Establishment of transformation protocol and regeneration potential comparison following introduction of the antiporter gene in peanut (*Arachis hypogaea* L.)

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Dedicated

To

My beloved parents

DECLARATION

I hereby solemnly declare that the research work embodying the results reported in this thesis entitled "**Establishment of transformation protocol and regeneration potential comparison following introduction of the antiporter gene in peanut (*Arachis hypogaea* L.)**" submitted by Kashmery Khan has been carried out under supervision of Dr. Aparna Islam, Associate Professor, Biotechnology Programme, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

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September 2015 **Kashmery Khan**

ABBREVIATIONS

A.	*Agrobacterium*
BAP	6-Benzylaminopurine
CaMV35S	Cauliflower mosaic virus 35 S
Conc.	Concentration
DNA	Deoxyribo nuclic acid
EDTA	Etylene diamine tetra acetic acid
gm	Gram
GUS	β-glucuronidase
h.	Hour
Ha.	Hectare
hpt	Hygromycin phosphotransferage
HCl	Hydrochloric Acid
IAA	Indole-3 Acetic Acid
IBA	Indole-3 Butyric Acid
Kg	Kilogram
Kn	6- furfurylaminopurine
mg/l	Milligram/Litre
mM	Milli mole
ml	Milli litre
MS	Murashige and Skoog (1962) medium
NaOH	Sodium Hydroxide
Na2-EDTA	Sodium salt of Ferric Ethylene Diamine Tetra Acetate
Ng	Nano gram
Nos	Nopaline synthetase
Npt	Neomycin phosphotransferase
OD	Optical Density
pH	-log [H+]
T-DNA	Transfer DNA

Ti-Plasmid	Tumor inducing plasmid
X- Gluc	5-Bromo-4-Chloro-3-indoyl-_-D-glucuronide
YMB	Yeast Manitol Broth
µl	Micro litre
µM	Micro Mole

CONTENTS

ABSTRACT

Peanut is a cash crop which is cultivated in 24 million ha all over the world for extracting edible oil. It is a great food having outstanding food values, specially for hunger and malnutrition affecting Asian countries like Bangladesh. But peanut yield is substantially reduced because of abiotic stresses like salinity. As a result, salt tolerance in peanut has to be improved to ensure better production where the success of conventional breeding severely hampered due to its cleistogamous nature of pollination. Therefore, *Agrobacterium*- mediated genetic transformation may lead the solution. On that context, present investigation was carried out with three Bangladeshi peanut varieties, such as, BINA Chinabadam 2, BINA Chinabadam 4 and BINA Chinabadam 6 using two explants, named, decapitated half embryo and embryonic leaflet. Factors influencing transformation and comparing the regeneration potential of explants on selection media `was done using three different vectors, named, *pBI121*, *pK7WG2_OsNHX1_1.6* & *pK7WG2_AtNHX1_1.6*. Decapitated half embryo showed better *in vitro* regeneration potential than leaflet explant. Hence, transformation study was conducted with 1 day old decapitated half embryo where highest percentage of GUS positive explants were found when OD_{600} was more than 1 at 60 minutes of infection and 3 days of co-cultivation duration in transient assay. Regeneration efficiency on selection media containing 50mg/l kanamycin was higher in almost all three varieties. In contrast to this, untransformed control explants were able to resist upto 20mg/l of kanamycin. Moreover, average number of putative transformed shoots was gradually dropped with the increased concentration of kanamycin. Shoots which survived upto 150mg/l kanamycin were transferred into rooting media for root induction. Finally transformation efficiency was evaluated by calculating the percentage of GUS positive plant parts, such as, leaf, stem and roots among the putatively transformed plantlets. Maximum transformation frequency (14.28%) was observed in BINA Chinabadam 4. In the current experiment, effect of salinity on peanut seed germination was also observed. The germination rate steadily decreased with the increase of NaCl in the media and finally no germination was observed in MS media supplemented with 150mM of NaCl and above.

Chapter 1

Introduction

1.1 Background

Peanut is an annual herbaceous legume plant. Its kernels are rich in protein (25−28%) and oil (48–50%), and are source of several vitamins, minerals, biologically active polyphenols, flavonoids, and isoflavones (Janila *et al*, 2013). Peanuts are naturally cholesterol-free and even they have a more anti-oxidants than grapes, green tea, tomatoes, spinach, broccoli and carrots. It is cultivated in 24 million ha all over the world for the purpose of extracting edible oil and also to be used as a source of healthy food (Rafiq, 2014) . Each 100 gm of peanut contain 600 kcal, 50gm fat, 800mg sodium and 10 mg fiber and no cholesterol. For this reason, World Health Organization recommends 2 servings of 100 gm of processed nuts as a survival base for African children per day (http://news.bbc.co.uk/2/hi/europe/8610427.stm, Date: November 6, 2012).

On botanical context of view, peanut is unique among most other cultivated crops due to its geocarpic growth habit. Geocarpy is the production of aerial flowers but subterranean fruits where the flowers contain both male and female reproductive parts as perfect flower.post- pollination, flowers produce an elongated ovarian structure known as peg or gynophores. The aerial gynophore grows vertically and penetrates the soil where the mature fruit which is known as pod develops (Burns, 2010) .

