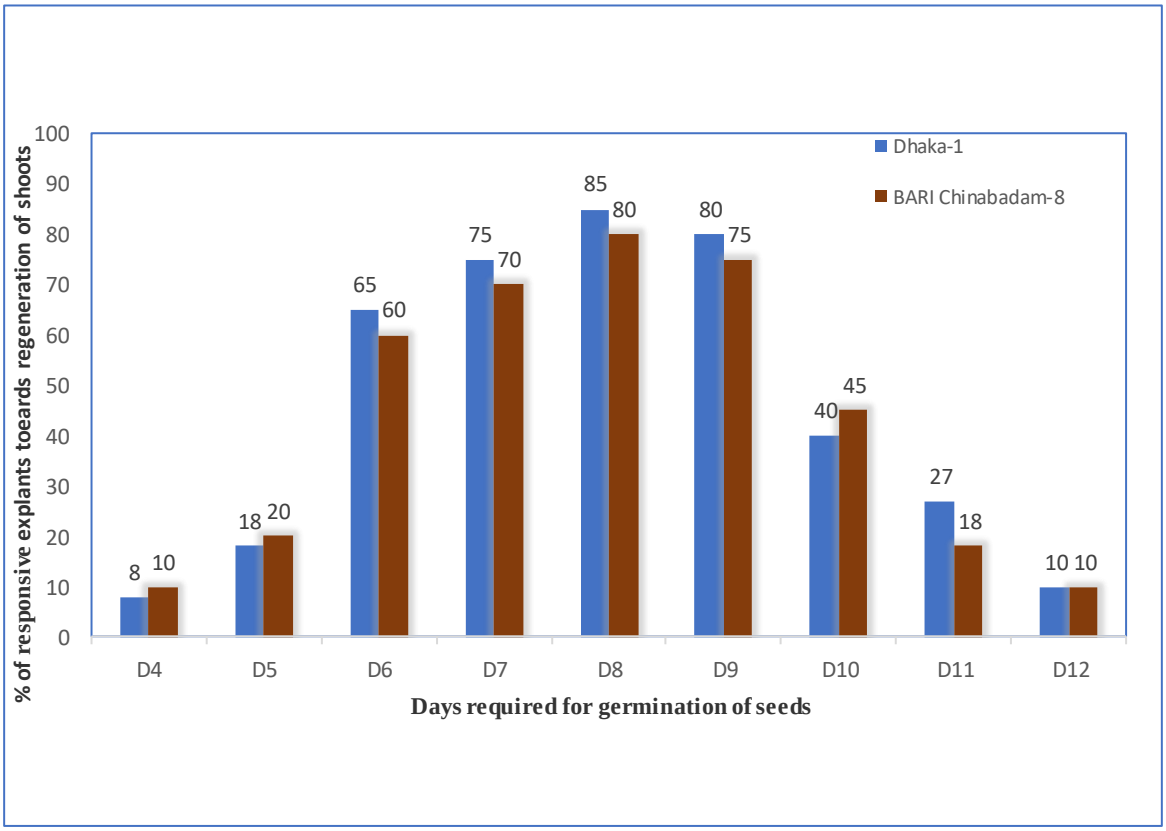


Fig. 13: Effect of germination period of peanut seeds in collecting the immature leaflet explants towards regeneration of shoots.

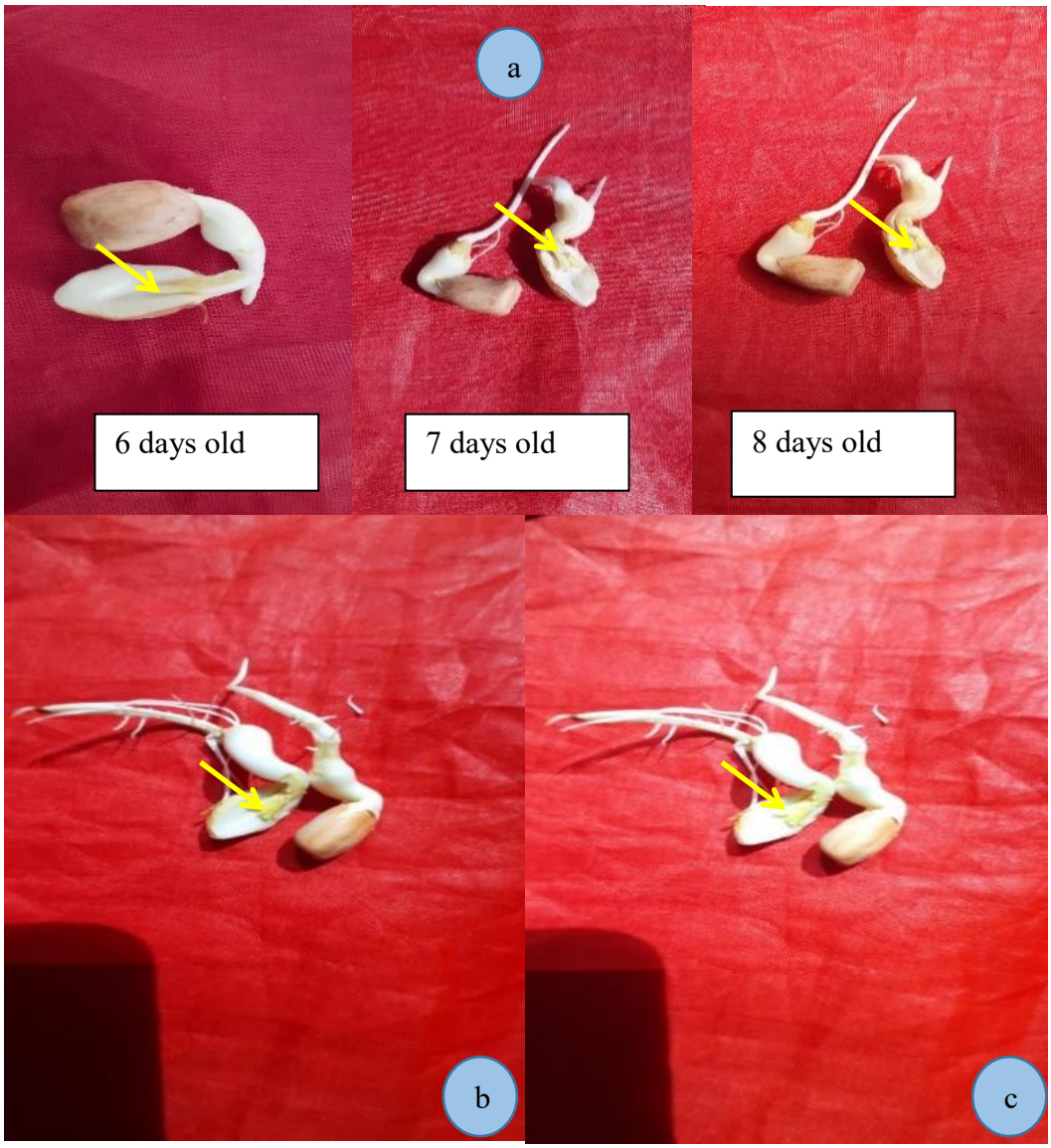


Fig. 14 (a - c): Collection of leaflet explants. (a) Various stages of seed germination (in days); (b) Immature leaflet explants (arrow) collected from 7 days old germinated seeds of Dhaka-1; (c) Leaflet explants (arrow) collected from 8 days old germinated seeds of BARI Badam-8.

4.1.1.3 Effect of culture medium and hormonal supplements on the regeneration of shoots from various explants

Each kind of explants needs a unique hormonal supplement to start organogenesis from *in vitro* produced cultures. The culture medium's composition is consistently regarded as a crucial component for the successful *in vitro* regeneration from any explant. For induction and elongation of shoots, MS medium containing various hormonal supplements and combinations was used. 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, DEHC and SCADE explants. The explants of immature leaflet and SCADE explants produced adequate responses towards the initiation and development of shoots (Tables 5, 6, 7, 8 and 9). In the present set of experiments, it was noticed that DEHC explants did not show any response towards regeneration on MS medium containing the above-mentioned hormonal supplements (Table 10). The SCADE explants also did not produced adequate responses for shoot development on MS with different hormonal supplements. For this reason, MSB (MS salts and Gamborg's B_5 vitamins**)** medium was used for the regeneration of shoots from the explants of DEHC and SCADE explants. 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 DEHC and SCADE 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 Effects of various hormone compositions on the regeneration of shoots from cotyledonary leaflet explants

In the current investigation, *in vitro* shoot regeneration from leaflet explants was carried out using MS media supplemented with cytokinins (BAP, Kn, NAA). According to the reports on previous work on peanut, 4.44, 11.1, 22.2, 44.4µM/l BAP alone, 22.2µM/l BAP + 2.3µM/l Kn and 44.4µM/lBAP+11.0 µM/l NAA were used for *in vitro* shoot regeneration.

4.2.2.1 Effect of various BAP concentrations in MS media on the regeneration of shoots from cotyledonary leaflet explant

To initiate shoot regeneration from leaflet explants of two peanut varieties, different concentrations of BAP were applied on MS media. For shoot development, the exact same media were also tried. To investigate the impact of BAP on the onset of regeneration and shoot growth from leaflet explants, MS media supplemented with various concentrations of BAP (4.44, 11.1, 22.2, and 44.4 M/l) was used. The responses of explants towards regeneration were more or less identical in all concentrations of hormones used with minor differences in the number of shoots/explants.

When leaflet explants were cultured on MS medium supplemented with 4.44, 11.1, 22.2, 44.4 µM/l; maximum callus induction was found on MS medium supplemented with 11.1 µM/l BAP and 22.2 µM/l BAP. When MS medium was supplemented with 22.2 µM/l BAP, callus was observed to form at the cut surfaces of the explants. These hormonal supplements were found to be the best for shoot initiation and development for the Dhaka-1 and BARI Badam-8 (Table - 5). MS media supplemented with 22.2 µM/l BAP produced 75% of responsive explants in case of Dhaka-1. On the other hand 22.2 µM/l BAP supplemented MS medium exhibited 74.80% responsive explants for BARI Badam-8. In case of Dhaka-1, the best regeneration response was obtained on MS medium supplemented with 22.2 M/l BAP. No. of shoots regenerated per explant was higher in variety Dhaka-1 than that of variety BARI Badam-8. In case of Dhaka-1 mean no. of shoots regenerated per explant was 5.5 whereas mean no. of shoots regenerated per explant was 5.2 in case of BARI Badam-8. The detailed results of this investigation are produced in Table 5, Fig.15 and 16.

Table 5. Effect of various concentrations of BAP in MS media on the regeneration of shoots from cotyledonary leaf explants of two varieties of peanut.

BAP (µM/l)	Varieties	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
4.44	Dhaka-1	40	81.33	09-14	62.44	4.3
	BARI Badam-8	40	80.00	10-14	60.00	4.2
11.1	Dhaka-1	40	86.66	10-15	70.00	4.5
	BARI Badam-8	40	85.55	10-16	72.00	4.4
22.2	Dhaka-1	40	93.00	11-16	75.00	5.5
	BARI Badam-8	40	91.11	10-15	74.80	5.2
44.4	Dhaka-1	40	82.11	12-16	61.00	4.1
	BARI Badam-8	40	81.00	11-16	55.33	4.0

Fig. 15 (a - f): Different stages of shoot formation from 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 with 22.2 µM BAP; (b) Initiation of multiple shoots from leaflet explants on the same medium as mentioned in Fig. 15a; (c) Development of multiple shoots formation on MS medium containing 22.2 µM BAP; (d) Elongated multiple shoots on the same medium as mentioned in Fig. 15c; (e) Shoot bud along with multiple shoots developed on MS with 11.1 µM BAP; (f) Multiple shoots formation on MS medium containing 44.4 µM BAP.

Fig. 16 (a - d): Various stages of shoot regeneration from leaflet explants of BARI Badam-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. 16a; (c) Elongation of multiple shoots on the same medium as mentioned in Fig. 16a; (d) Multiple shoot formation and elongation of shoots on MS medium containing 11.1 µM BAP.

4.2.2.2 Effect of various BAP and Kn concentrations in MS media on the regeneration of shoots from cotyledonary leaflets of two peanut varieties.

Using leaflet explants from two varieties of peanut, MS supplemented with various amounts and ratios of BAP and Kn was employed to regenerate new shoots. In this study, leaflet explants were grown into shoots on MS medium with varying concentrations of BAP (11.1 µM /l and 22.2 µM l) and Kn (2.3 µM /l and 4.6 µM /l). On MS media supplemented with BAP (11.1 µM - 22.2 µM) and Kn (2.3 µM - 4.6 µM), leaflet explants of two peanut types were found to generate quite a few shoots throughout this experiment. It was discovered that MS media supplemented with 22.2 µM BAP and 2.3 µM Kn was the most successful at producing shoots out of all the concentrations and combinations of BAP and Kn studied. Using these hormone concentrations, 94% of the explants for Dhaka-1 and 96% of the explants for BARI Badam-8 were shown to be responsive. 84% of the explants in MS media supplemented with 11.1 µM BAP and 4.6 µM Kn were shown to be responsive to variety in the case of BARI Badam-8, Dhaka-1 and 83% of explants were discovered to be responsive. The mean number of shoots obtained per explant when explants were cultivated on MS media supplemented with 22.2 µM BAP and 2.3 µM Kn was 6.2 in the case of Dhaka-1 and was 6.5 in the case of BARI Badam-8. On the other hand, the mean number of shoots obtained per explant when explants were cultivated on MS media supplemented with 11.1 µM BAP and 4.6 µM Kn was 4.2 in the case of Dhaka-1 and was 4.4 in the case of BARI Badam-8. As a result, in this experiment, MS medium supplemented with 22.2 µM BAP and 4.6 µM Kn and MS medium supplemented with 22.2 µM BAP and 2.3 µM Kn were shown to be the most effective for multiple shoot regeneration, respectively (Figs. 17 and 18). It was found that BARI Badam-8 had a better overall regeneration response than Dhaka-1. The detailed results of this investigation are produced in Table 6. for Dhaka-1 and BARI Badam-8.

Table 6. Effect of various BAP and Kn concentrations in MS media on the regeneration of shoots from explants of cotyledonary leaves of two varieties of peanut.

BAP + Kn (µM/l)	Varieties	No. of explants inoculated	% of explants formed callus	Days required for initiation of regeneration	% of explants showed shoot initiation	Mean no. of shoots/ explant after 3.5 months
11.1+2.3	Dhaka-1	50	82	15-19	68	4.0
	BARI Badam-8	50	81	14-18	65	3.9
11.1+4.6	Dhaka-1	50	84	16-20	70	4.2
	BARI Badam-8	50	83	15-19	68	4.4
22.2 +2.3	Dhaka-1	50	94	15-20	72	6.2
	BARI Badam-8	50	96	16-22	78	6.5
22.2+ 4.6	Dhaka-1	50	91	14-20	70	5.7
	BARI Badam-8	50	89	15-20	74	5.9

Fig. 17 (a - f): Various stages of shoot development from 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. 17a; (c) Multiple shoots formation via callus formation on medium as mentioned in Fig.17a; (d) Multiple shoot bud formation along with elongated shoots via callus formation on MS medium supplemented with 11.1 µM BAP and 4.6 µM Kn; (e) Elongation of shoots on medium as mentioned in Fig. 17d; (f) Elongated shoots developed on MS medium with 22.2 µM BAP and 4.6 µM Kn.

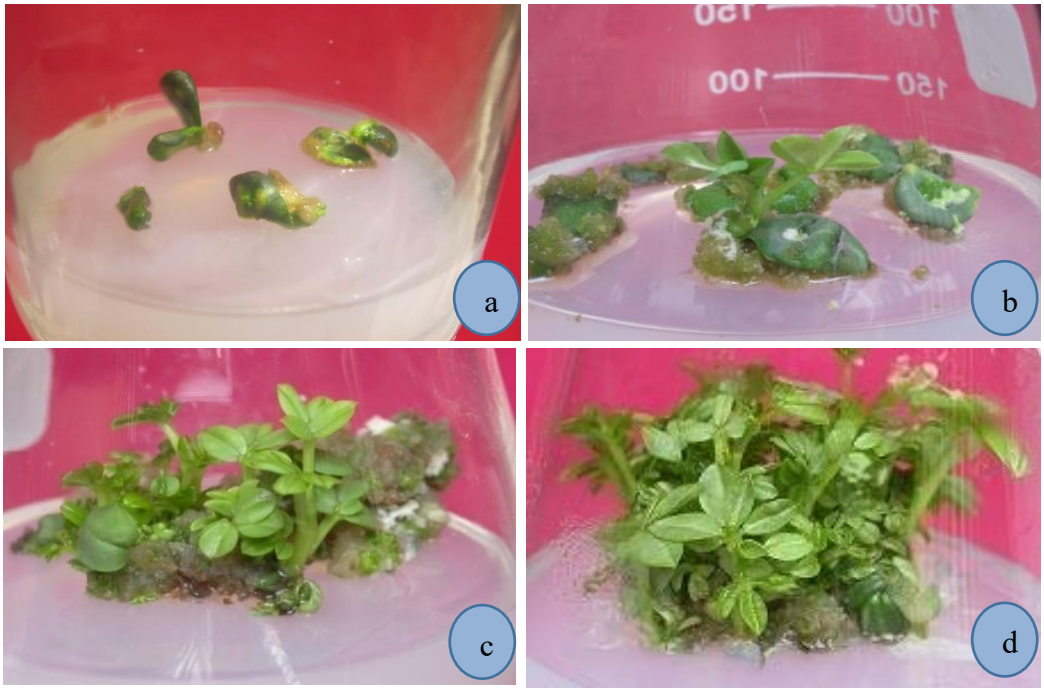


Fig. 18 (a - d): Various stages of shoot formation from leaflet explants of BARI Badam-8 on MS medium supplemented with different concentration of BAP and Kn. (a) Shoot formation from the callus (arrow) on MS medium supplemented with 22.2 µM BAP and 2.3 µM Kn; (b) Multiple shoot formation on the same medium as mentioned in Fig 18a; (c) Elongated multiple shoots developed on the same medium as mentioned in Fig 18a; (d) Fully developed elongated shoots on MS medium with 22.2 µM BAP and 4.6 µM Kn.

4.2.2.2.1 Effects of various BAP and NAA concentrations in the MS medium on the regeneration of shoots from cotyledonary leaflet explants of two different peanut varieties

Various combinations and concentrations of BAP and NAA were used for shoots regeneration using leaflet explants from two different peanut varieties. Shoots from leaflet explants were produced in this experiment on MS media with various concentrations and combinations of BAP (22.2 µM and 44.4 µM) and NAA (5.5 µM and 11.0 M/l). During this investigation quite a smaller number of shoots were found to develop from leaflet explants of two peanut varieties. The MS medium supplemented with 44.4 µM BAP and 11.0 µM NAA was shown to be more effective at producing shoots than any other concentrations and combinations of BAP and NAA employed. Using these hormonal concentrations 93.33% of explants were found to be responsive in case of Dhaka-1 and 91.11% of explants were found to be responsive in case of BARI Badam-8. When MS medium supplemented with 44.4 µM/l BAP and 5.5 µM/l NAA were used 88.88% of the explants were found to be responsive in case of variety Dhaka-1 and 86.66% of explants were found to be responsive in case of BARI Badam-8. The mean number of shoots obtained per explant when explants were cultivated on MS media supplemented with 44.4 µM BAP and 11.0 µM NAA was 4.2 in the case of Dhaka-1 and the mean number of shoots obtained per explant was 3.8 in the case of BARI Badam-8 in this same treatment. However, when explants were cultivated on MS media supplemented with 44.4 µM BAP and 5.5 µM NAA, the mean number of shoots obtained per explant was 3.8 in the case of Dhaka-1 as well as 3.6 in the case of BARI Badam-8 in this same treatment. As a result, in this experiment, it was discovered that MS medium supplemented with 44.4 µM BAP and 11.0 µM NAA was more beneficial for multiple shoot regeneration in the case of two peanut types (Figs. 19 and 20). It was observed that, the overall regeneration response was better in Dhaka -1 than that of BARI Badam-8. The detailed results of this investigation are produced in Table 7. for Dhaka-1 and BARI Badam-8.

Table 7. Combined effect of BAP and NAA in the MS medium on the regeneration of shoots from cotyledonary leaf explants of two varieties of peanut.

BAP + NAA (µM/l)	Varieties	No. of explants inoculated	% of explants formed callus	Days required for initiation of regeneration	% of explants showed shoot initiation	Mean no. of shoots/ explant after 3.5 months
22.2+5.5	Dhaka-1	45	83.33	09-13	22.66	2.6
	BARI Badam-8	45	81.00	10-14	23.00	2.2
22.2+11.0	Dhaka-1	45	86.66	09-14	35.00	2.8
	BARI Badam-8	45	84.44	08-13	32.00	2.6
44.4+5.5	Dhaka-1	45	88.88	08-12	35.00	3.6
	BARI Badam-8	45	86.66	09-11	38.00	3.4
44.4+ 11.0	Dhaka-1	45	93.33	08-13	48.00	4.2
	BARI Badam-8	45	91.11	08-12	40.00	3.8

Fig. 19 (a - d): Different stages of shoot formation from 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 11.0 µM NAA; (b) Elongated multiple shoots along with callus on the same medium as mentioned in Fig 19a; (c) Multiple shoots formation on MS medium with 44.4 µM BAP and 5.5 µM NAA; (d) Induction of profuse callus from leaflet explants on MS medium with 22.2 µM BAP and 11.0 µM NAA.

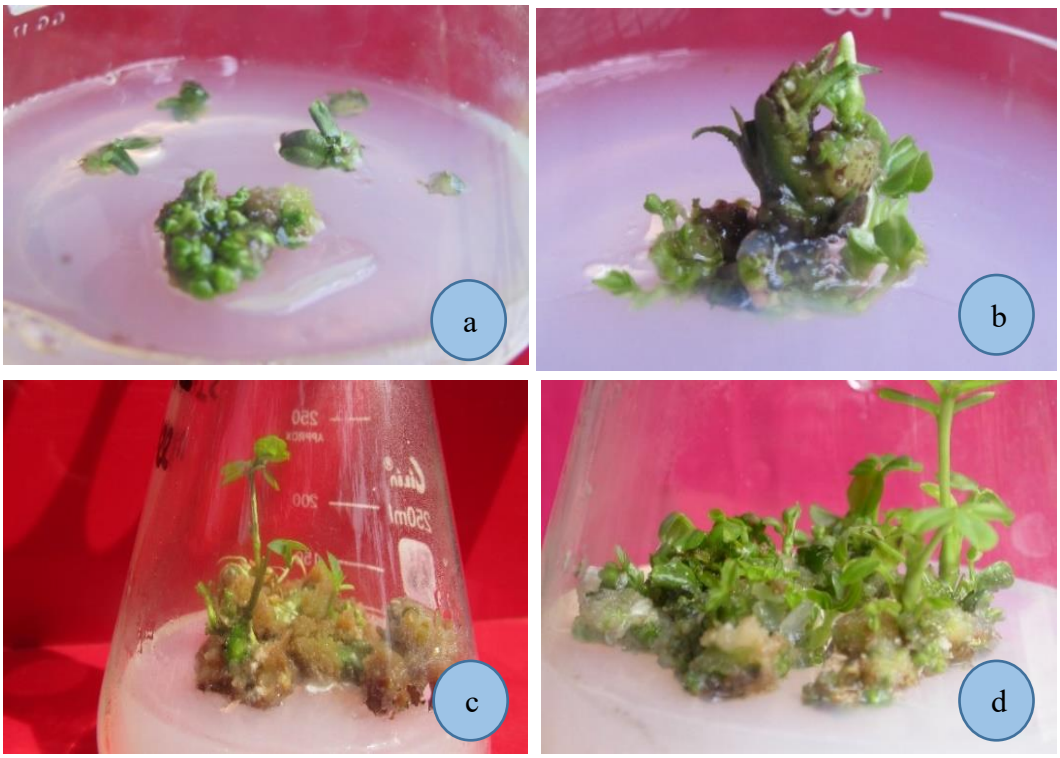


Fig. 20 (a - d): Regeneration of shoot from leaflet explant of BARI Badam-8 on MS medium supplemented with BAP and NAA. (a) Multiple shoot formation on MS medium supplemented with 44.4 µM BAP and 11.0 µM NAA; (b) Elongated multiple shoots along with callus on the same media composition as mentioned in Fig. 20a; (c) Shoot regeneration from the callus on MS medium with 44.4 µM BAP and 5.5 µM NAA; (d) Induction of profuse callus on MS medium containing 22.2 µM BAP and 11.0 µM NAA.

4.2.3 Effects of various hormonal compositions on the regeneration of shoots from a single cotyledon-attached decapitated half embryo explant

In the current investigation, explants of single cotyledon attached decapitated embryos (SCADE) were used to regenerate shoots *in vitro* using MS media enriched with cytokinins (BAP, Kn, and 2, 4-D). According to the report on the previous work on peanut, in the present experiment, 22.2 µM/l BAP alone, 22.2 µM/l BAP + 2.3µM/l Kn and 20 µM/l BAP+10 µM/l 2,4-D were used for *in vitro* shoot regeneration.

4.2.3.1 Effect of various BAP concentrations on the regeneration of two peanut varieties (Dhaka-1 and BARI Badam-8) from SCADE explants

Both the varieties, MS medium supplemented with various concentrations of BAP (4.44, 11.1, 22.2, 44.4 µM/l) were employed to examine its effect on initiation of regeneration and shoot development from SCADE explants. The responses of explants towards regeneration were same in all concentrations with minor differences in the number of shoots/explants.

In this instance, MS medium supplemented with 22.2 µM BAP showed the best regeneration. At this concentration Dhaka-1 variety required 9-10 days for shoot initiation. Different stages of shoot regeneration on this concentration were shown in Figs. 21 (a- d). In the case of 4.44 µM BAP, shoot initiation was seen after 10-14 days, that of 11.0 µM BAP after 9-14 days, and that of 44.4 µM BAP after 8 - 12 days. The regeneration of multiple shoots on MS media supplemented with various amounts of BAP is depicted in Figs. 21(c) through (e), respectively. Table 8. shows the result of this experiment. For the BARI Badam-8 variety, the best regeneration was similarly shown in this instance on MS medium supplemented with 22.2 µM BAP. At this concentration BARI Badam-8 variety required 10-12 days for shoot initiation. Different stages of shoot regeneration on this concentration were shown in Fig. 22 (a - d). Although in case of 4.44 µM/l BAP shoot initiation was observed after 10-15 days, at 11.1 µM/l BAP shoot initiation was observed earlier at about after 8-13 days, but the mean number of shoots per explants was higher in case of 22.2 µM/l BAP and at 44.4 µM/l BAP after 9-13 days shoot initiation was observed. In Figs. 22 (c-e), multiple shoot regeneration on MS media enriched with these concentrations of BAP was demonstrated. The outcomes of this experiment are presented in Table 8.

Table 8. Response of Dhaka-1 and BARI Badam-8 single cotyledon attached decapitated embryo (SCADE) explants to shoot regeneration at various BAP concentrations in MS media.

BAP (µM/l)	Varieties	No. of explants inoculated	% of responsive explants	Days required for initiation of regeneration	% of explants showed shoot initiation	Mean no. of shoots/ explant after 45 days
4.44	Dhaka-1	40	25	10-14	62.5	1.6
	BARI Badam-8	40	26	10-15	65.0	1.87
11.1	Dhaka-1	40	29	9-14	72.5	2.5
	BARI Badam-8	40	30	8-13	75.0	2.9
22.2	Dhaka-1	40	35	9-10	93	4.5
	BARI Badam-8	40	35	10-12	95	5.0
44.4	Dhaka-1	40	32	8-12	80.0	3.0
	BARI Badam-8	40	33	9-13	82.5	3.5

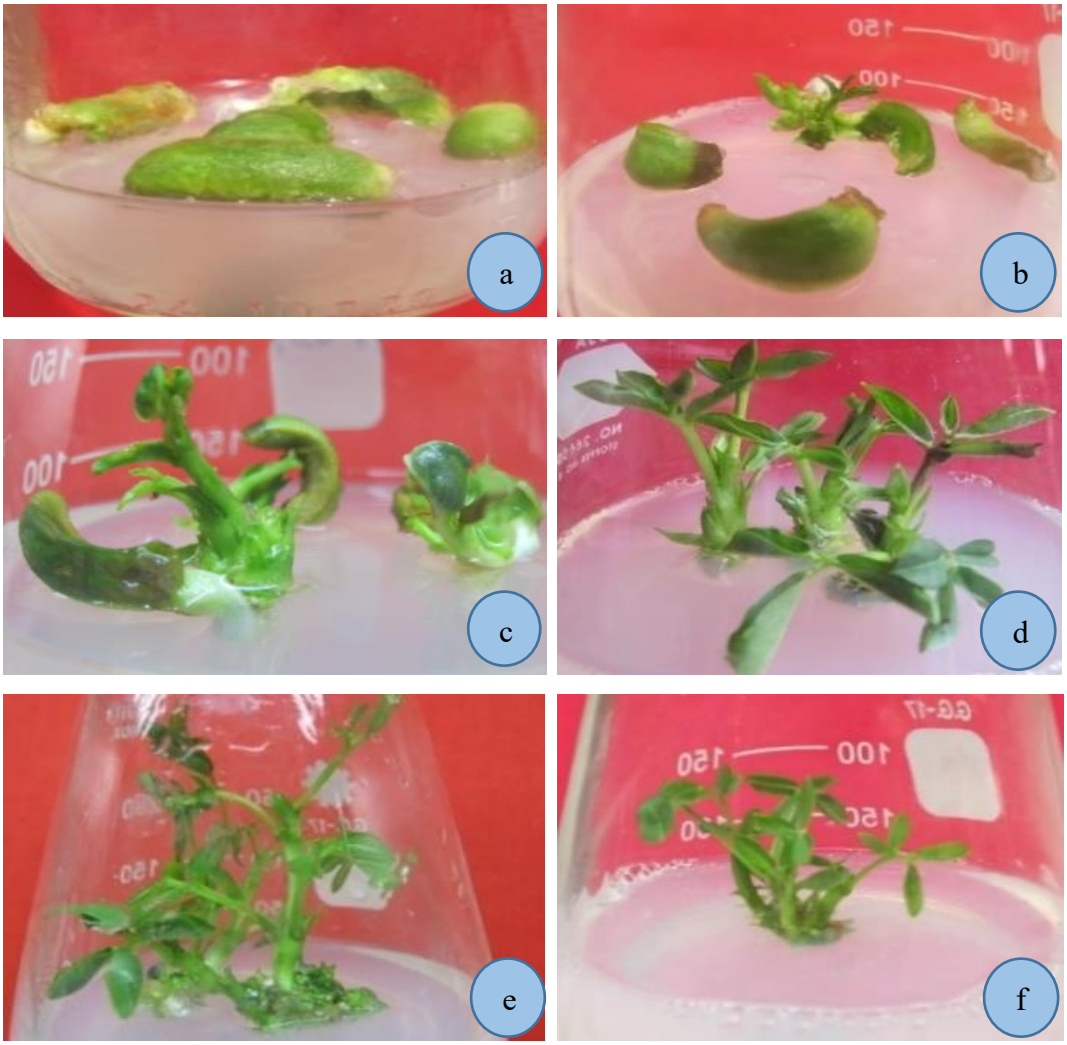


Fig. 21 (a - f): Different stages of shoot development from SCADE explant of Dhaka-1 of peanut on MS medium supplemented with BAP. (a) Greening of SCADE explants on shoot induction medium MS medium supplemented with 22.2 µM BAP; (b) Shoots initiation from SCADE explant in Dhaka-1 on the same medium as mentioned in Fig 21a; (c) Multiple shoots formation in Dhaka-1 on the same medium as mentioned in Fig 21a; (d) Multiple shoots formation in Dhaka-1 on the same medium as mentioned in Fig 21a; (e) Elongation of multiple shoots in Dhaka-1 on the same medium as mentioned in Fig 21a); (f) Elongation of multiple shoots in Dhaka-1 on MS medium supplemented with 44.4 µM. BAP.

Fig. 22 (a - f): Different stages of shoot development from SCADE explant of BARI Badam-8 of peanut on MS medium supplemented with BAP. (a) Greening of SCADE explants on SIM supplemented with 22.2 µM BAP; (b) Shoots initiation from SCADE explant in BARI Badam-8 on the same medium as mentioned in Fig 22a; (c) Multiple shoots formation in BARI Badam-8 on the same medium as mentioned in Fig 22a; (d) Multiple shoots formation in BARI Badam-8 on the same medium as mentioned in Fig 22a; (e) Elongation of multiple shoots in BARI Badam-8 on the same medium as mentioned in Fig 22a); (f) Elongation of multiple shoots in BARI Badam-8 on MS medium supplemented with 44.4 µM BAP.

4.2.2.3 Effect of various BAP and Kn combinations and concentrations on Dhaka-1 and BARI Badam-8 variety regeneration from SCADE explants

Depending on different concentration and combination of hormonal supplements used about 62-95% of cultured explants responded towards regeneration. Table 9 displays the findings of these observations. Four different combinations were tested in this experiment. Among these, MS medium supplemented with 22.2 µM BAP and 2.3 µM Kn showed the best regeneration. Although the quantity of shoots/explants varied amongst combinations, shoot regeneration was seen in every one of them. For both kinds, MS medium supplemented with 22.2 µM BAP and 2.3 µM Kn yielded the best mean number of shoots (4 shoots/explant). Figs. 23 and 24 depict various stages of shoot regeneration on this concentration (a-d). On the same medium, regenerated shoots were subcultured for periodic multiplication and elongation. The best regeneration in this variety was shown on MS medium supplemented with 22.2 µM BAP and 2.3 µM Kn; here, nearly 90% of explants responded to regeneration in the case of Dhaka-1 variety and 95% in the case of BARI Badam-8 variety. Although the quantity of shoots/explants varied amongst combinations, shoot regeneration was seen in every one of them. Table 9 displays the findings of these observations. The BARI Badam-8 variety's best mean number of shoots (7.0 shoots/explant) was likewise discovered on MS medium supplemented with 22.2 µM BAP and 2.3 µM Kn. In Figs. 23 and 24, several stages of shoot regeneration on this concentration were depicted (c-e). For their regular multiplication and elongation, regenerated shoots were sub-cultured on the same medium.

Table 9. Response of Dhaka-1 and BARI Badam-8 SCADE explants to varied combinations and concentrations of BAP and Kn in MS media towards shoot regeneration.

BAP + Kn (µM/l)	Varieties	No. of explants inoculated	% of responsive explants	Days required for initiation of regeneration	% of explants showed shoot initiation	Mean no. of shoots/ explant after 45 days
11.1+2.3	Dhaka-1	40	25	8-12	62.5	3.13
	BARI Badam-8	40	27	7-11	67.5	3.41
11.1+4.6	Dhaka-1	40	24	7-11	60.0	2.3
	BARI Badam-8	40	26	8-12	65.0	2.41
22.2+2.3	Dhaka-1	40	30	8-10	90	6.5
	BARI Badam-8	40	35	7-10	95	7.0
22.2+4.6	Dhaka-1	40	25	7-10	62.5	3.5
	BARI Badam-8	40	28	7-11	70.0	3.6

Fig. 23 (a - f): Different stages of shoot development from SCADE explant of Dhaka-1 on MS medium supplemented with BAP and Kn. (a) Greening of SCADE explants on SIM, MS supplemented with 22.2 µM BAP+ 2.3 µM Kn; (b) Shoots initiation from SCADE explant in Dhaka-1 on the same medium as mentioned in Fig 23a; (c) Multiple shoots formation in Dhaka-1 on the same medium as mentioned in Fig 23a; (d) Multiple shoots formation in Dhaka-1 on the same medium as mentioned in Fig 23a; (e) Elongation of multiple shoots in Dhaka-1 on the MS medium supplemented with 22.2 µM BAP+ 4.6 µM Kn); (f) Elongation of multiple shoots in Dhaka-1 on MS medium supplemented with 11.1 µM BAP+ 2.3 µM Kn.

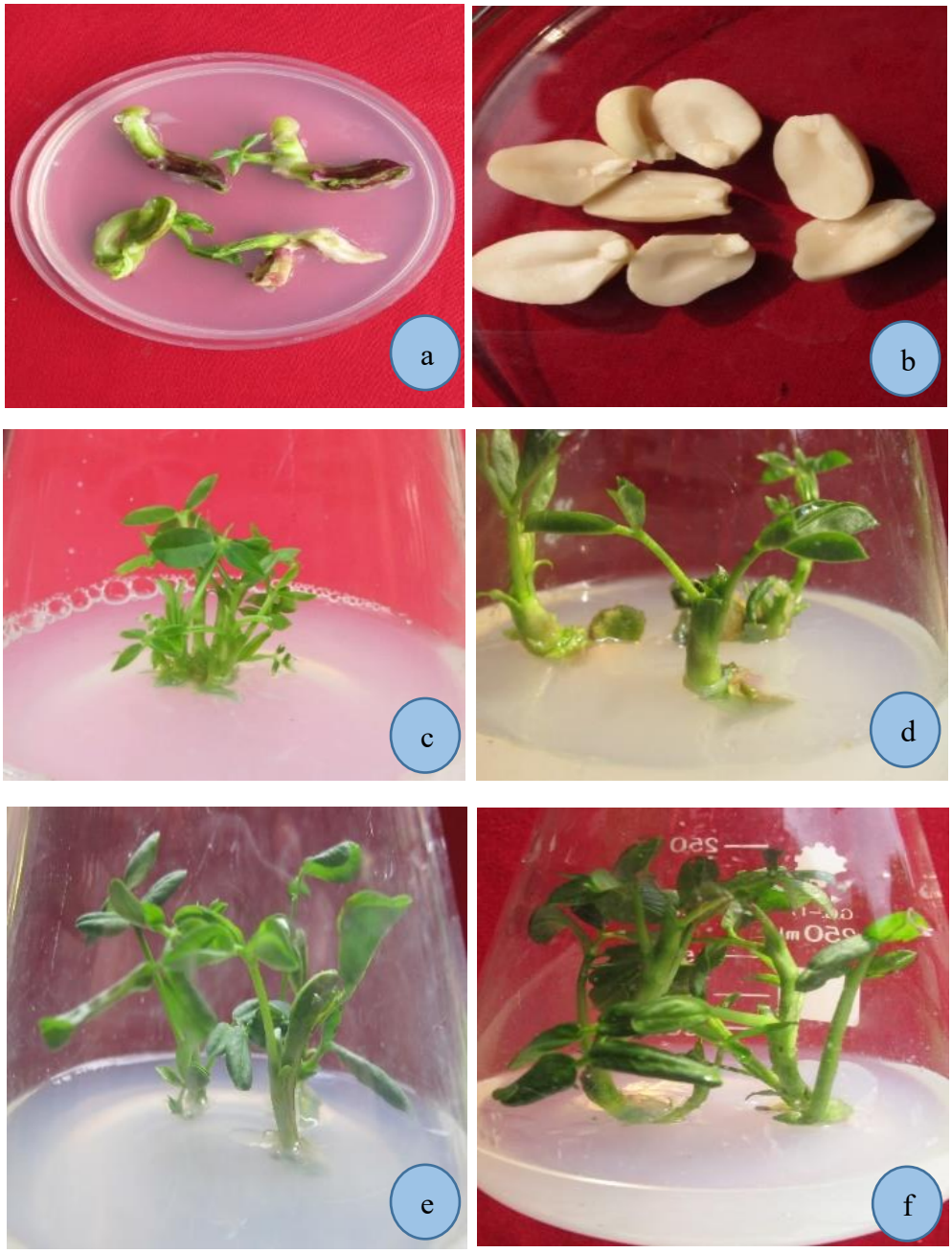


Fig. 24 (a - f): Different stages of shoot development from SCADE explant of BARI Badam-8 on MS medium supplemented with BAP with Kn. (a) Greening of SCADE explants on SIM, MS supplemented with 22.2 µM BAP+ 2.3 µM Kn; (b) Shoots initiation from SCADE explant in BARI Badam-8 on the same medium as mentioned in Fig 24a; (c) Multiple shoots formation in BARI Badam-8 on the same medium as mentioned in Fig 24a; (d) Multiple shoots formation in BARI Badam-8 on the same medium as mentioned in Fig 24a; (e) Elongation of multiple shoots in BARI Badam-8 on the MS supplemented with 22.2 µM BAP+ 4.6 µM Kn); (f) Elongation of multiple shoots in BARI Badam-8 on MS supplemented with 11.1 µM BAP+ 2.3 µM Kn.

4.1.1.3.11 Effect of MS medium with BAP alone and in combination of BAP and Kn from DEHC and SCADE from Dhaka-1 and BARI Badam-8

A set of experiments were conducted using de-embryonated half cotyledon (DEHC) and single cotyledon attached decapitated embryo (SCADE) explants from both the Dhaka-1 and BARI Badam-8 on MS medium with BAP alone and in combination of BAP and Kn. These results have been presented in Table 10. 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 10). In case of SCADE explants all showed responses towards multiple shoots formation with lower number of shoots/explants. The best response was noted on MS with 22.2 µM BAP and 2.3 µM Kn from SCADE explants of Dhaka-1, out of the two media combinations.

Table 10. Response of Dhaka-1 and BARI Badam-8 DEHC and SCADE explants 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	DEHC	50	-	-	No response
		SCADE	50	6-9	65	4.5
	BARI Badam-8	DEHC	50	-	-	No response
		SCADE	50	5-9	62	3.5
MS + 22.2 µM BAP + 2.3 µM Kn	Dhaka-1	DEHC	50	-	-	No response
		SCADE	50	5-9	70	4.5
	BARI Badam-8	DEHC	50	-	-	No response
		SCADE	50	6-9	65	3.9

DEHC= De-embryonated half cotyledon; SCADE= Single cotyledon attached decapitated embryo

4.1.1.3. Effects of BAP and 2, 4-D on MSB (MS salts and Gamborg's B5 vitamins) media on the regeneration of shoots from de-embryonated cotyledon (DEHC) explants of Dhaka-1

From the previous experiments (section, 4.1.3.11) it was observed that DEHC 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 were used for the initiation of regeneration from the DEHC explants. On MSB medium enriched with various concentrations of BAP and 2, 4-D, the response of DEHC explants toward shoot initiation via callus induction was studied. The results of the observation have been presented in Table 11. For this purpose, DEHC explants (Fig. 25a) were inoculated on shoot induction medium (SIM) which contained various concentration of BAP and 2, 4-D. After that, the explants with induced shoot buds were placed in the shoot elongation medium (SEM), which included varied BAP concentrations at lesser levels. For SIM, the induction of shoots by the formation of calluses was accomplished using MSB medium supplemented with various concentrations of BAP (10 M and 20 M) and 2, 4-D (5 M and 10 M). 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. 25b) and within 8-10 days on SIM, callus formation was initiated from the explants. After 11 - 16 days shoot bud formation was observed on MSB with 20 µM BAP and 10 µM 2, 4-D supplemented medium (SIM) (Fig. 25c). 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 DEHC explants were not turned green in colour, as initiation of callus and shoots was started from large green cotyledon.

Cotyledon explants were transferred to shoot elongation medium (SEM), which contained MSB supplemented with 2–4 M BAP, after two weeks. On MSB with 4 M BAP, the greatest response to the commencement of numerous shoot buds was seen. The proximal end (the de-embryonated section), distal ends (the opposite of the de-embryonated portion), and cut surfaces differentiated into callus when DEHC explants were cultured for 2 weeks on SIM. Upon subculture the explants on shoot elongation medium, multiple shoot buds were started to initiate from the proximal end of cotyledon (Fig. 25d), while

compact undifferentiated calli was formed on the rest of the sites (Fig. 25 e). In Dhaka-1, 62.57% explants showed shoot initiation response on MSB with 2 µM BAP whereas 70.28% shoot initiation was observed on MSB containing 4 µM BAP. The mean number of shoots/explants was 5.1 and 5.5 when cotyledon explants were cultured on MSB with 2 µM BAP and MSB with 4 µM BAP supplemented media, respectively (Fig. 25f, Table 11). 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 32.22% and 33.33% 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 5 µM 2, 4-D (Table 11). In this case mean number of shoots per explant was 2.0 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 5 µM 2,4-D supplemented media (SIM) explants produced profuse callus with slow initiation of shoots (Fig. 25f).