Peanut flower is a self pollinating flower and grows low on the plant. Peanut shows cleistogamous pollination which means the pollination occurs before the flowers open, because of which genetic variation doesn't occur in peanut (Chowdhury, 2014b). After pollination the flower stalk elongates and bends until the ovary touches the ground. Continued stalk growth then pushes the ovary underground where development of the mature fruit occurs by surrounding a legume shell which is further called peanut. Shells are 3 to 7 cm long, containing 1 to 4 seeds.

1.1.1 History of peanut cultivation

The cultivated peanut, *Arachis hypogaea* is an indeterminate and annual herbaceous legume crop of global importance. It is believed that peanut's center of genetic diversity was in South America, specifically northern paraguay and southern Brazil (Pattee and Young, 1982). Early Spanish and Portuguese explorers discovered indigenous people of central and South America cultivating peanut during the sixteenth and seventeenth centuries. Subsequently these explorers introduced peanut first to Europe and eventually to both African coasts, Asia, the pacific islands, and finally to North America (Burns, 2010).

Currently, peanut is grown on six continents and in over 100 countries (Nwokolo, 1996). China led the world in peanut production and value (13,079,363 metric tons (MT), Int. $6,112,785,000, followed by India (9,182,500 MT, Int. $4,205,879,000), Nigeria (estimated 3,835,600 MT, estimated Int. $1,778,082,000), and the U.S. (1,696,728 MT, Int. $778,851,000) (FAO 2010). Although the U.S. does not lead the world in peanut production, it has ranked first in yield per land unit for over 15 years (Chenault *et al*, 2008).According to FAO statistical report 2008, in Bangladesh, 31182 hectares land is utilized for peanut cultivation, where 16658 hectogram production is achieved per hectare. Total production is 51944 tons (with shell), 4054 tons of seeds per year (Chowdhury, 2014).

1.1.2 Genetic diversity of peanut

Among all the species of *Arachis, Arachis hypogaea* is the only species that has been domesticated as a manner that can be grown worldwide**.** Many studies have concluded that *Arachis hypogaea* has low genetic diversity due to extensive morphological and physiological variation. These studies have used pedigree analysis (Knauff and Gorbet,1989), protein profiles (Singh *et al*, 1991), isozymes (Grieshammer and Wynne, 1990; Lacks and Stalker, 1993; Lu and Pickersgill, 1993, Stalker *et al*, 1994), restriction fragment length polymorphism (RFLP) (Galgaro *et al*, 1998; Garcia *et al*, 1995; Halward *et al*, 1991,1993; Kochert *et al*, 1991, 1996; Paik-Ro *et al*, 1992) and random amplification of polymorphic DNA (RAPD) (Halward *et al*, 1992; Lanham *et al*, 1992;

Garcia *et al*, 1995; Galgaro *et al*, 1998; Subramanian *et al*, 2000; Raina *et al*, 2001). Moreover the genetic diversity that exists in domesticated peanut remains low compared to other important crops. Because most *Arachis* species are diploid and do not readily cross with tetraploid *A. hypogaea.* Moreover the limited genetic diversity found in cultivated peanut is most likely because of the single hybridization event between wild, diploid *Arachis* species (Halward *et al*, 1991). This type of lower genetic diversity in peanut has been further complexed by the self-pollinating nature of peanut and breeding programs using very few elite breeding lines (Hersellman, 2003).

1.2 Peanut diseases due to biotic stresses

Peanut is susceptible to a variety of biotic stresses. The varieties of peanuts which occupy the Asian lands have a narrow genetic base and minimal genetic diversity which resulted into their susceptibility to many pests and diseases (Anuradha *et al*, 2006; Iqbal *et al*, 2012). Fungal diseases are the main constraints behind peanut low productivity. The commonly seen diseases are late leaf spot (LLS) caused by *Phaeoisariopsis personata*, early leaf spot caused by *Cercospora arachidicola* and rust caused by *Puccinia arachidis* (Janila *et al*, 2013, Backman and Crawford, 1984, Khaleque *et al*, 1985). Iqbal and his colleagues (2012) have also reported about other diseases which hamper peanut yield, named, wilt caused by *Fusarium oxysporum* and stem and pod rot caused by a fungi named *Sclerotium rolfsii* . There are some viral diseases which frequently occur in peanut, such as, peanut bud necrosis disease (PBND) and peanut stem necrosis disease (PSND).Those diseases are very much common in India whereas peanut stripe potyvirus (PStV) is mainly common in East and South East Asia (Rafiq, 2014).

1.3 Peanut diseases due to abiotic stresses

Crop productivity reduces upto as much as 50% due to the hindrances of many abiotic stresses, such as, drought, salinity, water logging, high temperature, and chilling (Boyer *et al*, 1982, Bray *et al*, 2000). It is predicted that in the future peanut production will be extremely difficult due to the more adverse condition of drought in the tropical and subtropical regions of the world (Sun *et al*, 2013). A large portion of irrigated land in Asia

has badly affected by salinization (Rains and Goya, 2003). So along with biotic stresses, the abiotic stresses also challenge the betterment of peanut production.

1.4 Peanut tissue culture as a base of genetic manipulation

Introducing novel and beneficial genes into peanut can be possible through genetic manipulation that would not be available using conventional breeding methods. Attempts of conventional hybridization can be taken by several wild *Arachis* species having novel genes. But conventional breeding between cultivated peanut and *A. paraguariensis* have failed, as they are cliestogamous (Rao *et al,* 2003). The available cross with *Arachis hypogaea*, ploidy differences, evaluation and the germplasm have been reviewed by many researchers (Gregory and Gregory, 1979; Moss and Stalker, 1987; Singh *et al,* 1980; Halward *et al,* 1992).

Many studies reported the successful production of transgenic peanuts but efficient production of independent lines were missing there (Burns, 2010). Some studies recognized the causal factors behind this scenerio, such as, somaclonal variation due to long tissue culture requirements, explants availability, cultivar specificity, and poor regeneration into mature plants (Livingstone and Birch, 1999; Anuradha *et al*, 2008). As a result lack of prolifilic tissue culture system is considered as the major impediment of the routine production of transgenic peanut.

Peanut tissue culture protocol establishment work had been going from the early 18th century (Harvey and Schulz, 1943; Nuchowicz, 1955). Established tissue culture protocols have been used for the transformation of peanut which has found in many previous reports (Higgins and Dietzgen, 2000; Sarker *et al*, 2003; Sharma and Anjaiah, 2000, Anuradha *et al*, 2006; Tiwari and Tuli, 2009; Sarker and Islam, 1999). There are two major purposes for which tissue culture has been carried out, such as, production of large number of plantlets and propagation of the selected genotypes without inducing any genetic variation (Al-Joboury, 2012).

Micropropagation can be achieved while working with different parts of the plant (Chowdhury, 2014a) as the primary explants such as, the apical meristem, nodal bud,

shoot buds, axillary buds, or through production of somatic embryos, a process commonly known as somatic embryogenesis. In the previous study, Chowdhury (2014a), genotype independent regeneration protocol for peanut was established with different hormonal concentration using two types of explants named decapitated half embryo and embryonic leaflet. A lot of research work has been done on nodal culture of several plants (Sanjaya and Rai, 2005; Siddique and Jahan, 2006). A successful tissue culture protocol starts with effective explants sterilization. In some study a simple and fast protocol using commercial bleach (sodium hypochlorite, NaOCl) was evaluated for explant sterilization and *in vitro* establishment in comparison to mercuric chloride ($HgCl_2$) which is mostly used in reported groundnut tissue culture studies (Sharma and Anjaiah, 2000).

For any explant in tissue culture, the differentiation pathway is reliant primarily on the growth regulators added into the culture medium. In general, compounds with cytokinin activity such as benzylaminopurine (BAP), kinetin (K), and thidiazuron (TDZ) promote shoot initiation and development, and auxins such as 2,4-D (2,4-dichlorophenoxyacetic acid) and picloram induce somatic embryogenesis (Ozias-Akins and Gills, 2001). There are some other variables to influence the magnitude of response rather than the response pathway, such as, basal medium, light, and temperature (Pestana *et al*, 1999). Moreover, the rate of initiation and maintenance of embryogenic cultures clearly is influenced by peanut genotype (Ozias-Akins *et al*,1992).

1.5 Peanut genetic transformation

Success of conventional breeding greatly hampered because of cleistogamous pollination in case of agronomic trait improvement in peanut. As a result, introduction of novel traits into peanut using genetic engineering techniques will assist in the development of improved peanuts by allowing the introduction of genes from source other than *Arachis* germplasm directly into commercially important cultivars without altering other characteristics.

There are two common methods available which were applied for gene delivery, named, *Agrobacterium tumefaciens* mediated gene transfer and particle bombardment method

using gene gun. It has been earlier reported that higher rate of transformation (4.5%) was achieved using gene gun in zygotic embryo of peanut compared to only 1.8% with *Agrobacterium tumefaciens* mediated gene transfer (Higgins and Dietzgen, 2000). However, Agro-based transformation is considered as a cleaner approach, since the T-DNA which is thought to be the only DNA which is transferred into the plant's genome (Smith *et al*, 1992). Moreover, wider availability and cost effectiveness are also two facilities which can be achieved through this methodology of gene transfer (Rafiq, 2014). Numerous studies have focused on transforming peanuts using particle bombardment as well as *Agrobacterium*-mediated transformation systems (Table 1.1).

It has been previously stated that grain legumes are recalcitrant to genetic transformation (Hassan *et al*, 2007). It is justified due to their poor regeneration ability and shortage of compatible gene delivery method. However, success has been also been seen in some legume crops like soybean (Chandra and Pental, 2003). So development of consistent transformation protocol is a need of time (Sarker *et al*, 2003).

The *Agrobacterium*- mediated transformation technique is extensively affected by various factors at different stages of experiment. After agroinfection, shoot regeneration pattern from cotyledon or other meristematic explants was observed rapid and efficient in a number of legume species (Somers *et al*, 2003). Decapitated half embryo is one of those types of explant. Conversely, transformation efficiency was found better when work was done by leaf tissue as explants (Cheng *et al*, 1996). Moreover, regeneration and transformation frequency were explants wise analyzed among three types of explants namely leaflet, hypocotyl and epicotyl of peanut and found better regeneration in leaflet (Sarker and Islam, 1999). However, the use of decapitated half embryo as explants for transformation in peanut has not been reported yet whereas it is one of the finest explants for transformation of lentils, another recalcitrant crop (Hassan *et al*, 2007).

It is necessary to evaluate factors involves in gene transfer prior to taking any transformation project just after the identification of compatible explants. Factors affecting transformation in chickpea were optimized by Akbulut and his colleagues (2008).

Islam and her coworkers stated the effects of optical density at 600nm, incubation period for infection and co-cultivation period in three Bangladeshi tomato varieties together with one Indian tomato variety (Islam *et al*, 2010). Earlier, comparable study was reported by Sharma *et al* (2009), where they optimized factors, like, bacterial density and co-cultivation time in three Indian varieties of tomato.