Using this explant, 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 sub cultured on SEM.

4.1.1.3.13. Effect of BAP and 2, 4-D on the regeneration of shoots from DEHC explants of BARI Badam-8

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 (DEHC) explants of BARI Badam-8. 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 Badam-8, 60.22% explants responded towards callus induction on SIM and among the callus 68.28% showed regeneration of shoots on MSB with 4 µM BAP (SEM). The mean no of shoots/explant was 5.2 in SEM. The results of these observations are presented in Table 12. 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 Badam-8, it was also 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. Various stages of regeneration are presented in Figs. 26 (a – f).

Table 11. Response of Dhaka-1 DEHC explants to shoot regeneration in MSB media treated with various BAP and 2,4-D concentrations.

Composition of SIM		No. of explants formed callus in SIM	Days required for callus formation	Com. of SEM (BAP µM)	Days required for shoot initiation from callus	% of explants showed regeneration	Mean no. of shoots/ explant after 45 days of culture
BAP (µM)	2,4-D (µM)						
20	5	30	8-10	2	11 - 15	62.57	5.1
20	10	35	8-10	4	11 - 16	70.28	5.5
10	5	30	7-10	2	18 - 24	32.22	2.0
10	10	25	7-10	4	18 - 22	33.33	2.5

Table 12. Response of DEHC explants of BARI Badam-8 towards callus formation and multiple shoots regeneration on MSB medium supplemented with various 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 regeneration	Mean no. of shoots/ explant after 45 days of culture
BAP (μM)	2,4-D (μM)						
20	5	30	8-10	2	11 - 15	60.22	4.9
20	10	35	8-10	4	11 - 16	68.28	5.2
10	5	30	7-10	2	18 - 24	31.11	2.2
10	10	25	7-10	4	18 - 22	32.33	2.7

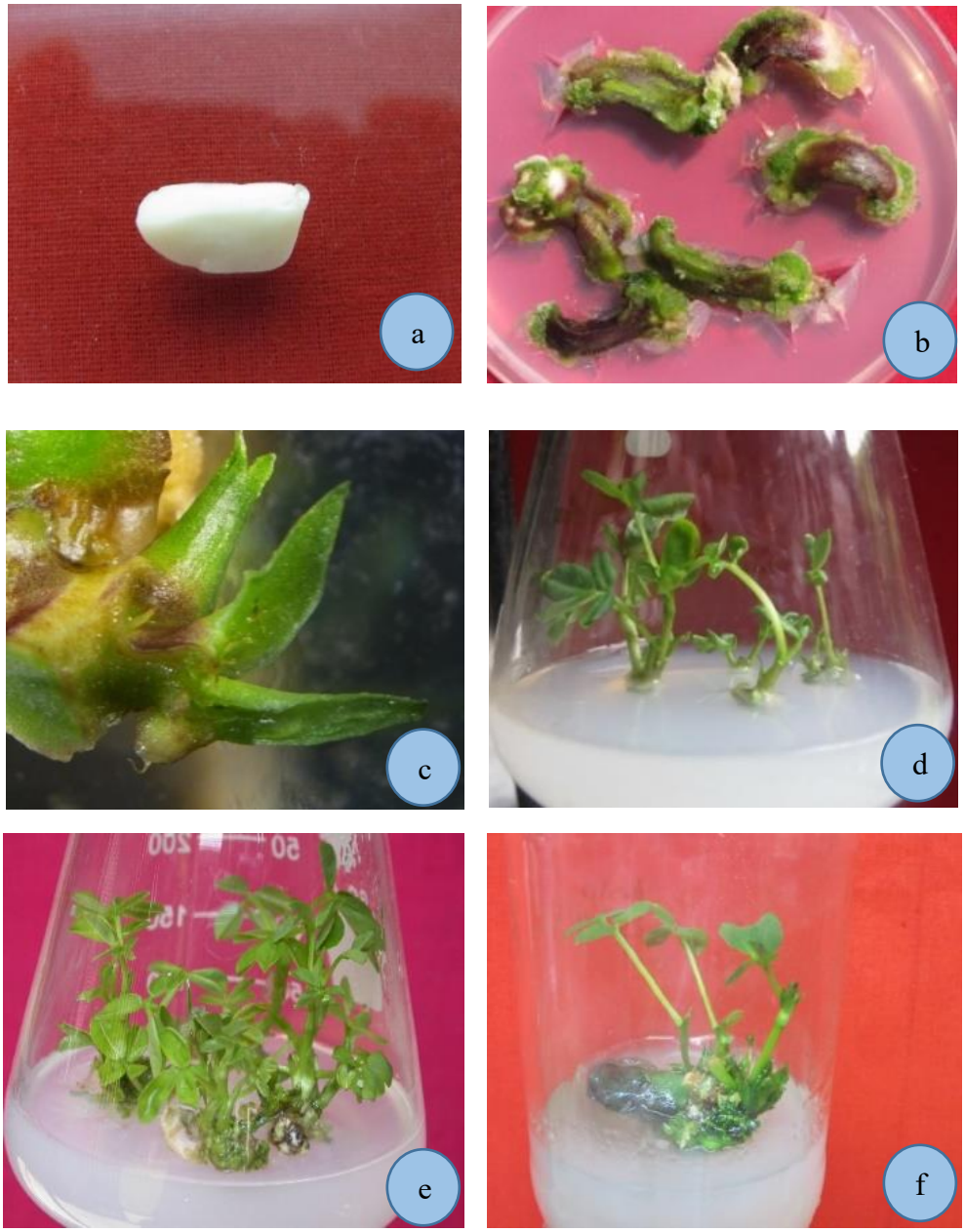


Fig. 25 (a - f): Different stages of shoot development from DEHC explant of Dhaka-1 on MSB with BAP and 2,4-D. (a) DEHC explants on shoot induction medium, SIM (MSB with 20 µM BAP and 10 µM 2,4-D); (b) Cotyledons became green on the same medium as mentioned in Fig 25a; (c) Stereomicroscopic view of formation of compact callus (arrow) in distal end instead of shoot formation on SEM (MSB with 4 µM BAP); (d) Multiple shoots formation in in Dhaka-1 on SEM; (e) Elongation of multiple shoots in Dhaka-1 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 10 µM 2,4-D.

Fig. 26 (a - f): Different stages of shoot regeneration from DEHC of BARI Badam-8 on MSB medium supplemented with BAP and 2,4-D. (a) DEHC explants on shoot induction medium, SIM (MSB with 20 µM BAP and 10 µM 2,4-D; (b) Cotyledons became green on the same medium as mentioned in fig 26b; (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 4 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 10 µM 2,4-D.

4.2.2.3 Effect of various BAP and 2, 4-D combinations and concentrations on the regeneration of the BARI Badam-8 and Dhaka-1 varieties from SCADE explants

In this set of experiment, SCADE explants of Dhaka-1, BARI Badam-8 were used to show their effects towards regeneration. The MSB medium, referred as shoot induction medium, was added with 20 µM BAP and 10 µM 2,4-D before the SCADE explants were inoculated (SIM). Healthy multiple shoots were initiated directly from the embryos of all the varieties. Results of this experiment have been presented in Table 13. 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, 7 - 9 days were required for Dhaka-1, 6-9 days for BARI Badam-8 (Table 13). 95% explants of Dhaka-1 as well as 98% BARI Badam-8 showed shoot regeneration response on the SIM (MSB with 20 µM BAP and 10 µM 2,4-D). The mean number of shoots/explants was recorded to be 7.5 in Dhaka-1 and 8.0 in BARI Badam-8 variety when the explants were subcultured on SEM which contained MSB with 4 µM BAP.

Table 13. Response of SCADE explants of Dhaka-1 and BARI Badam-8 varieties to varied combinations and concentrations of BAP and 2, 4-D in MSB medium in terms of shoot regeneration.

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	40	7-9	95	4 µM BAP	7.5
	BARI Badam-8	40	6-9	98		8.0

4.1.1.3.14 Effect of BAP with MSB on multiple shoot regeneration from de-embryonated cotyledon (DEHC) explants of two peanut varieties (MS salts and Gamborg's B5 vitamins)

A set of experiments were carried out with DEHC explants from two peanut varieties, namely, Dhaka-1 and BARI Badam-8. The effect of the BAP at various concentrations on the regeneration of shoots was investigated for this purpose using MSB medium. Firstly, shoot induction media was inoculated with DEHC explants (SIM). Then they were transferred to the shoot elongation medium (SEM) after shoot bud initiation (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. 27b). 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).

Though direct organogenesis was the norm in the majority of cases, callus development was occasionally used to initiate shoot initiation from DEHC explants. 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 subcultures in shoot elongation media (MSB with 13 µM BAP).

Among the two varieties Dhaka-1 showed best response towards multiple shoots formation on MSB with BAP supplemented medium. A total of 85.0% explants showed regeneration response in SIM1 (MSB with 88.8 µM BAP). Among the responded explants, 72.2% 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 combination of BAP in MSB. The results of this experiment are shown in Table 14. Mean number of shoots/explants on SEM (MSB with 13 μM BAP) was recorded as 7.0 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. 27 (a - f).

Among the two varieties, BARI Badam-8 showed comparatively lower number of shoots/explant (6.5) compared to other two varieties. Here, 80% explants showed regeneration response on SIM1 and among them 68.0% 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 Badam-8 are shown in Figs. 28 (a - d). Table 14 displays the findings of this experiment.

Table 14. Response of DEHC explants of two 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	50	85.0	72.2	13	7.0
88.8	66.6	BARI Badam-8	50	80.0	68.0	13	6.5

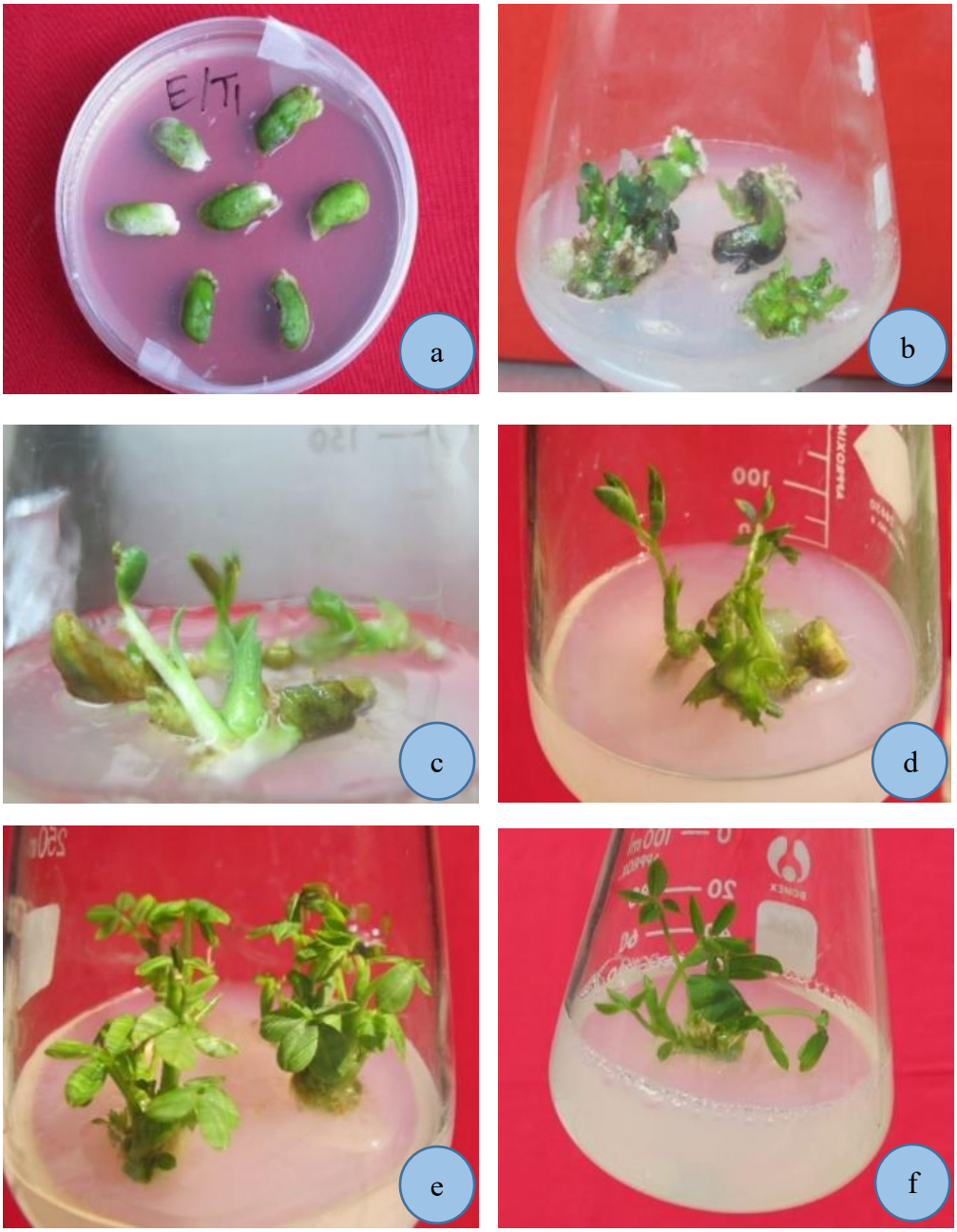


Fig. 27 (a - f). Different stages of shoot regeneration from DEHC explant of Dhaka-1 on MSB with BAP. (a) Cotyledons became green on shoot induction medium, SIM (MSB with 88.8 µM BAP; b) Shoot formation from DEHC after 11-13 days of culture on the same medium as mentioned in Fig.27a; (c) Proliferation of multiple shoots on the same medium as mentioned in Fig.27a; (d) Elongation of multiple shoot on SIM2 (MSB with 66.6 µM BAP); (e) Shoot development and elongation of shoots on SEM (MSB with 13 µM BAP); (f)) Elongation and multiplication on the same medium as mentioned in Fig.27e.

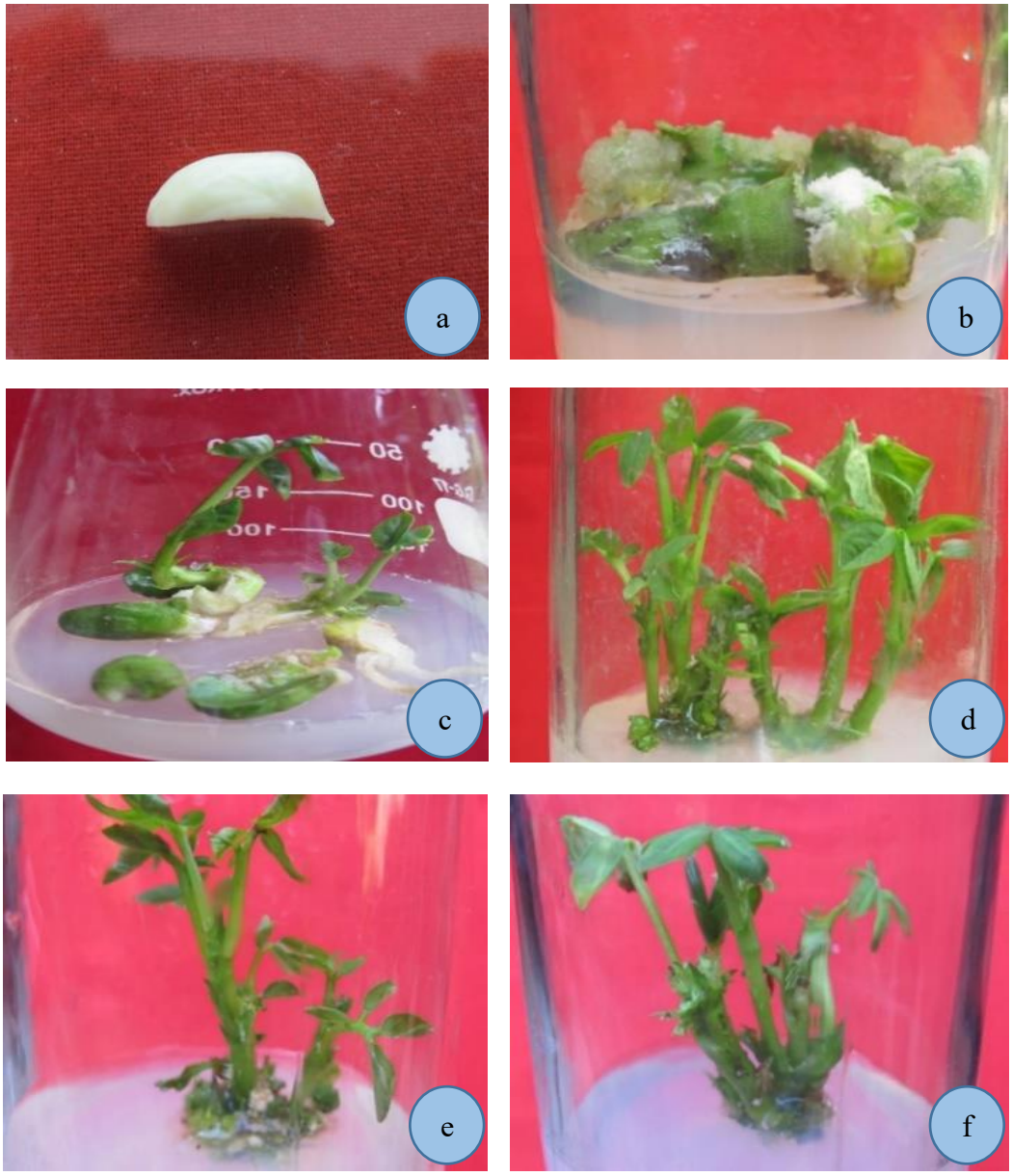


Fig. 28 (a - f): Stages of shoot regeneration from DEHC explant of BARI Badam-8 on MSB medium supplemented with BAP. (a) DEHC explants (b) Cotyledons became Green on Shoot induction medium 1, SIM1 (MSB with 88.8 µM); (c) Direct multiple shoot regeneration on SIM2 (MSB with 66.6 µM BAP) after 25-30 days 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.

4.1.1.3.16 Effect of BAP in MSB medium on the shoot regeneration from SCADE explants of Dhaka-1 and BARI Badam-8

From SCADE explants of two types of peanuts, Dhaka-1 and BARI Badam-8, the effect of BAP on MSB was seen in the regeneration of shoots. SCADE 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 Badam-8 where mean no of shoots/explant were 6.5 and 7.0 for both the varieties. The data of the present study are described in Table 15. Different stages of shoot regeneration are presented in Figs. 29 and 30 (a - f).

Table 15. Response of SCADE explants towards multiple shoot formation using BAP on MSB medium of two peanut varieties.

Composition of SIM	variety	No. of explants inoculated	Days required for initiation of regeneration	% of explants showed shoot initiation	Composition 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	50	4 - 8	90	13 µM BAP	6.5
	BARI Badam-8	50	5 - 8	92		7.0

Fig. 29 (a - f): Different stages of shoot development from SCADE explant of Dhaka-1 on MSB with BAP. (a) Shoot induction on SIM1 (MSB with 88.8 µM BAP); b) multiple shoot regeneration on SIM2 (MSB with 66.6 µM BAP) after 25-30 days culture; (c) Proliferation of multiple shoots along with callus in SEM (MSB with 13 µM BAP); (d) Multiple shoots along with elongated shoots on SEM; (e) Elongated shoots developed on SEM; (f) Elongated multiple shoots on the same medium as mentioned in Fig.30e.

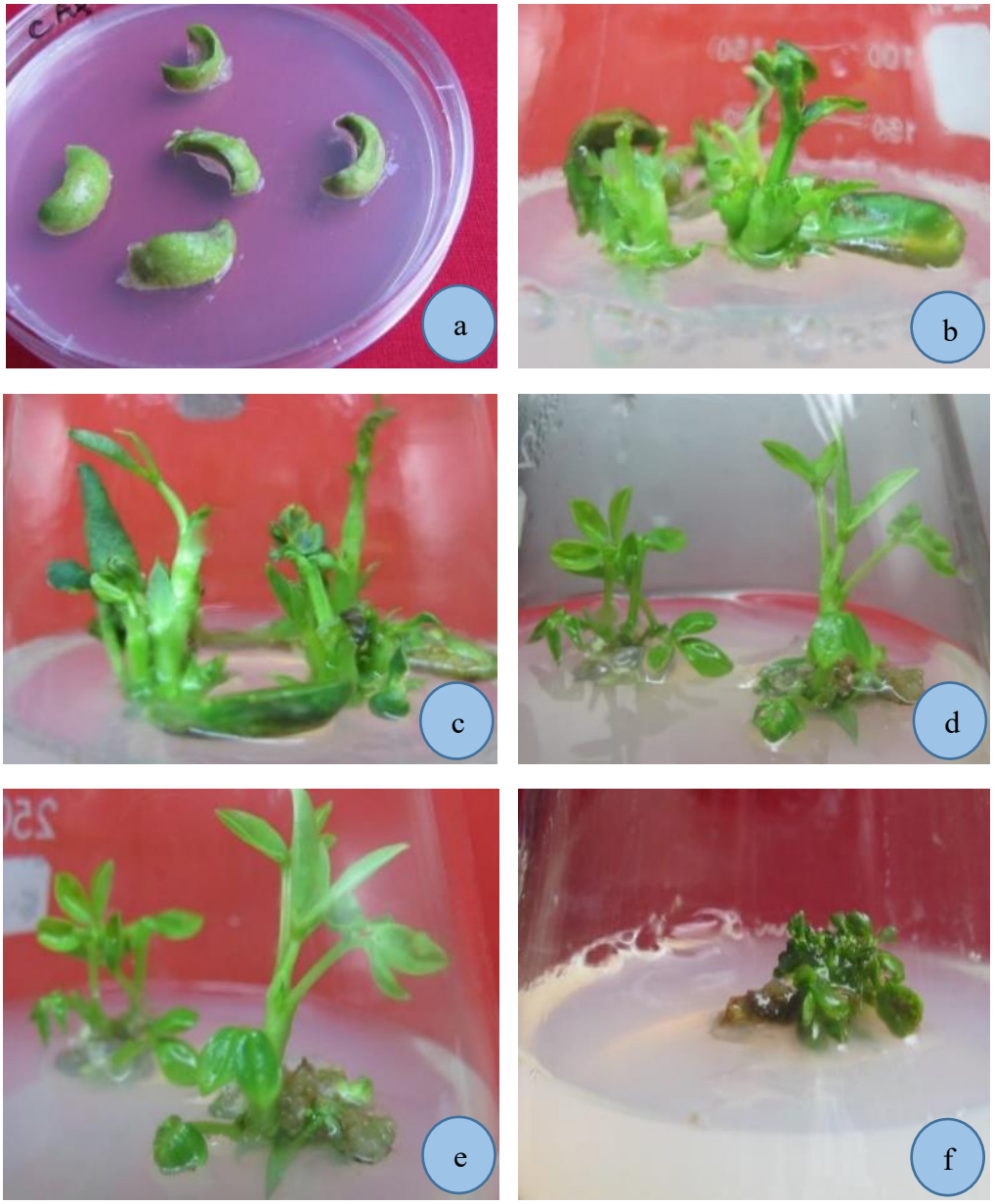


Fig. 30 (a - f): Different stages of shoot development from SCADE explant of BARI Badam-8 on MSB with BAP. (a) Cotyledons became Green on SIM1 (MSB with 88.8 µM); b) Multiple shoot initiation on SIM1; (c) Multiple shoots formation with callus on SIM2 (MSB with 66.6 µM BAP) after 25-30 days culture; (d) Proliferation of multiple shoots in the shoot elongation medium, SEM (MSB with 13 µM BAP); (e) Multiple shoots along with elongated shoots on SEM; (f) Elongated shoots developed on SEM.