Higher concentration of *Agrobacterium* during transformation may cause hypersensitive response of explants as well as it will be a difficult work to kill them after co-cultivation due to excessive aggregation of *Agrobacterium* cells (Saini and Jaiwal, 2007). Similar result found in *Nicotiana tabacum* and *Arabidopsis thaliana* (Lin *et al*, 1994) and in most of the grain legumes (Bean *et al*, 1997). As a result, determination of optical density into optimum level is a part of transformation factors analysis. It can be determined directly by direct enumeration of bacteria (Sharma *et al*, 2009) or by indirect method, such as, measuring optical density using a spectrophotometer (Pérez-Piñeiro *et al*, 2012).

Akbulut *et al.* (2008) varied infection time while carrying out *Agrobacterium*-mediated transformation of chickpea (*Cicer arietinum* L.) seedlings. They applied a variety of infection duration (2h, 8h, 16h and 24h) and concluded 16h and 24h as optimum. They also mentioned that the longer the infection period, higher is the rate of transformation but this also resulted in bacterial overgrowth which eventually lessened viability of seedlings It was seen for Blackgram that further increase of infection duration from the optimum value (20-30 minutes) was not helpful for the boost of transformation frequency and caused problems in eliminating the bacteria (Saini & Jaiwal, 2007). On the other hand, browning of the target tissue had been seen in gherkin due to extending infection time (De Clercq *et al*, 2002). Infection duration was also optimized the herb *Artemisia absinthium* where root, leaf and hypocotyls were used as explants (Mannan *et al*, 2009). Paramesh *et al* (2010) reported varying infection duration in tomato where they used cotyledonary leaf and hypocotyl explants for transformation whereas optimization of infection duration for decapitated half-embryo explant of peanut was not done yet. Although Rohini and Rao (2000) have optimized the necessary factors for regeneration of transgenic plants from whole-embryo of peanut.

Co-cultivation duration is an important factor in plant transformation. Lower co-cultivation duration was sufficient for batter transformation in canola (Opabode, 2006) whereas higher co-cultivation duration such as 4 days is needed for better transformation efficiency in peanut (Venkatachalam *et al*, 1998) and alfalfa (Chabaud *et al*, 1988). According to Venkatachalam (1998), more than 4 days caused explant abortion due to bacterial contamination. Similar result was observed in cowpea (Muthukumar *et al*, 1996) and in pea (Decathen and Jacobsen, 1990; Lulsdorf *et al*, 1991) whereas further extension of co-cultivation time from 2-3 days leads to lower the regeneration potential of the explants in Blackgram (Saini and Jaiwal, 2007).

Apart from explants, efficiency of plant transformation also depends on the genotype, technique employed and regeneration system (Gelvin *et al,* 2000). The successful production of transgenic plants relies on several factors:

i) method of plant regeneration directly or indirectly from transformed cell,
ii) means of stably introducing foreign DNA into the genome of a plant cell,
iii) method by which transgenic plants can be identified among population of untransformed individuals (Somers *et al*, 2003).

1.6 Soil salinity and its effect in crop production

Salinity is an increasingly important environmental stress of crop production worldwide. About 20% of the world's cultivated land and nearly half of all irrigated lands are unsuitable for growing crops due to salinity (Rhoades and Loveday, 1990). More than 800 million hectares of land are affected by salt throughout the world (FAO, 2008). A saline soil is generally defined as one in which the electrical conductivity (EC) of the saturation extract in the root zone exceeds 4 dSm−1 at 25^{o}C and has an exchangeable sodium percentage of 15 (FAO, 1997). Salinity is that condition of soil which is characterized by high concentration of soluble salts. The natural floras of highly saline soil are halophytes, which are able to tolerate salinity exclusion than glycophytes. Salinity induces a major change in the signaling pathways of root origin which further leads to root and shoot growth retardation due to hormonal imbalance (Lerner *et al*, 1994).

A large portion of irrigated land in Asia has badly affected by salinization (Rains and Goya, 2003). Moreover, the increasing rate of salinized soil is 10% annually. Salinity has an effect on plant differently based on the extent of salinity, age of plant and plant genotype. Some varieties are more prone to salinity than others. Salinity hinders plant growth for decrease of water accessibility to the plants and increase of salt or ion (Munns *et al*, 2006).

1.7 Salinity problem in Bangladesh

Mainly the cultivable areas of coastal districts in Bangladesh are distressed with varying degrees of soil salinity. The coastal and offshore area of Bangladesh includes tidal, estuaries and river floodplains in the south along the Bay of Bengal. The use of agricultural land in these areas is very low, which is roughly 50% of the country's average (Peterson and Shireen, 2001). In general, it is believed that soil salinity is responsible for low land use and cropping intensity of these areas (Rahman and Ahsan, 2001). Salinity is a recent area of concern in Bangladesh. Combating land salinization problem is vital for food security of the growing population in Bangladesh where it is needed to explore the possibilities of increasing use of saline lands for the acceleration of the production of food crops (Haque, 2006).

1.8 Strategy to make salt tolerant peanut

Salinity and drought are two major problems of Bangladesh which limit the agricultural productivity (Boyer, 1982; Bartels and Sunkar, 2005). Better farm management practices, such as, phase farming, better irrigation, intercropping and precision farming could be helpful to get rid of these problems (Munns, 2002). As it requires spending a lot of money and time, better breeding and advance technologies like genetic engineering has to be developed by the researchers to make crops more productive under high salinity conditions (Cushman and Bohnert, 2000). Development of conventional breeding strategies has been hindered due to complex salt tolerance mechanism and narrow genetic makeup of plants.