4.2.4 Effect of various growth regulators on the induction of roots from regenerated shoots

A crucial stage in the creation of plantlets is the induction of roots at the base of *in vitro* regenerated shoots. Generally, *in vitro* regenerated peanut shoots did not spontaneously develop roots. In the current investigation, different concentrations of IAA, IBA, and NAA, two different auxins, were utilized to induce roots. Shoots that were developed in vitro and ranged in length from 2.5 to 4.0 cm were removed and cultured on MS medium at half strength with different concentrations of the auxins. Rooted plantlets from all two varieties were transplanted to soil in small plastic pot for their establishment.

4.2.4.1 Effect of varying IBA concentrations on half-strength MS for root induction

To investigate their effects on the induction of roots from the *in vitro* regenerated shoots of two peanut varieties, half strength of MS media with three different concentrations of IBA (1.0, 2.5, and 5.0 µM) were utilized. Results on root induction in two peanut varieties using different concentrations of IBA have been shown in Table 16. On half strength of MS medium containing 2.5 M IBA, a high frequency of root induction was seen in both Dhaka-1 and BARI Badam-8 Figs. 31. (a -b). It was recorded that 85% regenerated shoots of Dhaka-1 and that of 80% of BARI Badam-8 formed well developed roots on 2.5 µM IBA supplemented media. Root initiation was noticed within 8 - 22 days of culture while it took about 22 - 30 days were required to get fully developed roots in Dhaka-1 variety. In BARI Badam-8 roots were initiated within 9 - 18 days of culture while it took about 23 - 30 days to get fully developed roots. The number of roots were 7 - 14 in case of Dhaka-1 and that of 6 - 10 for BARI Badam-8 (Fig. 31c). It was noted that no callus formed when roots were begun in IBA enriched media at the base of cut ends of in vitro regenerated shoots (Fig. 31d). Occasionally callus was found to form at the cut end after 20 – 35 days of culture (Figs. 31 (e -f).

4.2.4.2 Effect of various concentrations of IAA on half strength of MS for the root's induction

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 two varieties of peanut. Table 17. has been presented the effect of those concentrations of IAA. The best root induction for Dhaka-1 was seen on half-strength MS medium with 1.0 M of IAA. Higher percentage (80%) 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 were 8 - 14 when the shoots were cultured on 1.0 µM IAA supplemented media. For BARI Badam-8, the best induction of roots (75%) was seen on half-strength MS medium containing 1.0 M IAA, and there were 6 to 12 roots in total. In BARI Badam-8 roots were initiated within 10 - 16 days of culture while it took about 25 - 30 days to get fully developed roots.

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 Figs. 32 (a-f)

Table 16. Effect of different concentration of IBA on half strength of MS towards formation of roots from regenerated shoots of two 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	9 - 18	22 - 30	3 - 7
	2.5	**20**	**85**	**8 - 22**	**22 - 30**	**7 - 14**
	5.0	20	80	10 - 18	24 - 30	6- 11
BARI Badam-8	1.0	20	65	9 - 22	20 - 30	5 - 6
	2.5	**20**	**80**	**9 - 18**	**23 - 30**	**6 - 10**
	5.0	20	75	11- 20	25 - 30	5 - 9

Table 17. Effect of varying IAA concentrations on MS media at half strength for inducing roots from regenerated shoots of two peanut varieties.

Varieties	Concentration of IAA (µM)	Number of shoots 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**	**80**	**10 - 15**	**25 - 30**	**8 - 14**
	2.5	20	75	10 - 18	28 - 32	6 - 10
	5.0	20	75	13 - 20	28 - 30	5 - 10
BARI Badam-8	**1.0**	**20**	**75**	**10 - 16**	**25 - 30**	**6- 12**
	2.5	20	55	12 - 20	25 - 30	6 - 9
	5.0	20	65	12 - 18	25 - 28	4 - 10

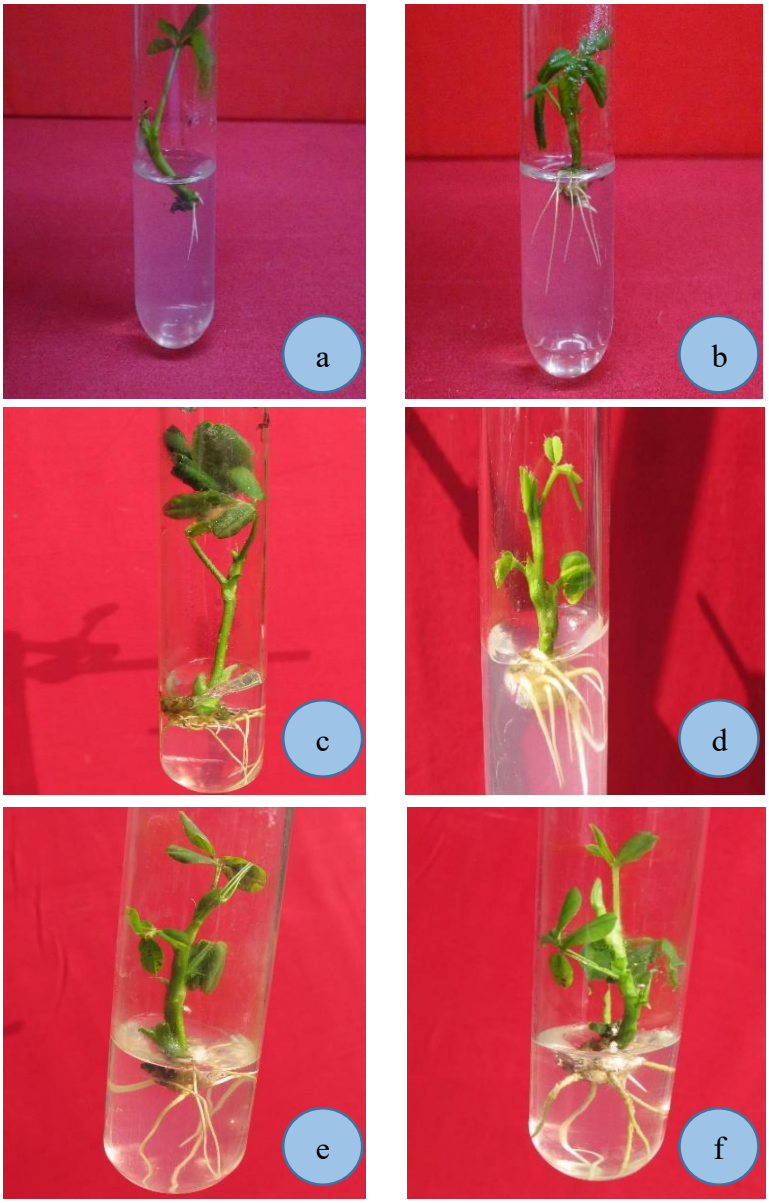


Fig. 31 (a - f): Formation on roots from the cut ends of the *in vitro* regenerated shoots on half strength of MS medium containing various concentrations of IBA in case of two varieties of peanut. (a) Initiation of roots from the shoots Dhaka-1 on 1/2MS medium containing 2.5 µM IBA; (b) Formation of roots from the shoots Dhaka-1 on the same medium as mentioned in Fig. 31a; (c) Fully developed roots of Dhaka-1 on 2.5 µM IBA containing medium; (d) Initiation of roots in BARI Badam-8 containing 2.5 µM IBA; (e) Formation of fully developed roots at the base of cut end of BARI Badam-8 on 2.5 µM IBA supplemented media; (f) Fully developed roots at the base of cut end of BARI Badam-8 on 2.5 µM IBA supplemented media.

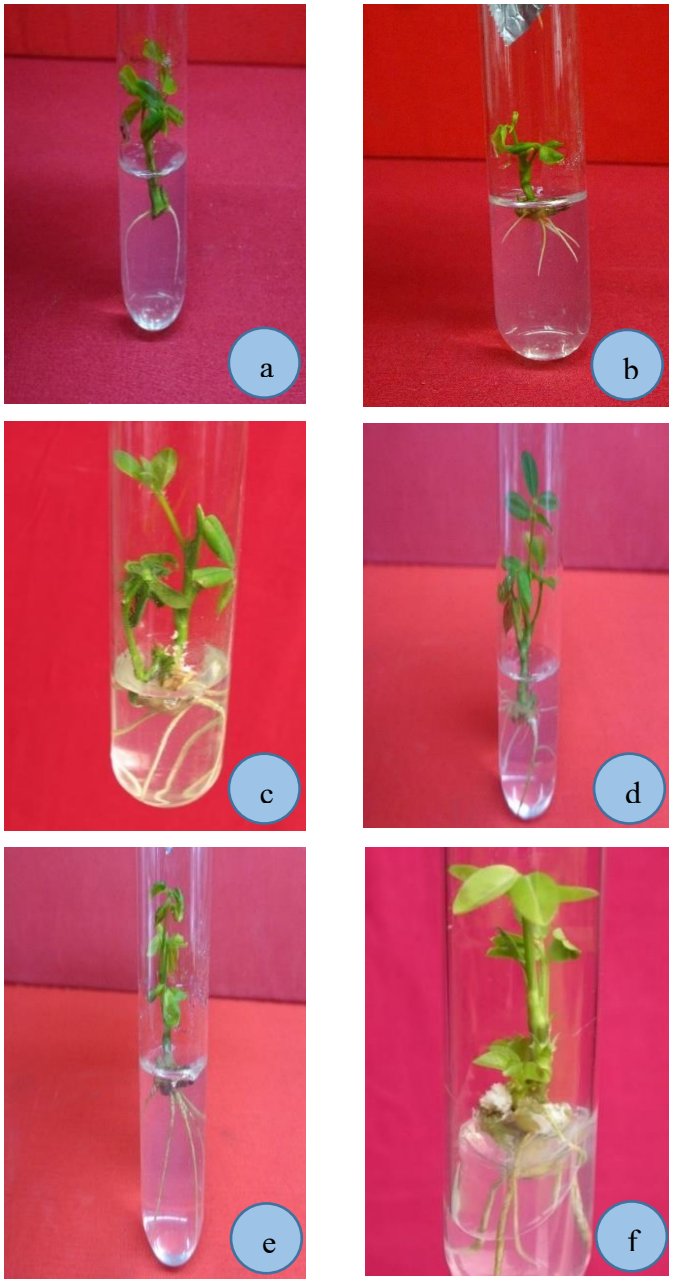


Fig. 32 (a - f): Formation of roots from the cut ends of the regenerated shoots on half strength of MS medium containing various concentrations of IAA in case of two varieties of peanut. (a) Initiation of roots from callus like structure of Dhaka-1 containing 1.0 µM IAA; (b) Initiation of multiple roots on 1.0 µM IAA in Dhaka-1; (c) Formation of multiple roots along with callus in Dhaka-1 on 1.0 µM IAA; (d) Formation of roots in BARI Badam-8 on 1.0 µM IAA supplemented media; (e) Fully developed roots of BARI Badam-8 on the same medium; (f) Fully developed roots in BARI Badam-8 on the same medium.

4.2.4.3 Effect of various NAA concentrations on the half strength of MS towards formation of roots in two peanut varieties

The growth of roots on half-strength MS media was the subject of a series of experiments to test the effects of NAA. For this purpose, three different concentrations of NAA (1.0, 2.5 and 5.0 µM NAA) were used for induction of roots in two varieties of peanut. In this case profuse callusing was observed along with induction of roots on various combinations of NAA. The outcomes of this experiment are shown in Table 18. It has been observed that all two 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, 60% of regenerated shoots were started to initiate roots within 13 - 19 days of culture in Dhaka-1. It was also observed that 22 - 27 days were required to get fully developed roots in the variety of Dhaka-1. In case of BARI Badam-8 roots were started to initiate within 15 - 19 days and highest percentage of (60%) 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 22 - 30 days of culture. Figs. 33 (a - f) shows the results of root formation on various combinations of NAA with half strength of MS media.

4.2.4 Transplantation of rooted plantlets and formation of pods

Dhaka-1 and BARI Badam-8 peanut variety plantlets with completely formed roots were successfully transplanted into small plastic pots filled with autoclaved soil (Figs. 35 and 36). (a-b). The section 3.10.5 of the document describes the transplantation process. The survival rate of the transplanted plantlets was determined using this method to be approximately 95% for Dhaka-1 and 90% for BARI Badam-8 (Table 19). The plantlets that made it were moved to earthen pots for further growth and establishment before being planted in the field. Figs. 34 and 35 (b - e) and Figs. 34 and 35 (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. 34f and Figs. 34 and 35(d- e).

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

Variety	Conc. of NAA (µM)	Number of shoots 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	40	16- 20	20 - 28	2 - 3
	2.5	**20**	**60**	**13 - 19**	**22 - 27**	**3 - 10**
	5.0	20	50	18 - 25	25 - 30	4 - 9
BARI Badam-8	1.0	20	35	17 - 20	22 - 32	2 - 4
	2.5	**20**	**60**	**15 - 19**	**22 - 30**	**5 - 12**
	5.0	20	45	20- 25	25 - 30	3 - 9

Table 19. Survival rates of rooted plantlets transplanted into soil.

Varieties	No. of plants transplanted in soil	No. of survival plants in soil	% Of survival plants in soil
Dhaka-1	10	9	95
BARI Badam-8	10	8	90

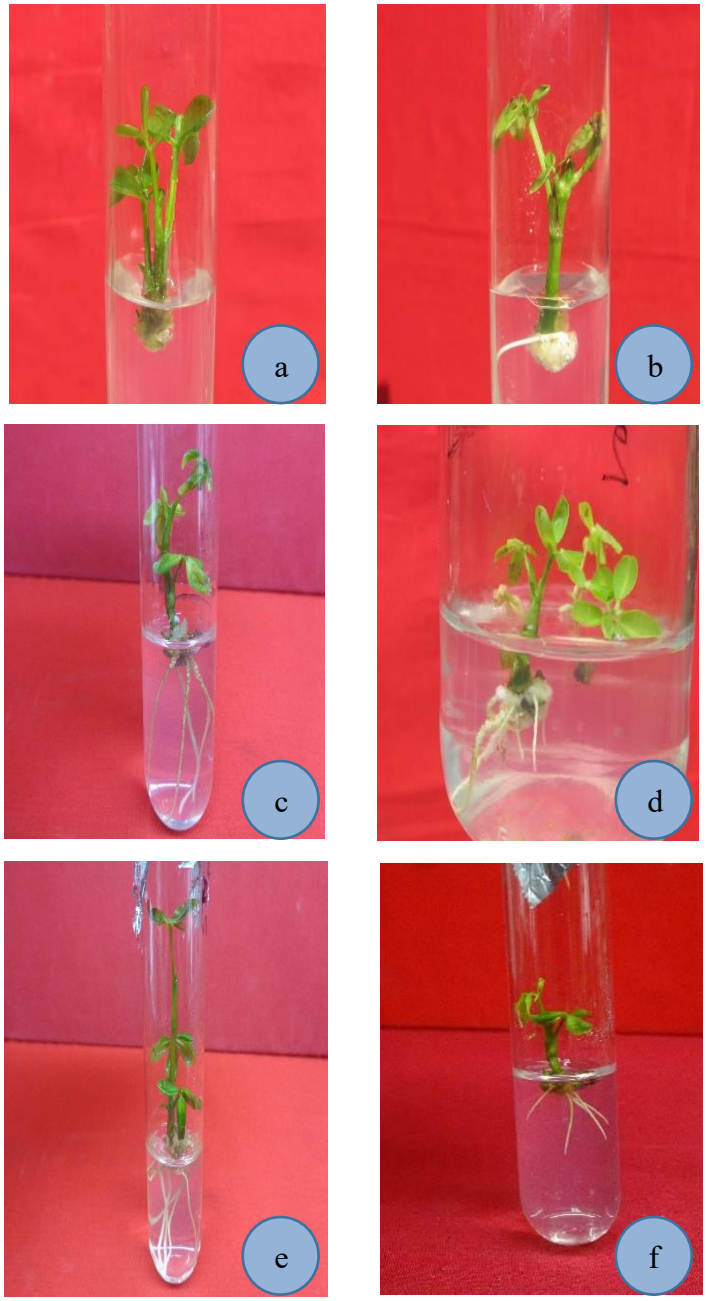


Fig. 33 (a - f): Formation of roots from the cut ends of the regenerated shoots on half strength of MS medium containing various concentrations of NAA in case of two varieties of peanut. (a) Formation of roots from callus like structure of Dhaka-1 containing 2.5 µM NAA; (b) Initiation of roots on 2.5 µM IAA in Dhaka-1; (c) Formation of multiple roots along with callus in Dhaka-1 on 2.5 µM IAA; (d) Formation of roots in BARI Badam-8 on 2.5 µM IAA supplemented media; (e) Fully developed roots of BARI Badam-8 on 2.5 µM IAA supplemented media; (f) Fully developed roots in BARI Badam-8 on the same medium.

Fig. 34 (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. 35 (a - f). Acclimatization as well as formation of pods from *in vitro* regenerated plantlets of BARI Badam-8; (a) *In vitro* regenerated plantlets of BARI Badam-8 transferred to soil in small pots; (b) Transplantation of BARI Badam-8 in large earthen pots; (c) Young plantlets with flower in large earthen pots; The peg (arrow) inserted into the soil; (e) Mature peanut plants before harvest; f) Harvested peanut plants (BARI Badam-8) with pods.

4.3 Genetic Transformation

Several experiments were conducted to integrate drought and salinity tolerance gene in peanut using *Agrobacterium*-mediated genetic transformation, for this purpose, two strains of genetically engineered *Agrobacterium*, namely, LBA4404 (pBI121) and EHA105/pCAMBIA1301- PDH45 (section 2.2) were used. This LBA4404 (pBI121) strain has the *GUS* reporter gene and the *nptII* gene (neomycin phosphotransferase II), which confers a selectable marker for kanamycin resistance. The *PDH45* or Pea DNA Helicase 45 gene confers drought and salt tolerance, and the *hptII* or hygromycin resistance gene serves as a selective marker in the EHA105/pCAMBIA1301-PDH45 strain (the gene of interest). Transformation experiments were undertaken using single cotyledon attached de-capitated embryo (SCADE) and de-embryonated half cotyledon (DEHC) explants of peanut variety, namely, Dhaka-1 and BARI Badam-8.

4.3.1 Transformation with SCADE and DEHC explants

Single cotyledon attached decapitated embryo (SCADE) and de-embryonated half cotyledon (DEHC) explants were used for genetic transformation using LBA4404 (pBI121) and EHA105/pCAMBIA1301-PDH45 strain of genetically engineered *Agrobacterium tumefaciens* in this investigation. The Dhaka-1 and BARI Badam-8 peanut varieties, as well as single cotyledon attached decapitated embryo (SCADE) and de-embryonated cotyledon (DEHC) explants, were used in the transformation tests. The findings of these experiments are reported below under different headings.

4.3.1.1 Optimization of various parameters influencing transformation efficiency using Agrobacterium strain (LBA4404 (pBI121)

It is evident from the previous report that *Agrobacterium*-mediated genetic transformation efficiency is impacted by several variables, such as optical density (OD) of *Agrobacterium* suspension, duration of co-cultivation period of transformation, etc. These parameters were optimized during this study. Optimization of these conditions was done by monitoring the transient expression of the *GUS* gene.

4.3.1.2 Influence of optical density of Agrobacterium LBA4404 (pBI121) on transformation

The optical density of the *Agrobacterium* suspension is one of the most significant influencing elements of transformation. Overnight grown *Agrobacterium* suspension was used to infect the explants for transformation. Optical density (OD) was measured at 600 nm. In this experiment, bacterial suspension with optical densities of 0.6, 0.8 and 1.0 with few seconds, 10, 15 minutes incubation periods were used. Tables 20 and 21 show the results of this set of experiment. The maximum transformation percentage of both the explants was observed at 1.0 OD in case of Dhaka-1. Simila results were observed in case of BARI Badam-8. But afterwards it was observed that at 1.0 OD the explants showed bacterial overgrowth. Therefore, *Agrobacterium* suspension with OD 0.6 and sometimes 0.8 were used for all the transformation experiments for both the varieties.

Table 20. Transient GUS histochemical assay for Dhaka-1 variety used to determine the effect of *Agrobacterium* (strain LBA4404pBI121) optical density (measured at 600 nm) on transformation of DEHC and SCADE explant.

Explants	OD600	No. of explants infected	No. of explants assayed for GUS	No. of explants +ve for GUS	% of GUS +ve explants
De-embryonated half cotyledon (DEHC)	0.6	50	25	18	72
	0.8	50	25	22	88
	1.0	50	25	23	92
Single cotyledon attached decapitated embryo (SCADE)	0.6	50	25	21	84
	0.8	50	25	20	80
	1.0	50	25	22	88

Table 21. Transient GUS histochemical assay for BARI Badam-8 variety used to determine the effect of *Agrobacterium* (strain LBA4404pBI121) optical density (measured at 600 nm) on transformation of DEHC and SCADE explant.

Explants	OD_{600}	No. of explants infected	No. of explants assayed for GUS	No. of explants +ve for GUS	% Of GUS +ve explants
De-embryonated half cotyledon (DEHC)	0.6	50	25	18	72
	0.8	**50**	**25**	**22**	**88**
	1.0	50	25	23	92
Single cotyledon attached decapitated embryo (SCADE)	**0.6**	**50**	**25**	**22**	**88**
	0.8	50	25	20	80
	1.0	50	25	23	92

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

Another critical factor was the length of time that the explants were cultured in an *Agrobacterium* suspension to allow the bacteria to infect the plant tissues. In this set of experiment, the effect of different incubation periods was investigated. For this, bacterial suspensions with consistent optical densities of 0.8 and 0.6 in the case of two types of explants for both varieties were used for incubation times of a few seconds, 10 minutes, and 15 minutes. 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 half cotyledon and single cotyledon attached decapitated embryo explants were incubated for few seconds, 10 and 15 minutes with *Agrobacterium* suspension. De-embryonated half cotyledon and single cotyledon attached decapitated embryo explants showed maximum GUS activity in 10 and 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 10 minutes for de-embryonated half cotyledon and 15 minutes for single cotyledon attached decapitated embryo explants at 0.8 and 0.6 OD of the *Agrobacterium* suspension (Tables 22 and 23).

Table 22. Influence of incubation period (measured at 600 nm) of *Agrobacterium* (strainLBA4404pBI121) on transformation of DEHC and SCADE explant analyzed by transient GUS histochemical assay for Dhaka-1 variety.

Explants	Incubation Period (mins)	No. of explants infected	No. of explants assayed for GUS	No. of explants +ve for GUS	% of GUS +ve explants
De-embryonated half cotyledon	Few seconds	50	25	18	72
	10 min	**50**	**25**	**23**	**92**
	15 min	50	25	08	32
Single cotyledon attached decapitated embryo	Few seconds	50	25	20	80
	10 min	50	25	10	40
	15 min	**50**	**25**	**21**	**84**

Table 23. Influence of incubation period (measured at 600 nm) of Agrobacterium (strainLBA4404pBI121) on transformation of DEHC and SCADE explant analyzed by transient GUS histochemical assay for BARI Badam-8 variety.

Explants	Incubation Period (mins)	No. of explants infected	No. of explants assayed for GUS	No. of explants +ve for GUS	% Of GUS +ve explants
De-embryonated half cotyledon (DEHC)	Few seconds	50	25	18	72
	10 min	**50**	**25**	**24**	**96**
	15 min	50	25	08	32
Single cotyledon attached decapitated embryo (SCADE)	Few seconds	50	25	20	80
	10 min	50	25	10	40
	15 min	**50**	**25**	**22**	**88**

4.3.1.3 Impact of co-cultivation period on transformation

Another important aspect that affects transformation is the co-cultivation duration. The altered explants in this experiment were co-cultured for 2, 3, and 4 days. It was observed that at the constant incubation period (few seconds, 10, 15 minutes and OD (0.6-0.8) the transformation efficiency was slightly different in all durations of co-cultivation. However, bacterial overgrowth was seen during a prolonged co-cultivation time (greater than 3 days). Therefore, when the transformation experiment was carried out under ideal conditions, a co-cultivation period of 3 days was shown to be the most appropriate (Table 24, 25).

Table 24. Effects of various co-cultivation times on the transformation of two explants for the Dhaka-1 variety while using a transient GUS histochemical assay at a fixed OD (600 nm).

Co-cultivation period (days)	No. of explants assayed for GUS expression	No. of GUS +ve explants	% Of GUS +ve explants
2	25	20	80
3	**25**	**22**	**88**
4	25	21	84

Table 25. Effects of various co-cultivation times on the transformation of two explants for the BARI Badam-8 variety when using a transient GUS histochemical assay at a fixed OD (600 nm).

Co-cultivation period (days)	No. of explants assayed for GUS expression	No. of GUS +ve explants	% Of GUS +ve explants
2	25	20	80
3	25	23	92
4	25	21	84

4.3.1.4 Determination of the transformation ability of two peanut varieties

In this set of experiment, the susceptibility of cotyledon explants of two peanut varieties against *Agrobacterium* strain LBA4404 (pBI121) were studied. Transient GUS histochemical assay was carried out to determine the transformation ability of the explants of two peanut varieties using the strain LBA4404 (pBI121) (Table 26). It was determined by GUS histochemical analysis that both types responded positive to transformation (Figs. 36, 37, 38, 39 and 40). However, among the two varieties BARI Badam-8 showed comparatively better transformation ability by showing 92% GUS positive explants in case of cotyledon explants. On the other hand, Dhaka-1 showed lowered transformation ability (88%).

Table 26. Responses of two varieties of peanut to a transient GUS histochemical test utilizing DEHC explants and a plasmid (pBI121) from Agrobacterium strain LBA4404.

Varieties	OD600 of the bacterial suspension	Incubation period	No. of explants inoculated	No. of explants assayed for GUS	No. of explants +ve for GUS	% Of GUS +ve explants
Dhaka-1	0.8	10 minutes	50	25	22	88
BARI Badam-8	0.8	10 minutes	50	25	23	92

4.3.1.5 Regeneration from co-cultured explants

Explants were transferred to regeneration medium on MMS containing 88.8 M BAP after co-cultivation to regenerate shoots. (Fig. 41a). To avoid the excess growth of *Agrobacterium*, 100 mg/1 carbenicillin was added to the medium. Selection pressure was not applied right away following co-cultivation in the current experiment. Rather, no selection agents were added to the medium where the explants were permitted to regenerate. After initiation of shoot-buds selection pressure using kanamycin (in case of LBA4404-pBI121) was applied. After 15 days when greening of explants were shown (Fig. 41b), explants were subcultured on SIM2 media containing 66.6 µM BAP. Regeneration of shoots was found to initiate after 15-20 days of culture on kanamycin free SIM2 medium (Fig. 41c). The regenerating explants were then sub-cultured on the SEM medium containing 13.0 µM BAP (Fig. 41d).

4.3.1.6 Selection of transformed shoots

For the development of transformed plantlets, it is important to select the transformants from the regenerating shoots. Kanamycin was used to select the transformed shoots in the case of LBA4404-pBI121.Various concentrations of kanamycin were applied gradually to select the transformed shoots.

4.3.1.7 Determination of optimum kanamycin concentration against LBA4404 /pBI121 for selection

Kanamycin resistant gene was present in the LBA4404/pBI121 strain as selectable gene. It may be assumed that the shoots developed from the transformed tissues were supposed to survive in the selection medium supplemented with optimum concentration of kanamycin. On the other hand, shoots regenerated from the non-transformed tissues could not survive on selection medium since they did not possess the kanamycin resistant gene (Figs. 41e and 41f). Optimization of selection pressure by applying proper concentration of kanamycin is necessary in obtaining transformed plantlets. Uninfected explants (those used as a negative control) were subjected to selection pressure in order to identify the ideal selection level. In this experiment, the explants were transferred to a regeneration medium containing kanamycin once the regeneration process had begun, specifically MS + 13.0 M BAP + 50.0 mg/1 kanamycin. Then, the kanamycin concentration was progressively raised in the following ways: 50, 100, 150, and 200 mg/1. The results of this experiment showed that the quantity of survived shoots decreased when kanamycin

concentration was raised. Kanamycin caused the shoots to turn albino and eventually died. All of the non-infected explants (negative controls) in this investigation died within 15 days of being exposed to 150-200 mg/1 kanamycin (Figs. 41g and 41h). In order to determine whether shoots from infected explants were transformed, they had to endure in selection medium containing 150 mg/1 kanamycin for more than 15 days. Thus, selecting real transformants surviving in the selection medium was made easier by tests using negative control shoots.

4.3.1.8 Transformation of shoots into roots and establishment in the soil

Separated and transferred to MS medium at half strength, the transformed shoots (2–3 cm in length) that had survived on the selection medium. The addition of 2.5 M IBA to the rooting medium was shown to cause the greatest number of shoots to form roots. On the medium containing half strength of MS + 2.5 M IBA, rooting could not begin for about 15–18 days. The transformed shoots on the rooting medium containing half the strength of MS with 2.5 mM/l IBA are shown in Fig. 42a. With a strong root system, the healthy plantlets were recovered. After being properly acclimated, these transformed plantlets were transplanted into soil and found to survive. (Figs. 41b and 41g).

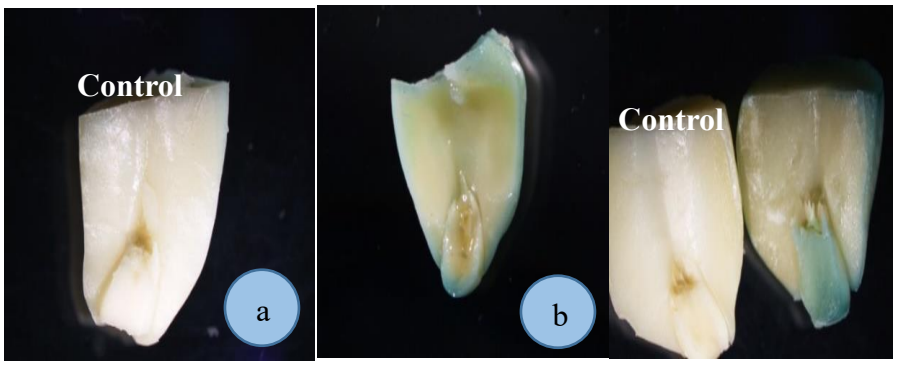


Fig. 36. GUS expression in SCADE explants of (a) Dhaka-1 and (b) BARI Badam-8

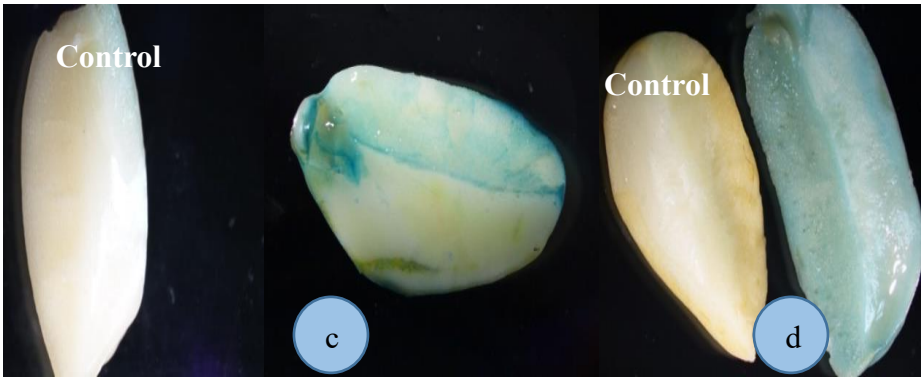


Fig. 36. GUS expression in DEHC explants of (c) Dhaka-1 and (d) BARI Badam-8

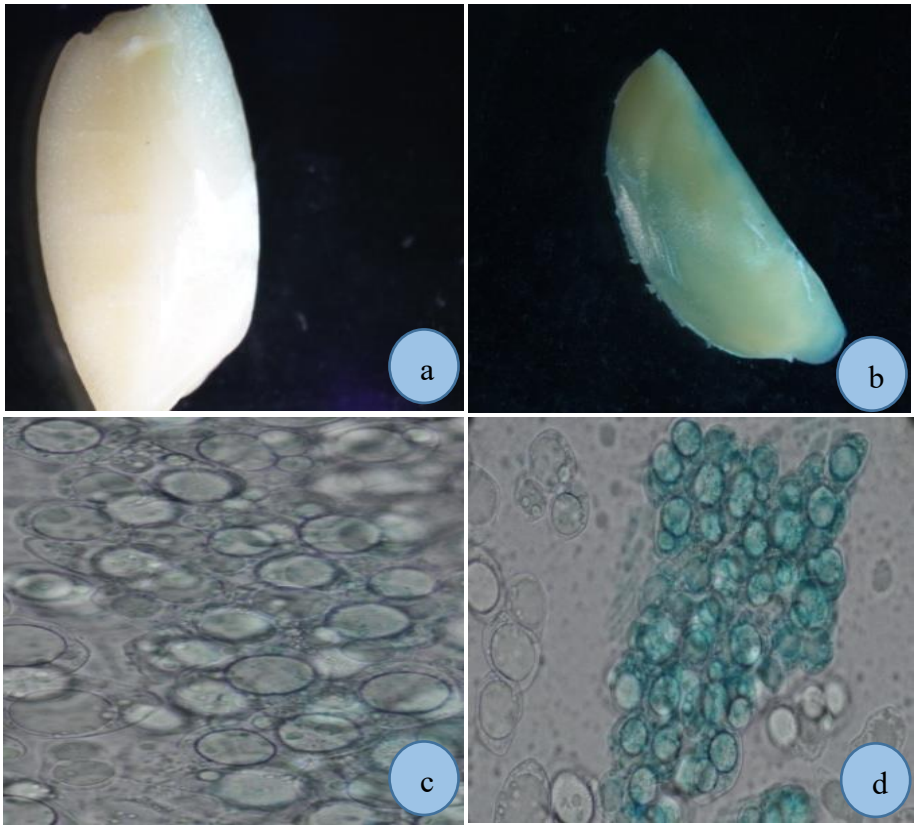


Fig. 37 (a-d): Histochemical localization of GUS activity of DEHC explants infected with LBA4404/pBI121 strain of *Agrobacterium* in case of variety Dhaka-1 with control. (a) Control explants; (b) Histochemical localization of GUS activity of cotyledon explants; (c) A part of macerated tissue of de-embryonated cotyledon explants of Dhaka-1 variety showing the absence of GUS positive blue color; (d) A part of macerated tissue of cotyledon explants of Dhaka-1 variety showing the presence of GUS positive blue color.

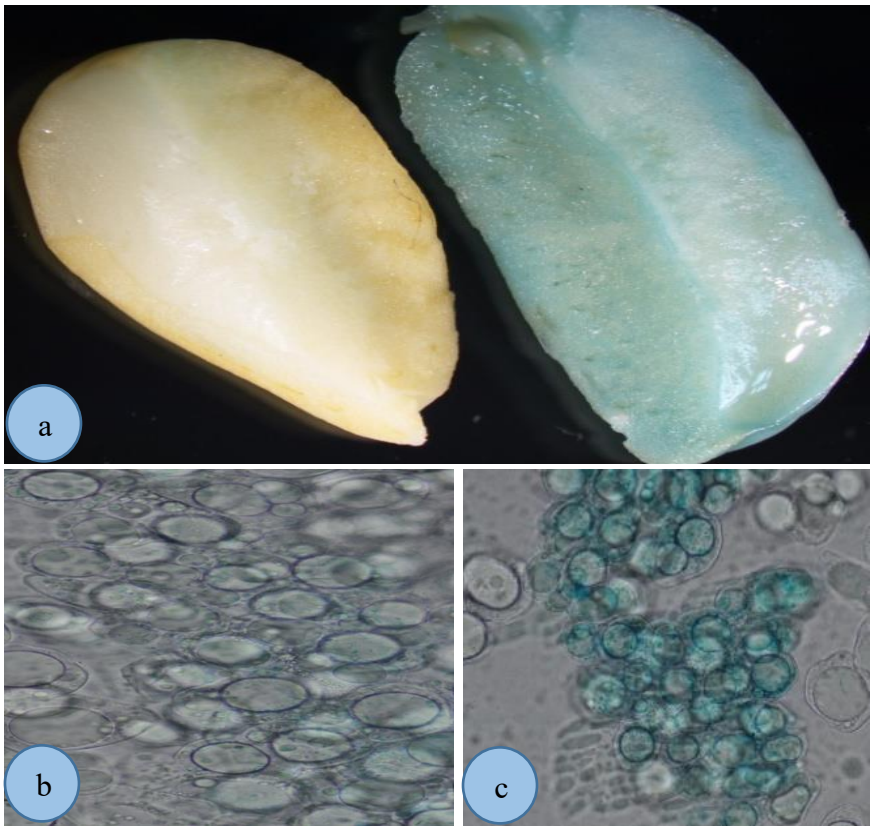


Fig. 38 (a-c): Histochemical localization of GUS activity of DEHC explants infected with LBA4404/pBI121 strain of *Agrobacterium* in case of variety BARI Badam-8 with control. (a) Control explants and histochemical localization of GUS activity of cotyledon explants; (b) A part of macerated tissue of de-embryonated cotyledon explants of BARI Badam-8 variety showing the absence of GUS positive blue color; (c) A part of macerated tissue of cotyledon explants of BARI Badam-8 variety showing the presence of GUS positive blue color.

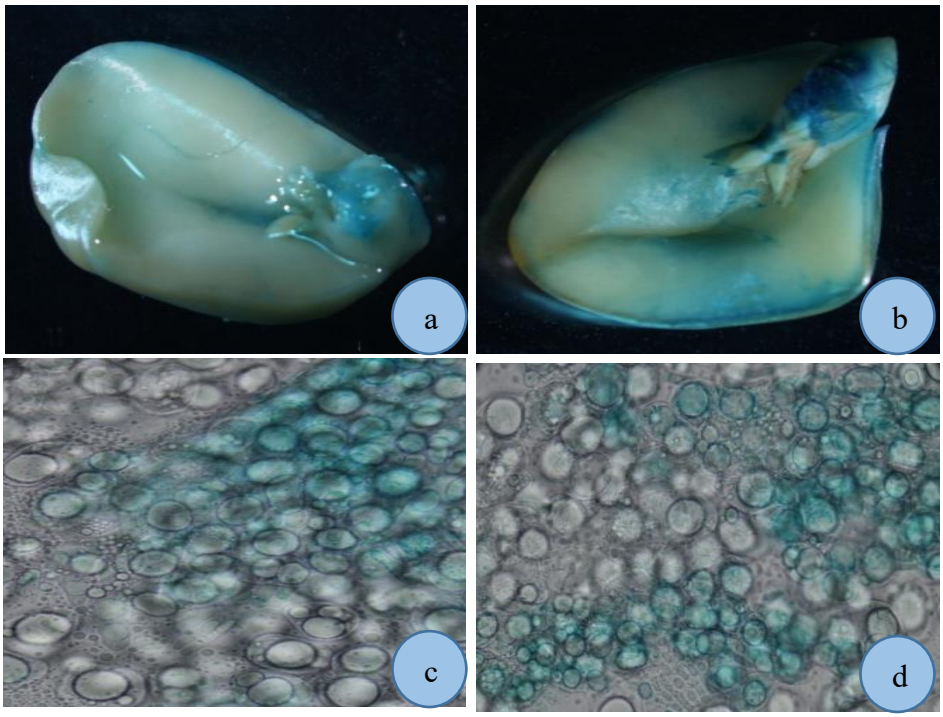


Fig. 39 (a-d): Histochemical localization of GUS activity of single cotyledon attached decapitated embryo explants infected with LBA4404/pBI121 strain of *Agrobacterium* of variety Dhaka-1 and BARI Badam-8. (a) Histochemical localization of GUS expression of explants in case of Dhaka-1 (b) Histochemical localization of GUS expression of explants in case of BARI Badam-8; (c) A part of macerated tissue of explants of Dhaka-1 variety showing the presence of GUS positive blue color; (d) A part of macerated tissue of explants of BARI Badam-8 variety showing the presence of GUS positive blue color.

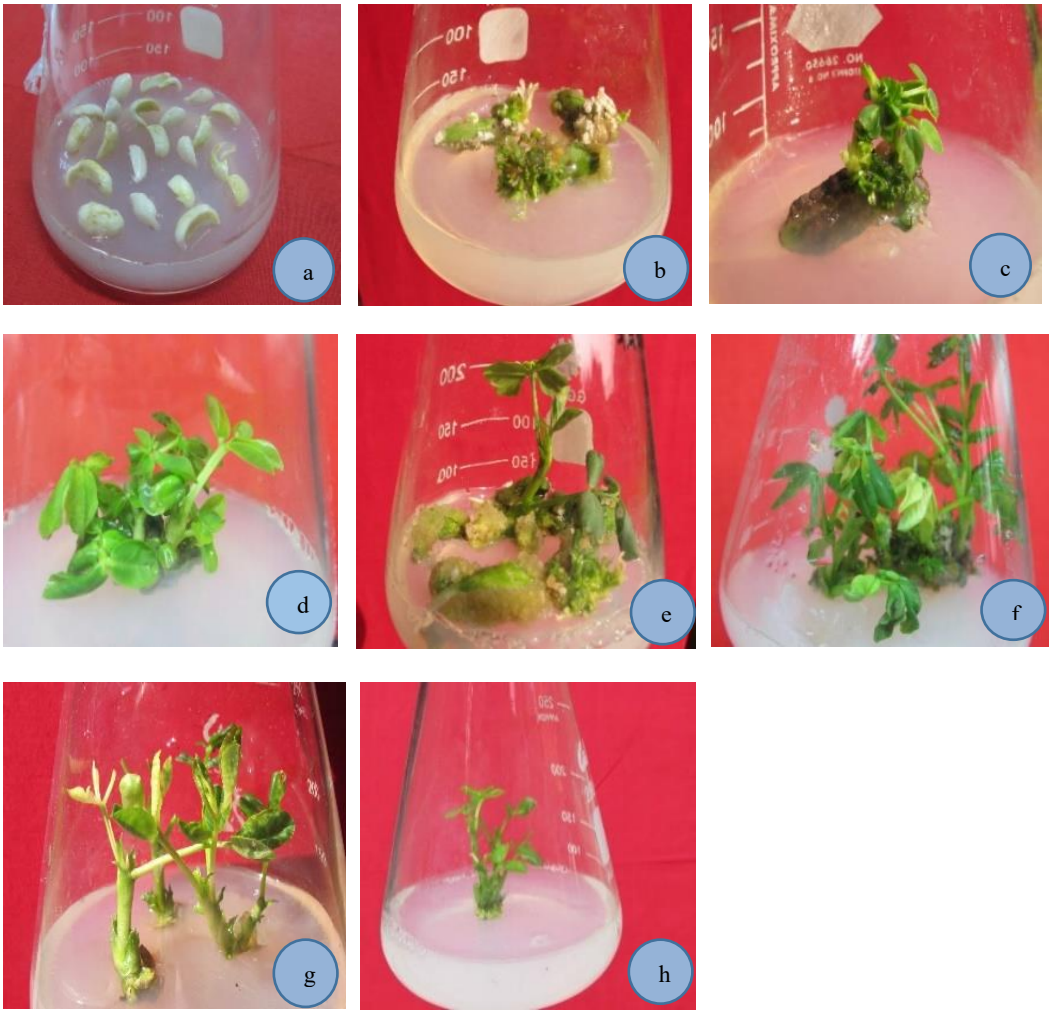


Fig. 40 (a - h): Different stages of shoot regeneration and selection of putatively transformed plants from DEHC explants of BARI Badam-8 variety. (a) Infected DEHC explants on selection free regeneration medium SIM1 (MMS with 88.8 µM BAP) after co-cultivation; (b) Greening and shoot formation from infected DEHC explants without selection pressure; (c). Induction of adventitious shoot buds from explants after 15 days of culture on SIM2 (MMS medium supplemented with 66.6 µM); (d) Development of multiple shoots from infected DEHC explants on 100 mg/l kanamycin supplemented; (e) Multiple shoot regeneration and Shoot elongation after 15 days on SEM (MMS medium supplemented with 13.0 µM); (f) Shoots of BARI Badam-8 on the selection medium containing 150 mg/l kanamycin ; (g) Putative transformed shoots survived on 200 mg/l kanamycin supplemented media; h) Shoots survived on selection medium.