However, it is possible to generate salt tolerant crops through molecular cloning of genes encoding major components in the signal transduction and metabolic pathway and the development of genetic transformation technology (Bohnert and Jensen, 1996).

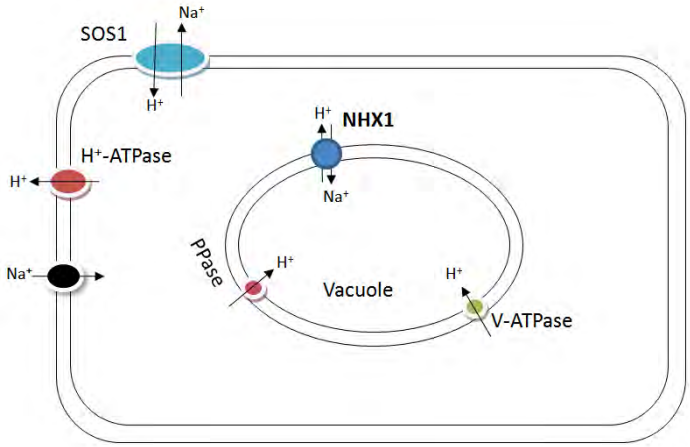
The transgenic technology has great potential to generate plants that are capable of growing in high salinity containing soil and improving agricultural productivity by manipulating the biosynthesis of compatible solutes to enhance ion homeostasis, increasing antioxidation to diminish oxidative damage, overexpressing Na^+/H^+ antiporter genes to reduce the Na^+ level in the cytoplasm (Apse *et al*, 1999; Zhang and Blumwald, 2001; Zhang *et al*, 2001; Shi *et al*, 2003; Ashraf and Harris, 2004).

In *Arabidopsis*, transportation of sodium out of the cell is carried out by a secondary active Na^+/H^+ antiporters, SOS1, which are responsible for salt tolerance (Zhu, 2003) Furthermore, the efficient compartmentalization of sodium is completed through the action of vacuolar Na^+/H^+ antiporters that remove potentially harmful Na^+ from the cytosol into the large tonoplast-bound vacuoles (Apse *et al*, 1999). Accumulation of ions in the vacuole helps to maintain water flow into the cell (Glenn *et al*, 1999; Gaxiola *et al*, 2002).

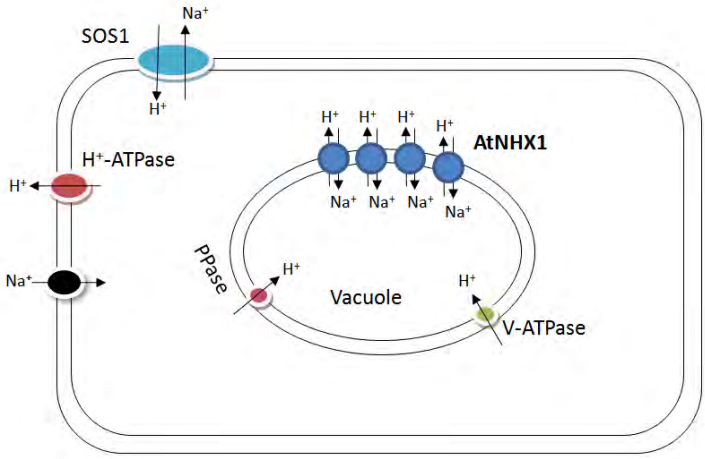
Na^+/H^+ exchangers are membrane proteins, which acts as enzymes for the exchange of Na^+ and H^+ across membranes. During the time of metabolism, cell volume, pH regulation, morphogenesis, and extruding the H^+ generation are maintained by these antiporters (Waditee *et al*, 2006). *Arabidopsis* genome sequencing suggested the presence of more than 38 Na^+/H^+ exchanger homologs, among which vocuolar *AtNHX1* and the plasma membrane *SOS1* are two extensively studied antiporters (Figure 1.1.).

The *Arabidopsis thaliana* genome-sequencing revealed a plant gene *AtNHX1,* which is similar to bacterial, fungal, and mammalian homologous (Apse *et al*, 1999; Frommer *et al,* 1999; Waditee *et a*l, 2006).Under salt stress condition, *AtNHX1*-overexpressing *Arabidopsis* showed some phenomena different compared to wild-type, such as, higher vacuolar Na^+/H^+ exchange activity and sequestering more Na^+ in plants (Apse *et al*,

1999), which provided convincing evidence that Na^+/H^+ antiporters in vacuoles play an important role in salt tolerance. In addition to *Arabidopsis* gene *AtNHX1*, overexpression of its homologous genes *GhNHX1* from cotton (*Gossypium hirsutum*), *OsNHX1* from rice (*Oryza sativa*), and *CgNHX1* from *Chenopodium glaucum* in tobacco and rice also can show the way to increase salt tolerance (Fukuda *et al*, 2004; Wu *et al*, 2004; Li *et al*, 2009). These studies indicate that overexpression of vacuolar Na^+/H^+ antiporters can confer increased salt tolerance in plants like peanut.



A.



B.