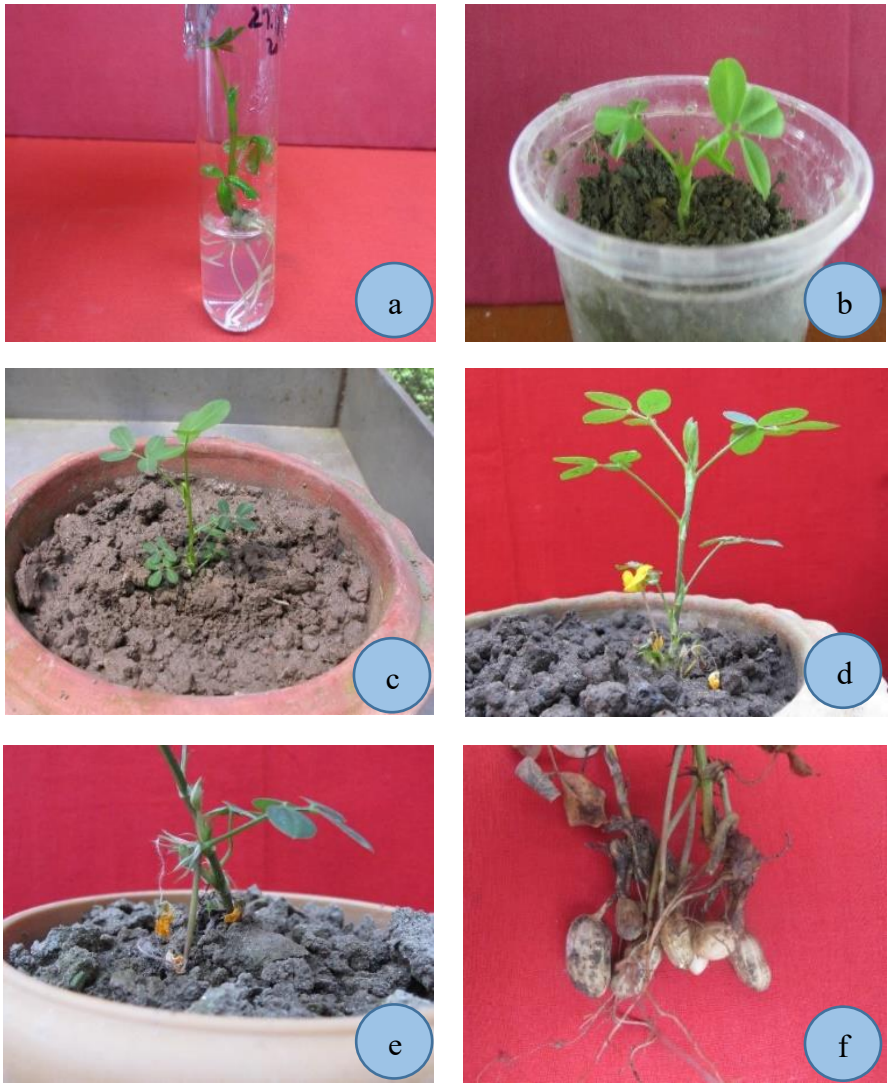


Fig. 41 (a - f): Formation of roots from base of the regenerated shoots and transplantation of putatively transformed plantlets of BARI Badam-8 in soil. a) Formation of roots from the base of the putatively transformed shoots of BARI Badam-8; (b) Transplantation of putatively transformed plantlets in small plastic pots containing soil; (c) Fully developed putatively transformed plantlet in large earthen pot; d) Young plantlets with flower in large earthen pot containing soil.; e) The peg (arrow) inserted into the soil; f) Harvested peanut plants (BARI Badam-8) with pods.

4.3.2 Employing a strain of *Agrobacterium* to optimize various parameters affecting transformation efficiency (pCAMBIA1301-PDH45)

Using the EHA105/pCAMBIA1301-PDH45 strain, *Agrobacterium*-mediated genetic transformation was used to incorporate the *PDH45* gene into the genome of peanut cultivars. (Section 4.3).

During the current experiment, the impact of optical density (OD) of the *Agrobacterium* suspension, incubation, and co-cultivation duration of the explants with the *Agrobacterium* suspension towards transformation was examined. Optical density (OD) was measured at 600 nm for this purpose, and bacterial suspensions with OD values of 0, 0.4, 0.5, 0.6, and 0.8 were utilized, with incubation times of 20, 25, and 30 minutes. It was discovered that bacteria overgrew in the co-cultivation media when the incubation time exceeded 25 minutes and the optical density (OD) of the bacterial suspension exceeded 0.5. When the explants were infected with the *Agrobacterium* suspension with an OD of 0.5 or less with an incubation period of 25 minutes or less, the overgrowth was minimal. Therefore, 25 minutes of incubation period with optical density of 0.5 was found to be optimum for the subsequent experiments.

The length of co-cultivation was crucial for recovering explants following transformation. To determine the ideal co-cultivation period, various co-cultivation periods ranging from 2-3 days with OD of 0.5 were explored. A co-cultivation lasting longer than two days was found to encourage bacterial proliferation. Because of this, most of the explants in co-culture media had poor health and did not regenerate. Based on the findings, it was shown that using this strain to transform the BARI Badam-8 type of peanut required 2 days of co-cultivation with an optical density of 0.5.

4.3.2.1 Regeneration from co-cultured explants

Transformation experiments were performed with DEHC and SCADE explants of BARI Badam-8 using *Agrobacterium* strain EHA105 containing plasmid pCAMBIA1301-PDH45. After co-cultivation, the explants were washed with sterile distilled water for 4-5 times and then washed for 10 minutes with 300 mg/l carbenicillin. The explants were soaked on a sterile Whatman filter paper to remove excess bacterial suspension. After that explant were transferred to SIM1(MSB with 88.8 µM BAP) for regeneration of BARI Badam-8. After 14-15 days the cotyledons were transferred to SIM2 (MSB with 66.6 µM

BAP). To control the overgrowth of *Agrobacterium* 300 mg/l carbenicillin was added to the regeneration media. After 14-15 days the explants were transferred to SEM (MSB with 13.0 µM BAP) containing 300 mg/l carbenicillin to control the overgrowth of *Agrobacterium*. Figs. 42 (a, d) and 44(a, d) show the results of these observations.

4.3.2.2 Selection of transformed shoots

The selection of transformed shoots is a crucial step in the development of a successful transformation protocol. Hygromycin was used as the selection agent in this investigation. After co-cultivation, the selection pressure was not used for the selection of transformed tissues. Instead, the explants were allowed to regenerate on SIM1, which contained 300 mg/l of carbenicillin but no hygromycin. Carbenicillin and 20 mg/l hygromycin were added to the subsequent SIM2 and SEM.

It has been observed that the initial shoot buds did not show any sign of further growth or even died when the selection agents were used during the first subculture. Following this result, the initiated shoot buds along with the cotyledons were sub-cultured on SEM along with the selection agents.

4.3.2.3 Elimination of non-transformed shoots

To choose putatively transformed shoots, non-transformed shoots must be eliminated. The shoots that were not transformed failed to survive under selection pressure. Hygromycin was used as the selection agent in these transformation experiments. Only green shoots and shoot buds were subcultured into fresh media containing hygromycin, a selective agent, leaving dead tissues and shoots in the SIM2 and SEM.

4.3.2.4 Using hygromycin to select transformed BARI Badam-8 shoots to combat strain EHA105 (pCAMBIA1301-PDH45)

As successfully transformed shoots have the capability to express the gene conferring hygromycin resistance, they were supposed to survive on the selection medium. Initially a few regenerated adventitious shoot buds were directly subjected to SIM2 containing 20 mg/l hygromycin immediately after the explants were co cultivated and maintained on SIM1 for 14-15 days.

Therefore, SIM2 containing the initial selection pressure (20 mg/l hygromycin) was applied when shoots regenerated on SIM1. After 14 days, the non-transformed shoots became pale and gradually died due to necrosis and only green shoots were sub-cultured on fresh medium containing 20 mg/l hygromycin (Figs. 42 (e, h) and 44 (e, h). Among approximately 150 regenerated shoots after transformation, 44 shoots survived on second subculture with 20 mg/l hygromycin concentration in case of BARI Badam-8 variety (Table 27). The percentage of survived shoots was 3.66 (DEHC explants) and 2.85 (SCADE explants) on selection medium.

Table 27. Effect of hygromycin on selection of shoots of BARI Badam-8 from de-embryonated half cotyledon (DEHC) explants and single cotyledon attached decapitated embryo explants (SCADE) infected with strain EHA105/pCAMBIA1301-PDH45.

No. of infected explants	No. of regenerated shoots on SIM after transformation	No. of shoots survived on SEM with hygromycin (20 mg/l)		% of survived shoots
		1 st subculture	2 nd subculture	
(DEHC)1200	150	75	44	3.66
(SCADE)700	75	60	20	2.85

4.3.2.5 Root induction from the transformed shoots and establishment in the soil

Separated and transferred to MS medium at half strength, the transformed shoots (2–3 cm in length) that had survived on the selection medium. The greatest number of shoots were found to induce roots, when the rooting medium was supplemented with 2.5 µM IBA. About 15-18 days was required to initiate rooting on the medium containing half strength of MS + 2.5 µM IBA. The transformed shoots on rooting media containing half the strength of MS with 2.5 mM/l IBA are shown in Figs. 43a and 45a.The healthy plantlets were recovered with adequate root system. The transformed shoots on rooting media containing half the strength of MS with 2.5 mM/l IBA are shown in Figs. 43a and 45a.

Table 28. Response of survived shoots of BARI Badam-8 towards *in vitro* root formation.

Explants	No. of shoots (under selection) inoculated for root induction	Days required for root initiation	No. of shoots showed rooting response	% of rooting response	
DEHC	44	21 – 42	30	68.18	43.33
SCADE	20	20-44	8	40.0	50.0

Fig. 42 (a-h): Different stages of regeneration of shoots and selection of putatively transformed plants DEHC explants of variety BARI Badam-8. (a) Infected DEHC explants on selection free regeneration medium after co-cultivation; b) DEHC explants being green in color and induction of adventitious shoot buds from explants after 15 days of culture on SIM1. (c) Stereomicroscopic view of formation of compact callus (arrow) in distal end instead of shoot formation on SEM; (d) Development of multiple shoots from infected DEHC explants on SEM; (e) Shoots survived on regeneration media containing 20mg/l hygromycin; (f) Multiple and elongated shoots survived on regeneration media containing 20mg/l hygromycin; (g) The non-transformed shoots became pale and gradually died due to necrosis after 14 days); (h) Non transformed shoots were leading to death on 20 mg/l hygromycin containing regeneration medium.

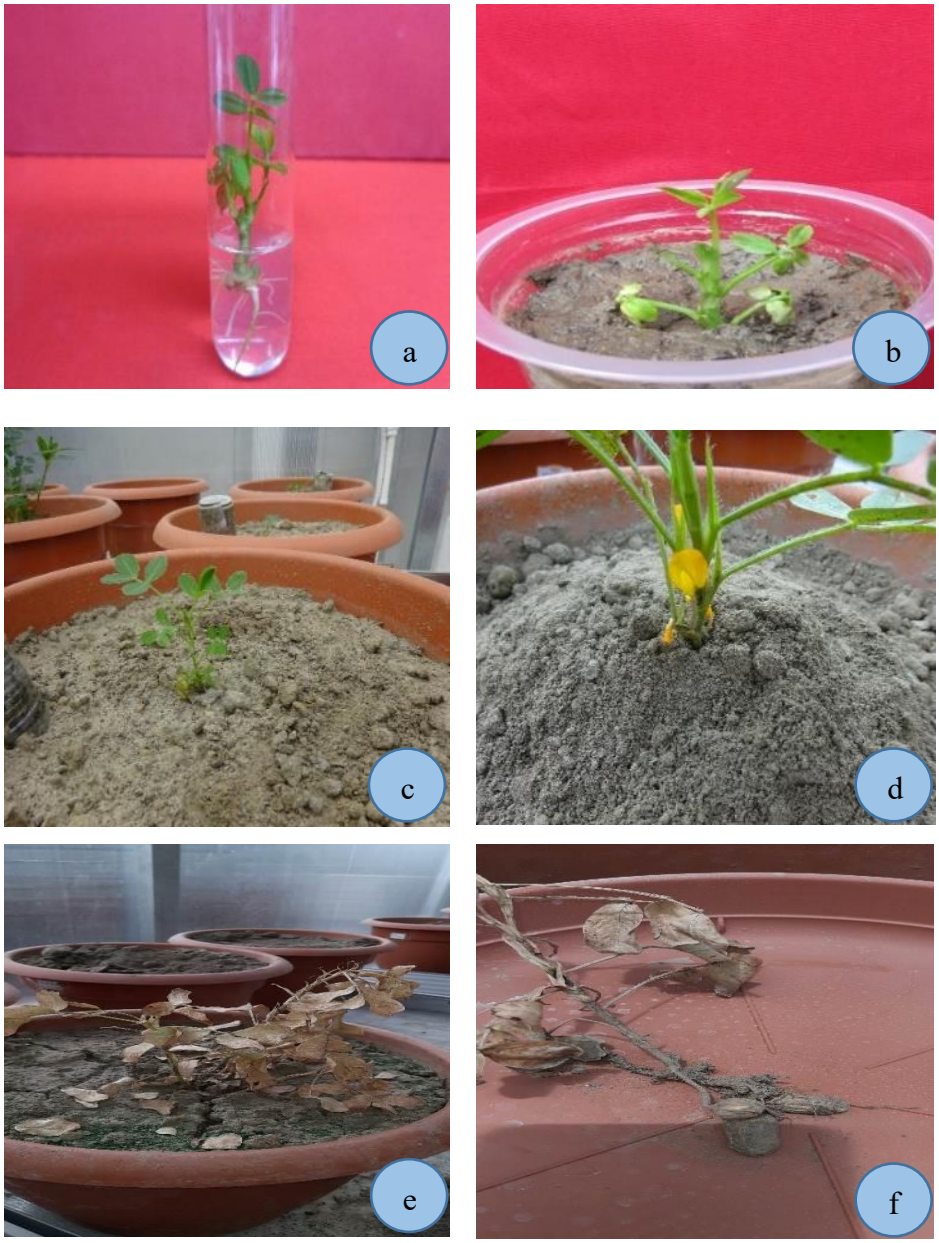


Fig. 43 (a - e): Formation of roots from base of the putatively transformed shoots and transplantation of transformed plants in soil. (a) The putatively transformed shoots were transferred on root induction medium (half strength of MS medium with 2.5 µM IBA) and formation of roots from the cut end of the putatively transformed shoots of BARI Badam-8; (b - c) Developed the T_0 plant in plastic and earthen pot maintained in double layered insect proof net house prepared for maintaining proper biosafety regulation. (d) Young plantlets with flower in large earthen pot containing soil; (e-f) Harvested peanut plants (BARI Badam-8) with pods.

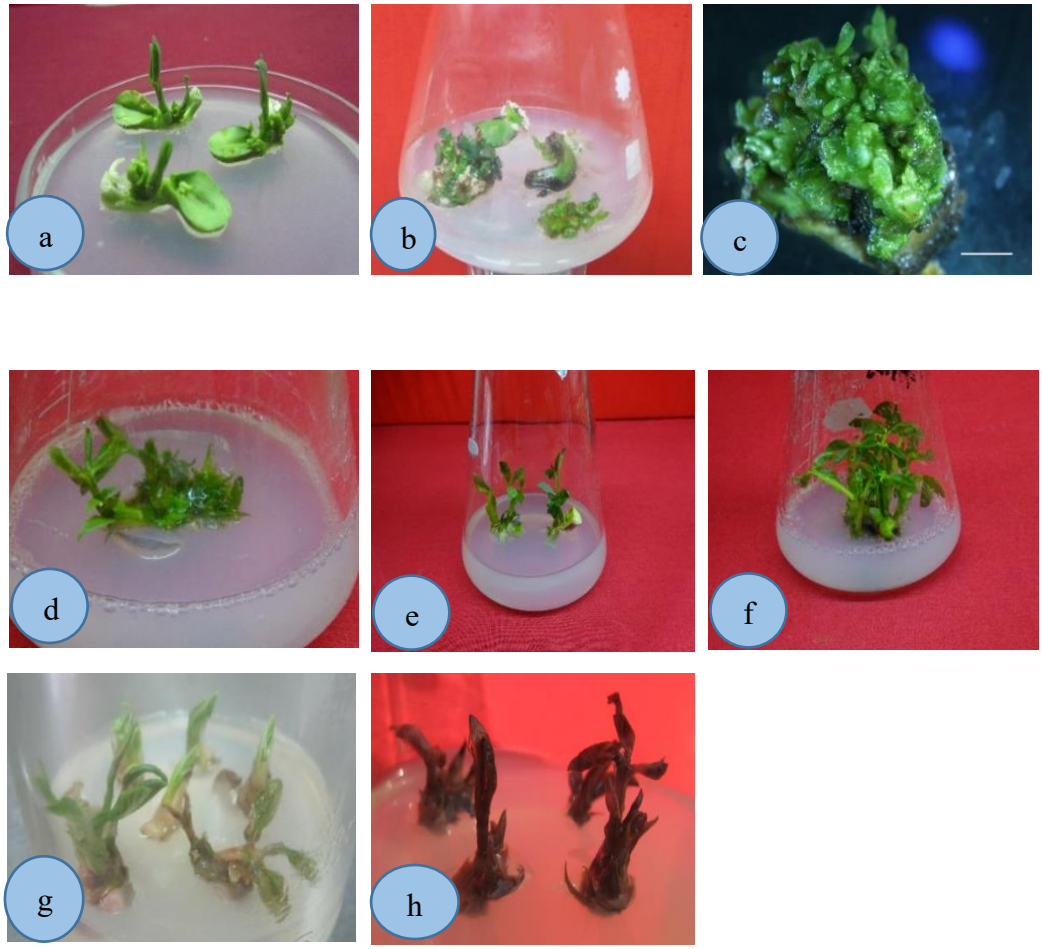


Fig. 44 (a-h): Different stages of regeneration of shoots and selection of putatively transformed plants SCADE explant of BARI Badam-8. (a) Infected SCADE explants on selection free regeneration medium after co-cultivation; b) SCADE explants being green in color and induction of adventitious shoot buds from explants after 15 days of culture on SIM1. (c) Stereomicroscopic view of formation of compact callus (arrow) in distal end instead of shoot formation on shoot elongation medium (SEM); d) Development of multiple shoots from infected SCADE explants on shoot elongation medium (SEM); e) Shoots survived on regeneration media containing 20mg/l hygromycin. (f) Multiple and elongated shoots survived on regeneration media containing 20mg/l hygromycin; g) The non-transformed shoots became pale and gradually died due to necrosis after 14 days); (h) Non transformed shoots were leading to death on 20 mg/l hygromycin containing regeneration medium.

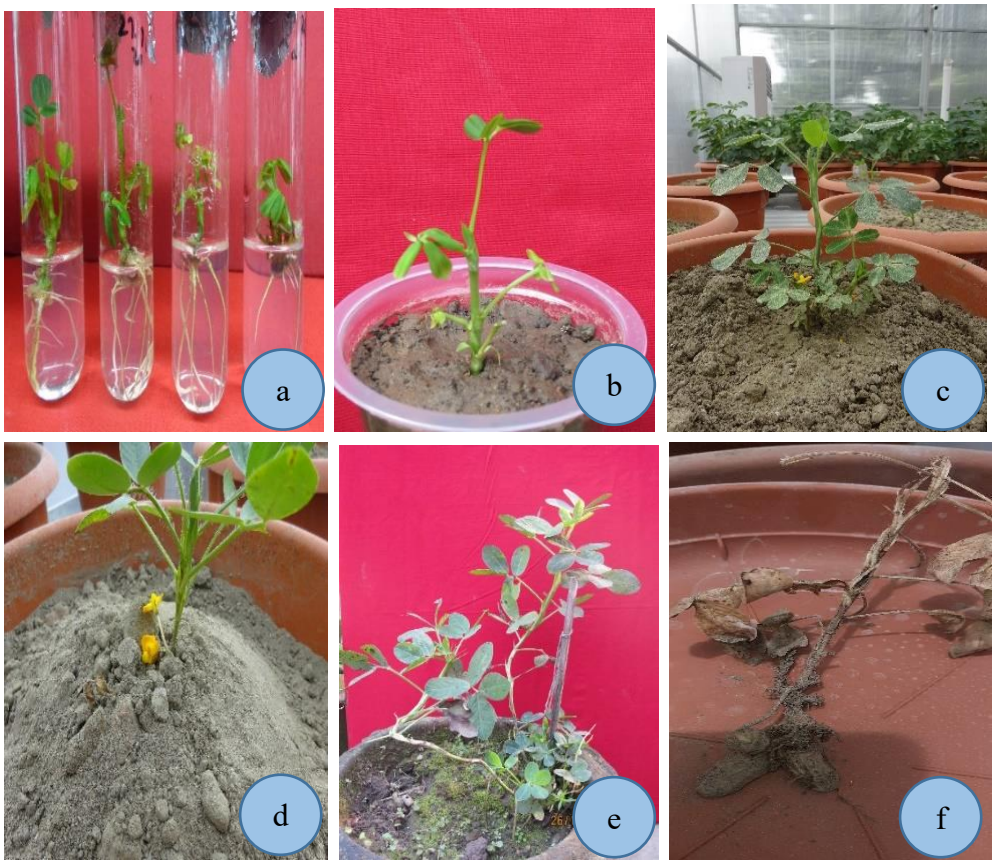


Fig. 45 (a - e): Formation of roots from base of the putatively transformed shoots and transplantation of transformed plants in soil. (a) The putatively transformed shoots were transferred on root induction medium (half strength of MS medium with 2.5 µM IBA) and formation of roots from the cut end of the putatively transformed shoots of BARI Badam-8; (b - c) Developed the T_0 plants in plastic and earthen pot maintained in double layered insect proof net house prepared for maintaining proper biosafety regulation. d)Young plantlets with flower in large earthen pot containing soil.; (e-f) Harvested peanut plants (BARI Badam-8) with pods.