Figure 1.1 Model for Na^+ regulation in plant cell. A. Transporters regulating Na^+ levels in a normal plant cell. B. Transporters regulating Na^+ levels in an *AtNHX1*-expressing plant cell (Banjara, 2010).

Table 1.1: List of published *Agrobacterium*-mediated peanut transformation studies (Source: Burns, 2010)

Cultivar	Explant	Traits	Promoter	Reference
Tatu, Tatui, Tatu branco, Tupa, Penapolis	epicotyls	β-Glucuronidase	ATC1	Lacorte *et al*, 1991
Okrun	hypocotyls	β-Glucuronidase	CaMV 35S	Franklin *et al*, 1993
New Mexico 'A'	leaf sections	β-Glucuronidase	CaMV 35S	Eapen and George, 1994
Florigiant, NC-7, Florunner, F435AT	embryo axes	β-Glucuronidase	MAS	McKently *et al,* 1995
New Mexico 'A'	leaf sections	β-Glucuronidase	CaMV 35S	Cheng *et al*, 1996, 1997
New Mexico 'A'	leaf sections	Nucleocapsid gene from TSWV	CaMV 35S	Li *et al*, 1997
New Mexico 'A', Florunner, Georgia Runner, Sunrunner, Southrunner	Epicotyls	β-Glucuronidase	CaMV 35S	Egnin *et al*, 1998
VRI-2, TMV-7	cotyledon	β-Glucuronidase	CaMV 35S	Venkatachalam *et al,* 1998, 2000
TMV-2	embryo axis attached to one cotyledon	β-Glucuronidase	CaMV 35S	Rohini and Rao, 2000

Cultivar	Explant	Traits	Promoter	Reference
TMV 2	embryo axis attached to one cotyledon	Tobacco chitinase	CaMV 35S	Rohini and Rao, 2000
JL-24	de-embryonated cotyledon	β-Glucuronidase	CaMV 35S	Sharma and Anjaiah 2000
TMV 2	embryo axis attached to one cotyledon	Tobacco chitinase	CaMV 35S	Rohini and Rao, 2001
JL-24	embryo axis attached to one cotyledon	β-Glucuronidase	none	Anuradha *et al*, 2006
JL-24	embryo axes	mustard defensin (BjD)	CaMV 35S	Anuradha *et al*, 2008
Florunner, Florigiant	Somatic embryos	β-Glucuronidase	CaMV 35S	Ozias-Akins *et al*, 1993
Gajah, NC-7	Somatic embryos	β-Glucuronidase	CaMV 35S	Livingstone and Birch, 1999

1.9 Objectives of the present study

Development of a salinity tolerant transgenic peanut plant is the main objective. To develop a robust and reproducible protocol for this purpose, this study was designed to establish the following divisions:

- Evaluation of the established tissue culture protocol using two different explants, named, decapitated half embryo and embryonic leaflets.
- *Agrobacterium*-mediated transformation using best responding explants to determine the optimum level of transformation factors, such as, genotypes, age of embryo, bacterial density, infection duration, co-cultivation duration and antibiotic tolerance of the putative transgenic peanuts.
- Determination of regeneration potential when transformed with three different vectors, named, *pBI121, pK7WG2_OsNHX1_1.6* and *pK7WG2_AtNHX1_1.6.*

Chapter 2

Materials

In the present research work, following plant explants and *Agrobacterium* strains were used as experimental materials.

2.1 Plant Materials

Matured seeds of three different varieties of peanut, named, BINA Chinabadam 2, BINA Chinabadam 4 and BINA Chinabadam 6 were used in tissue culture and transformation study. These were collected from Bangladesh Institute of Nuclear Agriculture (BINA). Seeds were preserved at 4°C temperature in Plant Biotechnology Laboratory, BRAC University, Mohakhali, Dhaka, Bangladesh. Important characteristics of all the varieties are given below:

2.1.1 BINA Chinabadam 2

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

2.1.2 BINA Chinabadam 4

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

2.1.3 BINA Chinabadam 6

Plants are dwarf (av. 28cm); leaves are small, darker green and oval shape; this variety is also a mutated product achieved through gamma irradiation. Pods are shiny, without constriction; maximum pod yield potential is 3.2 tons/ha in winter and 1.7 tons/ha in summer; maturity period ranges between 150-160 days in winter and 140-150 days in summer; seeds contain oil-48.51% and protein-28.68 %. This variety is moderately resistant to collar rot and rust diseases.

2.2 Engineered *Agrobacterium* vectors

Agrobacterium tumefaciens strain LBA4404 with plasmids constructs, *pBI121*, *pK7WG2_OsNHX1_1.6* and *pK7WG2_AtNHX1_1.6* were used for infection in the transformation experiment.

2.2.1 Plasmid *pBI121*

The total size of *pBI121* is 12.8 kb according to its construction map. The T-DNA of Ti plasmid contains a plant selectable marker gene neomycin phosphotransferase II (*npt* II) conferring resistance to kanamycin and a *uidA* gene encoding β-gluduronidase (*GUS*) reporter gene (1812 bp). These two genes were separately fused under the control of the nopaline synthase promoter (NOS-pro) and CaMV 35S promoter (CaMV 35S-pro) within the left and right border region (Fig. 2.1).

2.2.2 Plasmid *pK7WG2_OsNHX1_1.6*

The Na^+/H^+ antiporter gene (*OsNHX1_1.6*) cloned from rice was immobilized to gateway vector, *pK7WG2_OsNHX1_1.6*. This final construct *pK7WG2_OsNHX1_1.6* was transformed into *A. tumefaciens* LBA4404 to be used in peanut transformation. It contains kanamycin resistance for selection in plants and spectinomycin and streptomycin resistance for selection in bacteria (Fig. 2.1).

2.2.3 Plasmid *pK7WG2_AtNHX1_1.6*

The Na^+/H^+ antiporter gene (*AtNHX1_1.6*) cloned from *Arabidopsis thaliana* was immobilized to gateway vector, *pK7WG2_AtNHX1_1.6*. This final construct

pK7WG2_AtNHX1_1.6 was transformed into *A. tumefaciens* LBA4404 to be used in peanut transformation. It contains kanamycin resistance for selection in plants and spectinomycin and streptomycin resistance for selection in bacteria (Fig. 2.1).

2.3 Different culture media used

2.3.1 Tissue culture media

In the present study, for tissue culture experiment Murashige and Skoog (MS) medium (1962) along with various concentration of different growth hormones were used for different types of experiments, such as,

2.3.1.1 Seed germination medium

For seed germination MS basal medium solidified with agar was used.

2.3.1.2 Regeneration initiation and shoot differentiation media

For regeneration initiation, cotyledonary leaf as well as decapitated half embryo was cultured on MS media supplemented with different concentrations and combinations of various growth regulators, such as, BAP and Kn. After shoot initiation same concentration of hormone containing MS media were used for shoot elongation.

2.3.1.3. Root induction media

For induction of root from the *in vitro* grown multiple shoots, half strength of MS basal medium supplemented with IBA was used.

2.3.2 Transformation media

2.3.2.1 *Agrobacterium* culture media

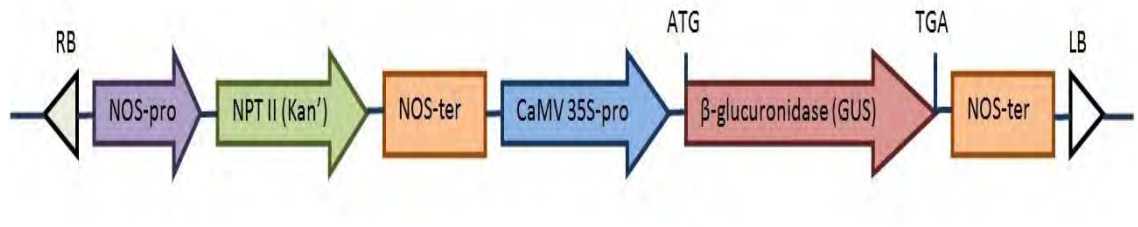
Two state of YEP (Yeast Extract Peptone Broth) with appropriate concentrations of antibiotics were used for bacterial culture. Liquid YEP medium was used for growing *Agrobacterium tumefaciens* strain LBA4404. This bacterial suspension was used as working culture for infection. Agar solidified YEP medium were used for maintenance of bacterial pure culture.

2.3.2.2. Co-culture media

Best shoot regeneration media without antibiotics were used as co-cultivation medium.

2.3.2.3 Selection media

For *Agrobacterium* culture, three antibiotics, such as kanamycin for *Agrobacterium* containing *pBI121*, *pK7WG2_OsNHX1_1.6* and *pK7WG2_AtNHX1_1.6* plasmid and streptomycin and spectinomycin for *Agrobacterium* strain LBA4404 were used. Cefotaxime (Duchefa Bioc) was used after co-cultivation as bacteriostatic agent against *Agrobacterium*. Appropriate concentration of kanamycin (Duchefa Bioc) obtained from kanamycin sensitivity test was used as selectable agents with the regeneration media containing best hormonal combination.



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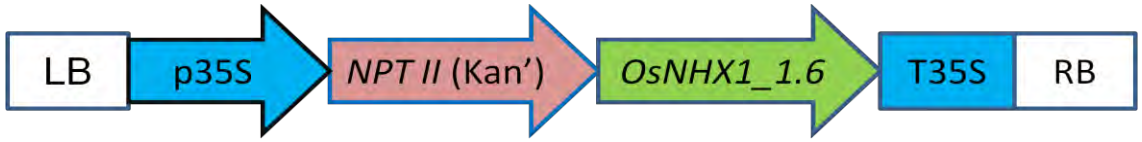

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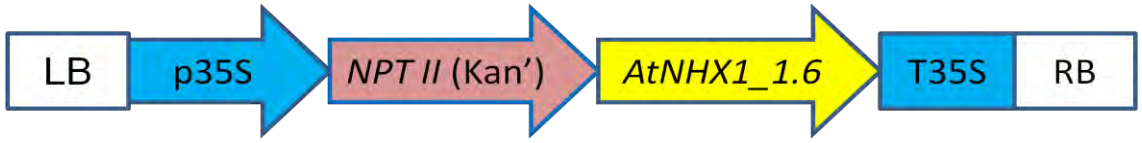

C.

Fig. 2.1 Schematic diagram of A. the T-DNA region of the binary *pBI121*, B. Constructed vector *pK7WG2_OsNHX1_1.6*, C. Constructed vector *pK7WG2_AtNHX1_1.6*

Chapter 3

Methods

3.1 Stock solution preparation

The various constituents of the medium were prepared by making different stock solutions as the first step for the preparation of the medium. As different constituents were required in different concentrations, separate stock solutions for macro- nutrients and micro-nutrients, plant growth regulators etc. for *in vitro* regeneration as well as antibiotics stock solutions for transformant selection were prepared.