4.4 Analysis of transgene integration

The genomic DNA from the putative transformed shoots along with their control shoots were isolated and visualized through agarose gel electrophoresis. This isolated DNA was used for PCR amplification of *PDH45* and *hptII* genes using gene specific primers. During the PCR study, the *Agrobacterium* (strain II) plasmid was employed as a positive control. Analyses of the amplified DNA were performed using agarose gel electrophoresis.

4.4.1 PCR for the amplification of *PDH45* gene

To amplify the *PDH45* gene, the DNA extracted from the leaves of both transformed and non-transformed shoots was subjected to PCR. The Primers were forward 5'-ATGGCGACAACTTCTGTGG-3' and reverse 5' GAGTCTAGATTATATAAGAT CACCAATATC-3'. For the amplification of DNA, 35 PCR cycles were maintained and analyzed through agarose gel electrophoresis. Thirty-eight (38) putatively transformed plants from developed from DEHC & SCADE explants were examined, and following PCR amplification, thirteen (13) bands corresponding to the positive control band were found (Fig. 46a). This finding suggested that thirteen (13) transformed plants had the *PDH45* gene inserted into their genomic DNA.

4.4.2 PCR for the amplification of *hptII* gene

The primers forward (5'- CGAAGAATCTCGTGCTTTCAGC-3') and reverse (5'-AGCATATACGCCCGGAGTCG-3') were used for the amplification of *hptII* gene. For the amplification of DNA, 30 PCR cycles were maintained and analyzed through agarose gel electrophoresis. Thirty-eight (38) putatively transformed plants developed from DEHC & SCADE explants were examined, and following PCR amplification, thirteen (13) bands corresponding to the positive control band were found (Fig. 46b). This finding suggested that the thirteen (13) transformed plants had the *hptII* gene inserted into their genomic DNA.

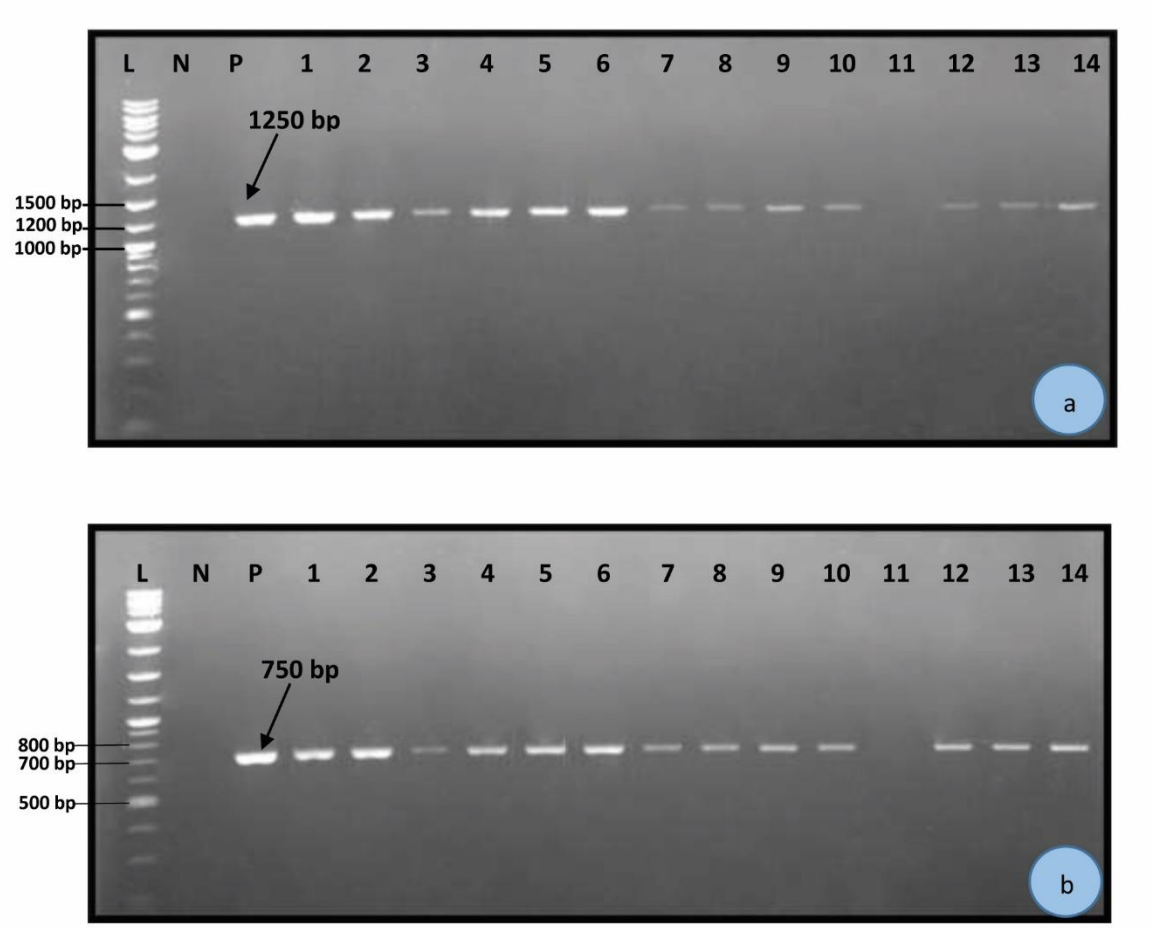


Fig. 46 (a-b): PCR amplification of the putative transformants of BARI Badam-8. (a) PCR amplification of *PDH45* gene from transformed shoots (lane L-1.2 kb ladder; lane N- negative control lane P- positive control lane (plasmid DNA same mentioned as fig 46a lanes 1-10,12, 13,14 genomic DNA of transformed shoots of BARI Badam-8; note that lanes 1-10,12,13,14 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 *hptII* gene from transformed shoots (lane L- 1 kb ladder; lane P - plasmid DNA as positive control; lane - N negative control; lanes 1-10,12,13,14 genomic DNA; note that lanes 1-10,12,13,14 amplified corresponding band, identical to the band amplified in positive control lane P, no band was amplified in negative control lane N.

5. DISCUSSION

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Legumes are regarded as the most significant crops after cereals for their nutritional quality. They are particularly considered as the essential sources for nutritional proteins and edible oil for human consumption. After soybean, cotton, sunflower, and rapeseed, peanut (*Arachis hypogaea* L.) is the fifth-most significant oil seed crop in the world (Rao *et al*. 2016). It is also an important industrial crop. However, abiotic stress such as salinity, drought, and heat causing significant loss of peanut productivity worldwide. Stress due to salinity and drought is also a major problem for better production of peanut in Bangladesh. Apart from abiotic factors, several biotic pressures like diseases due to fungus, mites, aphids and pests causes serious problems for the production of peanut (Kumar *et al*. 2015). It is generally believed that due to climate change issues these problems associated with peanut production will continue in many countries including Bangladesh. However, since peanut is a marginal crop, it can be grown with little input.

Under these circumstances, improved varieties of peanut are required to enhance the productivity of peanut specifically to obtain better protein quality and quantity as well as cultivars that are resistant to biotic and abiotic stresses. To develop improved varieties of crops with high consistent yields, superior quality, widespread adaptation, and resistance to abiotic stress, conventional breeding procedures, including hybridization techniques and selection, are being used. Due to the tetraploidy nature and conserved genome of the cultivated peanut, the majority of traditional breeding initiatives appear to have failed (Pandey *et al*. 2012). The lack of sources for desired features and low levels of polymorphism in cultivated varieties have slowed down the advancement of peanut breeding.

If the abiotic stress tolerance genes/characters be incorporated in local varieties of peanut, stress tolerance peanut can be cultivated in the salinity and drought prone areas of Bangladesh. Thus, the peanut production can be enhanced and marginal lands can be included for cultivation of peanut. It is understood that this crop cannot be improved without incorporating genes resistant to various biotic and abiotic stresses (Agrios, 2005).

One strategy offered for the improvement of peanut characteristic is the integration of genetic engineering and molecular breeding with traditional breeding (Varshney *et al*.

2011). The allotetraploid species *A*. *monitcola* and *A. hypogaea* are found in the section *Arachis* of the genus *Arachis,* which also includes several wild relatives of the domesticated peanut. A vast collection of genes for resistance to diverse biotic and abiotic challenges can be found in diploid species. Due to the genetic burden carried by the introgression lines resulting from the crossings of the wild ancestors with the farmed peanuts, breeders were unable to access these genes. Since the introgression was linked to unfavorable gene blocks, peanut improvement has not yet benefited from it. This issue can be solved by finding the appropriate genes in wild peanuts using studies on differential gene expression, and genetically modifying these genes is the greatest solution for long-term peanut improvement (Kumar *et al*. 2015). The introduction of desired genes across species barriers is made possible by genetic transformation (Sharma and Anjaiah, 2000). Using *Agrobacterium tumefaciens*, numerous effective genetic transformation techniques have been documented in peanut (Tiwari *et al*. 2008, 2011; Bhatnagar *et al*. 2010; Rao *et al*. 2016).

The goal of the current study was to incorporate *PDH45* gene, which confers both drought and salinity tolerant in local peanut varieties through *Agrobacterium*-mediated genetic transformation. In the current investigation, two regional peanut varieties namely Dhaka-1 and BARI Badam-8 were employed. These cultivars were brought to light by the Bangladesh Agricultural Research Institute (BARI) and are currently extensively grown in Bangladesh.

The overall experiments were performed in three phases. In the first phase, several experiments were carried out to determine the salt tolerance of local peanut varieties. To develop a suitable methodology for an *in vitro* regeneration system, a number of studies were carried out in the second phase. Regeneration experiments were conducted using the cotyledonary leaflet explants, de-embryonated half cotyledon (DEHC) and single cotyledon attached decapitated embryo (SCADE). Establishment of regenerated plants to soil was also carried out following the development of sufficient root system. In the second phase a genetic transformation methodology was developed using *Agrobacterium tumefaciens* strain LBA4404 (strain I) having the binary plasmid pBI121 conferring Glucoronidase (*GUS*) and the *nptII* genes. And plasmid pCAMBIA1301-PDH45 was present in *Agrobacterium* strain EHA105.

In the third phase of this investigation genetically engineered *Agrobacterium* strain EHA105 harboring pCAMBIA1301 plasmid with drought and salinity tolerant gene *PDH45* (strain II) was used. This gene construct was used to integrate *PDH45* gene in the peanut genome. In this case selection of transformed shoots was carried out using hygromycin resistance *hpt* gene. Strain I was used for the optimization of suitable condition of transformation in peanut whereas strain II was used for the integration of drought and salinity tolerant gene *PDH45* into peanut genome. The transformants developed through this study were characterized through PCR analysis to confirm the integration of foreign genes.

Almost every element of peanut growth and development is negatively impacted by salinity stress, including seed germination, early seedling establishment, photosynthesis, pod formation, total biomass, and yield output (Salwa *et al.* 2010; Qin *et al.* 2011; El-Akhal *et al*. 2013). Salinity limits the growth of legumes and controls how widely they are disseminated in the environment (Giannakoula *et al*. 2012). The ability of plants to thrive in salty soil varies from species to species and is influenced by the salt content of the root zone. The most practical factors for choosing salt tolerance are germination and seedling characteristics (Sharma *et al*. 2013). Several experiments were conducted as part of the current study to ascertain the salt tolerance of local peanut types. These tests are run to see which peanut types are tolerant or vulnerable to salinity.

The establishment of plants growing in saline soils depends on their ability to tolerate salt during germination, which is a pivotal stage in the life history of each individual plant. According to Almansouri *et al*. (2001), osmotic and salt stressors are too responsible for delaying and inhibiting seed germination as well as for seedling growth.

In the current study, cotton beds containing 0, 50, 100, 200 and 300 mM of NaCl solutions were used to allow the seeds of two local peanut types, Dhaka-1 and BARI Badam-8 to germinate. It was observed that the rate of germination decreased with the increase of salinity in case of both the varieties. Moreover, the germination rate was lowered to 0 % at 300 mM NaCl solution in Dhaka-1 as well as BARI Badam-8 varieties. Additionally, it was discovered that the length of the seeds' radicles decreased as the saline level rose. The observed decrease in peanut germination percentage can be related

to the germination medium's decreased osmotic potential, which reduces the amount of water that the seeds can absorb (Ambede *et al*. 2012).

In order to observe the effects of salt stress on seedlings, 8-day-old seedlings were cultivated in MS supplemented with 50, 100, 200 or 300 mM NaCl and simply MS medium (experimental control) in white light at 25±2°C under a 16-hour photoperiod (Sanan-Mishra *et al*. 2005.) The effect of the seedlings under the conditions of salt stress was scored. From each treatment, the plantlets were removed and divided into roots and branches. In addition to measuring the length of the roots and shoots, the number of roots and leaves was also counted.

It was shown that as salt increased, roots and shoots of the plantlets steadily shrank in length. The plantlets cultivated on MS medium without any salt supplements had the longest shoots, measuring 6.0 and 5.2 inches for the Dhaka-1 and BARI Badam-8 varieties respectively. When plantlets were cultivated on MS medium with 300 mM NaCl solution added, no shoot growth was seen. Again, extensive root formation occurred in MS medium alone and the number of roots gradually decreased with the increase of NaCl solution and at 300 mM only one root was observed in Dhaka-1 variety. Here it should also be mentioned that the root tips of plantlets cultured above 200 mM was blunted whereas the root tip of control plants (cultured in MS medium alone) was pointed. Shi *et al.* (2003) reported that, the root growth was inhibited under NaCl stress and root tips of NaCl-treated salt-hypersensitive mutants of *Arabidopsis* seedlings were swollen. In the present investigation the number of roots and leaf was also found to be decreased with the increase of salt concentration.

For successful plant genetic transformation, a transformation compatible *in vitro* regeneration method is necessary for effective plant genetic transformation. To create a transformation technique, an effective and repeatable *in vitro* regeneration system must be created for the specific plant species (Gardner *et al*. 1993). According to previous research, peanut is recalcitrant to transformation and *in vitro* regeneration, both of which are highly genotype-specific stages. (Guimaraes *et al.* 2017). Due to their passivity toward *in vitro* methods, grain legumes have been labeled as recalcitrant (Mroginski and Kartha, 1984). Leguminous crop transformation has a limited degree of success (Nisbet and Webb, 1990). Despite this, attempts have been undertaken to regenerate plantlets

from a variety of legumes, such as *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 (Kartha *et al*. 1981; Rubluo *et al*. 1984; Allavena and Rossetti, 1986; Wright *et al*. 1987; Barwale *et al*. 1986; Bose 1991; Khanam 1994; Khanam *et al*. 1995; Ahmad *et al*. 1997). There has been significant progress in the regeneration of legumes, there are still very few effective regeneration techniques that may be used to use legumes in transformation experiments. As a result, efforts to use gene transfer technologies to enhance leguminous crops were substantially hampered. Many attempts had been made in the past to design an appropriate procedure for plant regeneration in Peanut. Organogenesis has been employed previously to regenerate peanuts from a variety of explants (Palanivel *et al*. 2001; Palanivel *et al.* 2002; Tiwari *et al*. 2008; Bhatnagar 2010; Srinivasan *et al.* 2010). The plants have been regenerated through tissue culture using a variety of explants, including leaflets, mature leaves, cotyledons, epicotyls, and hypocotyls (Cheng *et al.* 1992; Bhuiyan 1994; Sarker *et al.* 1997; Sarker *et al*. 1999) and from immature zygotic embryos, additional seedling explants, and leaflets by somatic embryogenesis (Hazra *et al.* 1989; Ozias-Akins *et al*. 1992; Baker *et al*. 1992; Victor *et al.* 1999).

During the current study, regeneration was obtained via callus from leaflet explants. Direct regeneration was also observed in case of regeneration initiated from de-embryonated half cotyledons and single cotyledon attached decapitated embryo explants. From aseptically germinated seeds leaflet explants were collected. For *in vitro* seed germination, cotton soaked with sterile distilled water was found to be most effective. Plantlets were taken from the seedlings cultivated in vitro. For Dhaka-1 and BARI Badam-8, leaf explants from seedlings that were 7 and 8 days old were suitable for callus induction.

In case of immature cotyledonary 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 explants. However, Sharma and Anjaiah (2000) Tiwari and Tuli (2008) and Anjaiah and Sharma (2000) employed comparable explants from surface sterilized seeds steeped in sterile distilled water for two to three hours.

The induction of calluses in culture and plant regeneration are influenced by a number of variables, including the genotype of the plant material, the concentration of growth regulators, the response of explants and the make-up of the culture media. The study's findings showed that MS and MSB medium with various concentrations and combinations of BAP and Kn were useful for either direct regeneration of numerous shoots or for callus phase intervention. This outcome is consistent with those of Kartha *et al*. (1981); 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; 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, on MS medium supplemented with 22.2 µM BAP, it was found that the immature leaflet explants of Dhaka-1 and BARI Badam-8 produced the greatest number of healthy multiple shoots. In this instance, 75% to 74.80% of the explants responded by initiating shoot growth. The impact of various hormone supplements, including BAP, Kn, and NAA, on in vitro shoot regeneration from leaflet explants was also the subject of a number of experiments. Although the mean number of shoots/explants was lower than the number of shoots regenerated on MS with 22.2 µM BAP, 22.2 µM BAP and 2.3 µM Kn demonstrated the best response towards regeneration of numerous shoots. The best shoot regeneration was previously seen by Sarker and Islam (1999, 2000) and Sarker and Nahar (2003) using leaflet explants of the DM-1, ACC-12, DG-2, and Dhaka-1 types on MS with 5.0 mg/l BAP and 0.5 mg/l Kn, which are the concentrations of BAP and Kn employed in the current study.

The ability of leaflet explants to regenerate multiple shoots was also tested in experiments on MS that contained different amounts of BAP and NAA. The best responses to the regeneration of shoots were found on MS medium supplemented with 44.4 µM BAP and 11.0 µM NAA were seen in Dhaka-1 and BARI Badam-8. Forty to forty eight percent of

the explants in this mixture of BAP and NAA exhibited responses to the start of shoots. The various mixtures of BAP and NAA created a significant amount of callus. According to Vadawale *et al*. (2011) immature leaflet explants with 13.32 µM BAP and 2.68 µM NAA demonstrated up to 70% direct shoot growth. In order to create somatic embryos from leaflet explants, Joshi *et al*. (2008) employed 2,4-D, TDZ, and NAA. By using organogenesis on MS media with 10 mg/l NAA and 1 mg/l BA, Rey *et al*. (2000) discovered the best combination for regeneration from fully grown leaves. However, it was found in this experiment that leaflet explants in all mixtures of BAP and NAA with MS media did not exhibit a discernible response to shoot initiation. For the Dhaka-1 variety, the MS with 44.4 µM BAP and 11.0 µM NAA produced the highest mean number of shoots per explant (4.2) which was less than the number of shoots produced on MS with BAP alone or in conjunction with Kn.

A number of reports on the regeneration of peanut using various explants are also available. The majority of the study used de-embryonated half cotyledons and de-capitated embryo connected cotyledons as explants (Tiwari and Tuli 2008; Tiwari and Tuli 2011; Manjulatha *et al*. 2014).

The development of transgenic peanuts has utilized a variety of explants, including as leaf disks, leaf sections, and hypocotyl and epicotyl explants, however these methods have only produced modest frequencies of transformants, ranging from 0.2 to 6.7 percent (Eapen and George 1994; Cheng *et al*. 1996; Tiwari and Tuli, 2011) reported higher frequencies of transformants using cotyledonary node explants in peanut as compared to the de-capitated cotyledon. Considering the above reports, single cotyledon attached decapitated embryo (SCADE) and de-embryonated half cotyledon (DEHC) explants were used in the current investigation for the production of high frequency transgenic peanut.

During the present study the regeneration experiment was conducted by using both DEHC and SCADE explants. According to Sharma and Anjaiah (2000), adventitious shoot buds with high frequencies (90%) was obtained using DEHC explants from mature peanut seeds. In several different species of legumes, including *Vigna radiata* (Chandra *et al.* 1995), *Dalbergia sissoo* (Roxb.) (Chand *et al.* 2004) and *Lens culinaris* (Medik), cotyledon segments have been employed to establish *in vitro* regeneration techniques (Khawar *et al.* 2004). Direct regeneration was seen when a half of an embryo was

severed. Regeneration utilizing a single cotyledon disc and a half embryo decapitated at the shoot end has been documented for the pea (Schroeder *et al.* 1993), chickpea, and other plants (Tewari-Singh *et al*. 2004, Jayanand *et al.* 2003). Halbach *et al*. (1998) employed half or whole lentil embryos, as well as slices of embryos with or without cotyledons, and discovered that the latter embryo responded better for regeneration. The number of competent cells at the wound site rose when the cotyledon was halved. Cotyledon usage in similar research has been linked to rates of up to 55% in peanuts (Sharma and Anjaiah 2000). Using cotyledon explants from other grain legumes, including rapeseed, pea, and pea (Li, 2011), transgenic plants have been created (Moloney *et al*. 1989).

Regeneration experiments were also carried out in the current investigation employing SCADE explants of the Dhaka-1 and BARI Badam-8 types. In the instance of SCADE explants, it was found that Dhaka-1 and BARI Badam-8 peanut kinds of MSB medium containing the aforementioned BAP concentrations were all capable of direct multiple shoot regeneration. There are numerous publications that show BAP and Kn in various concentrations and combinations in modified MS medium to be equally effective (Tiwari *et al*. 2014; Pittman and Dunbar, 1992; Bhuiyan, 1994; Sarker *et al*. 1997). This differential response on shoot regeneration on different media may be due to the use of different genotypes by the above workers in both the hormonal combinations, all varieties showed 90 - 95% response towards regeneration of shoots. On MSB and BAP supplemented with Kn medium, a greater response toward the production of mean number of shoots/explants was seen.

On diverse hormonal combinations, no notable variations in regeneration or mean number of shoots were seen. From overnight socked embryo axes on a medium containing 1 mg/l BAP and 1 mg/l NAA from Bambara peanut plant, Lacroix *et al.* (2003) discovered an effective regeneration system. MS with 4.4 μM BAP enriched medium; Pacheco *et al.* (2007) demonstrated different shoot regeneration pattern from embryo axis explants. But in this work, several concentrations of BAP alone were utilized for the regeneration of shoots, and the best response was obtained on a medium combination supplemented with 22.2 μM BAP, with a rather low mean number of shoots/explants (5.2 – 5.5).

Among the three explants used for the regeneration of shoots, the highest multiple shoots were obtained within the shortest period from DEHC explants. It was noticed that regeneration of shoots was not observed from DEHC explants on MS with various hormonal combinations. In case of SCADE explants all showed responses towards multiple shoots formation with lower number of shoots/explants. The best response to the regeneration of shoots from de-embryonated cotyledon explants of two kinds of peanut was seen in MSB medium supplemented with varied concentrations of BAP. Dhaka-1 showed the best result where 85% explants responded towards multiple shoot formation from the DEHC 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). According to Tiwari *et al*. (2014) and Sharma and Anjaiah (2000), where regeneration frequency was 37.29 percent and 95.5 percent, respectively, MSB supplemented with 13.0 µM BAP (SEM) was adequate in the case of DEHC explants. A combination of BAP and other PGRs was used by Iqbal *et al.* (2011) to regenerate peanut, with an 87 percent regeneration frequency. Dhaka-1 also responded favorably to the regeneration of shoots from the DEHC explants in this concentration of BAP. Compared to the Dhaka-1 variant, BARI Badam-8 responded slowly. In the regeneration of peanuts and other plant species, the influence of cytokinins on the production of shoot buds has been extensively reported (Palanivel and Jayabalan, 2001; Kakani *et al.* 2009; Verma, 2009). De-embryonated cotyledon explants were utilized by Tiwari *et al*. (2008) to optimize a genotype independent methodology for peanut, which performed better in terms of shoot bud development and high regeneration, which is consistent with the current experiment.

Investigations were also conducted on the impact of MSB medium supplemented with BAP and 2, 4-D on the regeneration of shoots from de-embryonated cotyledon explants. On MSB with BAP and 2, 4-D supplemented media, de-embryonated half cotyledon explants responded moderately in terms of shoot initiation efficiency, but the mean number of shoots/explants was lower than on MSB with BAP supplemented media in both types of peanuts. For the regeneration of de-embryonated half cotyledon explants, Tiwari *et al.* (2015) employed three different 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) then sub-cultured on 66.6 µM BAP (SIM2) were used for the regeneration of de-embryonated half 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/explants 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/explants were 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).

On modified MS containing BAP and 2,4-D, Sharma and Anjaiah (2000) showed high rates (90 percent) of adventitious shoot bud development from de-embryonated half cotyledon explants from mature peanut seeds. However, the MSB medium supplemented with BAP and 2,4-D in the current study did not provide the best response to shoot regeneration. At the proximal end of explants, adventitious shoot buds were developed, but in the majority of cases, they were not fully elongated even after two to three subcultures. Several species of legumes, including *Vigna radiata* (Chandra *et al.* 1995) Dalbergia sissoo (Roxb.) (Chand and Singh, 2004), Lens culinaris (Medik.) (Khawar *et al.* 2004), and *Cicer arietinum* L., have shown the use of cotyledon segments as explant for *in vitro* regeneration (Tripathi *et al*. 2013).

It was noticed that the orientation of the explants on the shoot induction medium affects the regeneration of adventitious shoot buds from DEHC explants. In order for the proximal cut end to stay in contact with the media for at least the first two weeks of culture, it is crucial that it be embedded into the medium. Similar findings were also found by Sharma and Anjaiah (2000), Beena *et al.* (2008), and Tiwari *et al*. (2008) in their research with de-embryonated half cotyledon explants of peanut. According to Tiwari *et al*. (2008), effective regeneration was greatly influenced by the orientation of de-embryonated half cotyledon explants and auxin polarity on shoot induction media. For the correct elongation of the shoots, the proximal portions of the explants containing numerous adventitious shoot buds were cut off and transferred to shoot elongation media (Sharma and Anjaiah 2000; Tiwari *et al.* 2015). However, it was found in the current study that partial sub-culture of de-embryonated half cotyledon explants caused a delay in the development and elongation of shoots. Frequently, it takes 8 to 12 weeks for shoots to elongate. In the majority of situations, several shoots joined together to form a compact

structure that was impossible to separate from. Cotyledons were also used by Robinson *et al*. (2011) as explants in order to generate viable plants through embryogenesis.

After successful regeneration of shoot, induction of a healthy root is crucial for the effective development of plantlets. In earlier studies, root formation of transgenic peanut and other legumes was successfully accomplished using rooting medium supplemented with IBA or NAA alone (Anuradha *et al.* 2006; Sharma and Anjaiah 2000; Sharma and Ortiz 2000; Sharma and Bhatnagar-Mathur 2006; Iqbal *et al.* 2012), though the success rate varied. The findings of this investigation showed that, while roots were induced essentially in all three combinations of IBA, IAA, and NAA, 2.5 µM concentration of IBA was determined to be the most efficient for root induction in regenerated shoots of Dhaka-1 and BARI Badam-8. The greatest results for root production from kanamycin-resistant shoots on MS were reported by Venkatachalam *et al*. (2000) with 1.0 mg/l IBA, which is equivalent to 5.0 µM IBA used in the current study. According to Vadawale *et al.* (2011), MS medium containing 3.5 µM IBA was ideal for peanut root induction. However, Verma *et al.* (2009) found that peanut responded best to MS containing 1.0 mg/l NAA, and that some types of peanuts responded best to MS containing 1.0 mg/l NAA and 0.5 mg/l IBA. As such Bhuiyan *et al.* (1992), 0.2 mg/l IBA was shown to be appropriate for peanut rooting. Nguyen & Le Tran (2012) found a similar outcome, observing the best rooting response in peanut with 0.3 mg/l of IBA.

For root induction, Eapen and George (1994) employed 0.2 mg/l NAA. According to McKently *et al.* (1989, 1991) and Bhuiyan *et al.* (1992), 1.0 mg/l NAA and 0.2 mg/l IBA were determined to be acceptable for rooting, respectively. On MS with 0.57 µM and 2.85 µM IAA, Pacheco *et al*. (2007) demonstrated the highest incidence of root development from peanut shoots originating from mature somatic embryos. In both the Dhaka-1 and BARI Badam-6 types, Kabir (2008) found that half MS with 0.2 mg/l IAA produced the greatest results.

The impact of IAA on root induction was also investigated in this experiment. The results showed that among the various IAA concentrations, Dhaka-1 showed the best response (80%) on half strength MS medium supplemented with 1.0 µM IAA, whereas BARI Badam-8 displayed the best response (75%) in the same medium. IBA was seen to be the best because in IBA-containing medium the root developed at the cut end of the shoot

without any callus, which is desirable. IAA supplemented medium was more preferable for early root induction than IBA, but IBA was still deemed best (Afroza and Hoque, 2013). In the current investigation, it was also found that there was no callus formation when IBA was present. In comparison to IBA and IAA, root induction by NAA developed callus at the cutting end of the shoots. The two kinds of MS media with 2.5 µM NAA at half strength produced the greatest results when different NAA concentrations were employed to induce roots. On NAA supplemented medium, the root induction was seen to be slower than on IBA and IAA-supplemented medium. Additionally, it was discovered that the cut ends of the shoots had begun to develop roots and calluses. On half strength of MS with 0.5 mg/l or 2.5 µM NAA supplemented media were able to get the best root induction in the past (Sarker and Nahar 2003 and Venkatachelam, *et al*. 1998). *In vitro* regenerated peanut shoots responded best to MS with 1.0 mg/l NAA for root induction, according to Banerjee *et al.* (2007) and Tiwari *et al.* (2008). The best root induction in peanut on MS with 0.8 mg/l NAA was obtained by Anuradha *et al.* (2006). On Modified MS with 5 µM NAA, Sharma and Anjaiah (2000) found that *in vitro* regenerated peanut shoots had the best rooting response. On half strength MS medium supplemented with 1.07 µM or 0.2 mg/l NAA, the best root induction in peanut was obtained by Eapen and George (1994), Sarker and Islam (1999), and others. According to McKently *et al.* (1989, 1991), 0.54 µM NAA was sufficient for peanut root induction.

After proper hardening, *in vitro* regenerated shoots with well-developed roots of Dhaka-1 and BARI Badam-8 were successfully transplanted into soil. Almost 90-95% transplanted plantlets were survived in soil. The survived plantlets grew well in large clay pots and afterwards they were transferred in experimental field.

The genetic modification of the peanut varieties Dhaka-1 and BARI Badam-8 is the subject of the third stage of the investigation. The most popular and effective method among the various techniques has been deemed to be *Agrobacterium*-mediated genetic transformation, which has been applied to a variety of leguminous crop plants, including soybean (Zhang *et al*. 2016; Li *et al*. 2017), chickpea (Kar *et al*. 1996; Mehrotra *et al.* 2011; Tripathi *et al.* 2013), and peanut (Eapen and George, 1994; Sarker *et al*. 2000; Chen *et al.* 2015).

The target tissues used in the majority of earlier studies of *Agrobacterium*-mediated transformation of peanut were leaf discs, cotyledons, cotyledonary nodes, and embryo axes (Eapen and George, 1994; McKently *et al.* 1995; Cheng *et al*. 1996; Gelvin, 2003; Anuradha *et al*. 2006; Iqbal *et al.* 2012). Explants generated from cotyledons can be used to transform and regenerate viable plants, including agricultural species like chickpea, rapeseed, and peanut (Moloney *et al.* 1989; Sharma and Anjaiah 2000; Sharma and Ortiz 2000; Bhattacharjee *et al*. 2010; Iqbal *et al*. 2012). Sarker and Islam (1999) had established an efficient regeneration protocol for peanut through callus as well as without the intervention of callus from leaflet explants. There are other reports on the use of a number of additional techniques for genetically modifying plants, such as microprojectile bombardment, electroporation, sonication, chemical methods of transformation, etc (Singsit *et al.* 1997; Livingstone *et al*. 2005; Athmaram *et al.* 2006) In general, all alternative procedures have been compared to *Agrobacterium*-mediated genetic transformation, which has been found to be the most practical and economical. Due to the significant co-expression of inserted genes, potential low copy number, and preferential integration into active transcription areas, it is the most common approach of genetic transformation (Sharma and Anjaiah, 2000; Tiwari *et al.* 2008, 2011.

The transformation efficiencies of these studies, however, ranged from 0.3 percent to less than 10 percent, which is quite low. A technique developed by Sharma and Anjaiah in 2000 significantly raised the efficiency of peanut transformation to at least 55%. The transformation of peanuts was comparatively simple and highly reproducible according to this technique.

The SCADE and DEHC explants from Dhaka-1 and BARI Badam-8 were employed for transformation in the current study. In this investigation, two genetically modified *Agrobacterium* strains were used: LBA4404 with the gus and *nptII* genes conferred by pBI121 and EHA105 with the *hptII* gene (of interest) conferred by pCMBIA1301-PDH45. This construct was primarily utilized for the optimization of an appropriate transformation protocol, which includes a number of parameters necessary for *Agrobacterium*-mediated genetic transformation, such as the optical density of the *Agrobacterium* suspension and the ideal incubation and co-cultivation times. Additionally, efforts were made to select and regenerate putatively altered plantlets. Sarker *et al.* (2000), Sarker and Naher (2003), Venkatachalam *et al.* (2000), Tiwari and

Tuli (2012) and others have described transformation using LBA4404 harboring plasmid pBI121GUS-NPTII from a variety of explants, including immature leaflets, cotyledonary nodes, de-embryonated cotyledons, etc. The influence of many physical and chemical parameters on transformation and regeneration, including genotype selection, explant use, co-cultivation time, virulence-inducing substances, hormone combinations, and selectable markers, is well established (Manjulatha *et al*. 2014).

A perfect transformation protocol should be quick, genotype independent, low chimerism in the regenerated transgenic plants, and high frequency of transformation. Explant type, polarity, and orientation, hormonal mixtures, addition of different antioxidants, co-cultivation period, temperature, pre-culture, bacterial strains, nature of the genes and promoters used, use of sonication, selective agent, selection pressure, and timing of application, binary vectors with enhanced virulence as well as phenolic compounds are reported to increase transformation efficiency in peanut as well as many recalcitrant species (Tiwari and Tuli, 2012; Tripathi *et al*., 2013; Rustagi *et al.,* 2015; Tiwari *et al.*, 2015).

For transformation studies with the *Agrobacterium* strain EHA105/pCAMBIA1301, bacterial solution with an optical density of 0.5 at 600 nm was selected. In order to achieve effective transformation, a 25-minute incubation period was followed by 48 hours of co-cultivation. The *hpt* gene is found in the T-DNA of *Agrobacterium* strain EHA105/pCAMBIA1301, and it imparts hygromycin resistance to the transformed cells. Hygromycin was therefore employed as a selectable marker.

It has been shown that a 0.6 OD600 *Agrobacterium* suspension is suitable for transformation (Ribas *et al*. 2011). The effectiveness of various inoculation techniques, however, does not appear to have been studied, and the immersion technique is usually often utilized (Jones *et al*. 2005; Bhattacharjee *et al*. 2010). Density of bacterial suspension was optimized using a spectrophotometer (OD at 600 nm). In the present investigation, it was observed that bacterial overgrowth started to increase above OD of 0.5. Therefore, 0.5 OD was preferred for the transformation experiments. The effect of co-cultivation varies on the species because co-cultivation has been shown to be genotype dependent (Zhang *et al.* 1997) In terms of transitory transformation efficiency determined after co-cultivation, the technique described by Tiwari and Tuli (2008) performed better

than others included in the study. Previous studies on the peanut variety JL-24 with tomato cotyledon explants and cotyledonary nodes as explants revealed that 72 hours of co-cultivation was the ideal time for transformation (Sharma and Anjaiah 2000; Anuradha *et al.* 2006; Sharma *et al*. 2009). Eapen and George (1994) injected peanut leaflet explants for five minutes, followed by three days of co-culture. Additionally, they claimed to get regeneration from altered tissues after 3 to 4 weeks. Sarker *et al*. (2000) co-cultured the peanut variety Dhaka-1 for 72 hours after applying a 60-minute incubation time for transformation. In order to produce transgenic plants in chickpea, Krishnamurthy *et al.* (2000) co-cultured mature embryo explants for three days after incubating them for 20 minutes.

Antibiotic use throughout the transformation process depends on the species and type of explant (Saini *et al*. 2003). The *hpt* gene, which is located within the T-DNA of the *Agrobacterium* strain EHA105/pCAMBIA1301-PDH45 employed in this study, gives hygromycin resistance to the transformed cells. Hygromycin was therefore employed in this experiment as a selective marker. After co-cultivation, Kar *et al.* (1996) applied selection pressure. In a number of agricultural plants, Sharma and Ortiz (2000) recommended applying selection pressure right after co-cultivation. When selection pressure was given to chickpea immediately following co-cultivation, the converted explants showed no signs of regeneration because it prevented explant regeneration (Tiwari-Singh *et al.* 2004). However, it was found in the current study that when selection pressure was given just after co-cultivation, it impeded the regeneration of explants and the transformed explants did not show any signs of regeneration. Regeneration from the co-cultivated explants was encouraged for the aforementioned reason. After two weeks, selection pressure was applied, beginning with smaller concentrations. In 2011, Liu *et al*. created a transgenic plant under a 3 mg/l hygromycin selection pressure using a hypocotyl explant of *B. napus,* Kong *et al*. (2009) created transgenic plants from cotyledonary nodes under a selection pressure of 10 mg/l hygromycin. Hypocotyl explants were employed by Dutta *et al*. (2008) and Das *et al*. (2006) to identify transgenic plants of *B. juncea.* Their view is also supported by the findings of the current investigation. As a result, the explants in this investigation were retained on regeneration medium for growth before being moved to medium containing the selection agent.

Hygromycin has been demonstrated to be very effective in the selection of putative transformants, and DEHC explants have been shown to be excellent for transformation and regeneration of peanut. The opinion is supported by the prior report by Tiwari *et al*. (2015). Pre-culture of inoculated explants for two weeks in the absence of selection, as previously described by Nehra *et al.* (1990), was crucial for improving the efficiency of transformation, albeit absence of selection at the beginning may also lead to a very low recovery of transformants (Mathews *et al*. 1995). Hygromycin was therefore added to the regeneration medium at a concentration higher than the corresponding explant tissue's inherent tolerance in order to select the transformed tissue. Different concentrations of selection agents (hygromycin) were evaluated in the selection medium for explants to assess the level of each. It was noted that in the selection medium containing 30 mg/l hygromycin, all control shoots perished. Shoots that endured this pressure of selection for 15 to 20 days were regarded as potential changed shoots. The selection pressure used in this investigation's initial selection medium was mild (20 mg/l hygromycin), and it steadily rose in successive subcultures. With the exception of the co-cultivation media, all of these culture media were added with 300 mg/l carbenicillin to prevent bacterial overgrowth.

Therefore, hygromycin was added to the selection medium at a higher concentration than the individual explant tissues' inherent tolerance threshold. In the present investigation, 20 mg/l hygromycin was used to impose selection pressure on the non-transformed shoots following a 15-days pre-culture of inoculated explants in a selection free medium. This caused the subsequent necrosis and death of non-transformed cells. The putative transformants remained green as they survived under the selection pressure. Therefore, SIM2 containing the initial selection pressure (20 mg/l hygromycin) was applied when shoots regenerated on SIM1. Only green shoots were subcultured on fresh media with 20 mg/l hygromycin after 14 days, at which point the non-transformed shoots turned pale and eventually died from necrosis (Fig. 42, 44). Among approximately 150 regenerated shoots after transformation, 44 shoots survived on second subculture with 20 mg/l hygromycin concentration in case of BARI Badam-8 variety (Table 27). The percentage of survived shoots was 3.66 (DEHC explants) and 2.85 (SCADE explants) on selection medium. All the culture medium except the co-cultivation medium contained 300 mg/l carbenicillin to check the bacterial overgrowth. The percentage of survived shoots on selection pressure containing medium was 3.66% in de-embryonated cotyledon explants

and 2.85% in single cotyledon attached decapitated embryo explants in BARI Badam-8 variety. Based on the results of survived shoots on selection medium of two explants, it was observed that the transformation efficiency was higher for hygromycin selection in DEHC explants than SCADE explants of BARI Badam-8 variety.

In the present investigation, for PCR analysis DNA was isolated from shoots which survived in medium containing selection pressure (20 mg/l hygromycin). The Chloroform-Isoamyl Alcohol DNA Extraction Protocol, also known as the CTAB method (Doyle and Doyle, 1987; Cullings, 1992), was used to extract DNA. Extracted DNA was measured and amplified for the *PDH45* gene using specific primers. Altogether 13 shoots showed positive band in PCR analysis which was identical to positive band amplified from bacterial plasmid. This result indicated that the gene *PDH45* was inserted in the genomic DNA of plantlets.

From the above discussion it can be concluded that, the two local varieties of peanuts are more or less susceptible to higher NaCl concentrations. So, the development of salinity tolerant peanut variety is very essential. The *in vitro* regeneration system established in this investigation and also reported in the earlier studies is reliable and reproducible. For this reason, this protocol was used to develop transgenic peanut plants from transformed tissue following proper selection pressure. However, it is exciting that this research has enabled the development of transgenic peanut plants employing the EHA105 pCAMBIA-1301-PDH45 gene construct, which is also *PDH45* gene within its T-DNA. The optimization of various factors influencing transformation will help to obtain transgenic peanut plants having stable integration of drought and salinity tolerant gene. It has been possible to obtain 13 plantlets using this gene and the integrated *PDH45* gene has been confirmed through PCR analysis.

The developed transgenic peanut plants need further confirmation using other molecular techniques, namely, Southern, Northern and Western blotting techniques. Due to time constraints, it has not been possible to perform the above experiments, however, considering the importance of this crop it is our hope that we shall continue these experiments in the future to obtain genetically engineered stable transgenic peanut plants towards the development of drought and salinity tolerance.

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*
B_5	:	B_5 basal medium
BAP	:	6-benzylaminopurine
BARI	:	Bangladesh Agriculture Research Institute
BBS	:	Bangladesh Bureau of Statistics
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
DEHC	:	De-embryonated half 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	:	Neomycin phosphotransferase II
OD	:	Optical density
PDH 45	:	Pea DNA Helicase 45
PCR	:	Polymerase Chain Reaction
pH	:	Negative logarithm of Hydrogen
PPT	:	Phosphinothricin
rpm	:	Rotation per minute.
RT-PCR	:	Reverse transcription polymerase chain reaction
SCADE	:	Single Cotyledon Attached Decapitated 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	:	Transgenic lines
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 glucuronide
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.

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
Pyridoxine-HCl	1.00
Thiamine-HCl	10.00
Inositol	100.00
Sucrose	30,000.00

PH adjusted to 5.8 before autoclave