3.1.1 Macro nutrients stock solution preparation

This stock solution was made 10 times the concentration of the full medium. The components of macro-nutrients (mentioned in Appendix-1) were serially added to distilled water in a volumetric flask and magnetic stirrer was used to mix them well. Then desired volume (500ml) was made by adding distilled water. After that the solution was poured into a clean container and tagged. Finally, the solution was autoclaved (Model: WAC-47, Korea/ ALP, Japan) and stored in a refrigerator at 4ºC for several weeks.

3.1.2 Micro nutrients stock solution preparation

The solution was made 100 times of the full strength of the media. The components of micronutrients (mentioned in Appendix-1) were mixed in a flask with distilled water by using a magnetic stirrer. Then the total 500 ml of the solution was autoclaved. Once cooled down, stored it at 4ºC for some weeks.

3.1.3 Iron EDTA stock solution preparation

The solution was made 100 times of their full strength. $FeSO_4.7H_2O$ (27.8 mg/l) was added and stirred in hot plate till dissolved and then $Na_2EDTA.2H_2O$ (37.3 mg/l) was

added. Magnetic stirrer was used for making this solution. This solution was made and preserved at 4ºC in amber bottle as it is light sensitive.

3.1.4 Organic stock solution preparation

The stock solution was made 100 times of their full strength. Components (mentioned in Appendix-1) were added one by one and stirred some more time before adding the next. Then it was stored at 4ºC.

3.1.5 Stock solutions of growth hormones

3.1.5.1 BAP stock solution preparation

The BAP (Sigma) stock solution was prepared by dissolving 10 mg of BAP in 1 ml or 2 ml of 1N NaOH and made up to 100 ml by additional distilled water (Table 3.1). The final concentration of the stock was (10mg/100ml). The stock solution was then filtered, labeled and stored at 4ºC for up to 2 months.

3.1.5.2 Kinetin stock solution preparation

The Kinetin (Sigma) stock solution was prepared by dissolving 10 mg of kinetin in 1 ml or 2 ml of 1N NaOH and made up to 100 ml by additional distilled water. The final concentration of the stock was (10mg/100ml). The stock solution was then filtered, labeled and stored at 4ºC for up to 2 months.

3.1.5.3 IBA stock solution preparation

First, 200 mg of IBA (Sigma) was dissolved with 1N NaOH. The total volume 200 ml was made by using double-distilled water. The final concentration of the stock was 1mg/ml. Finally, it was filtered and labeled and was stored at -20°C for several months.

3.1.6 Antibiotics stock solution preparation (25 mg/ml)

Kanamycin, streptomycin, spectinomycin and cefotaxime stock solutions were prepared. Any one of these antibiotic powder of 1g was dissolved in 35 ml of ddH_2O.

Volume was made up to 40 ml with ddH_2O and sterilized by filtration and finally stored at -20ºC.

3.2 Preparation of one liter of MS medium

Murashige and Skoog (MS) medium (1962) was used as basal tissue culture medium for peanut *in vitro* regeneration. MS medium was prepared from these stock solutions. The amounts of using prepared stock solutions are given below in Table 3.2. The following steps were carried out successively:

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

II. 100 ml of stock solution Macro-nutrients, 10 ml of Micro-nutrients, Iron-EDTA and Organic stock solution was added sequentially and mixed thoroughly.

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

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

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

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

VII. The pH of the medium was adjust to 5.8 using a digital pH meter with the help of 1N NaOH or 1N HCl, whichever was required.

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

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

3.3 Media sterilization

Fixed volume of hot medium was dispensed into conical flasks. The flasks were plugged with non-absorbent cotton plugs and covered with aluminium foil and marked with the help of glass marker to indicate specific hormonal supplements. The conical flasks were then autoclaved (ALP-32, Japan) at 15 lb/sq inch at 121º C temperature for 20 minutes.

Table 3.1 Growth regulators, their solvents and molecular weights

Growth regulator	Solvent	Molecular weight
IBA	1 N NaOH	203.24
BAP	1 N NaOH	225.3
Kn	1 N NaOH	215.2

Table 3.2 Different components needed for preparation of 1 litre of full strength MS media

Components (stock conc.)	Amount (for 1000 ml)
Macronutrients (10x)	100ml
Micronutrients (100x)	10 ml
Organic (100x)	10ml
Fe- EDTA (100x)	10 ml
Myo-inositol	0.1g
Sucrose	30g

3.4 Preparation of seed germination media

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

3.5 Precaution to maintain aseptic condition

To maintain aseptic condition, all inoculation was carried out under the laminar air flow hood (SCV, Singapore). UV light of laminar hood was switched on for 15 minutes before manipulation. Then the laminar hood was cleaned with 70% ethanol spray and hands were washed with antimicrobial hand wash (Hexisol®, ACI Ltd.). The instruments (forceps, scalpel, Petri-dish etc.) were sterilized by using a Bunsen burner to prevent air borne bacteria and immersed into absolute alcohol during the experiment taking place. The flask and Petri-dish cover were flamed twice, once after opening and again before closing them. All pipettes were disposed and reused after autoclaved. Filter sterilized antibiotics were added to the media under laminar air flow hood, when required. All contaminants and old bacterial culture were discarded after autoclaving to maintain biosafety procedure.

3.6 Culture techniques

Techniques implemented in this present study are presented below:

(i) Axenic Culture

(ii) Explant designing and explant culture

(iii) Subculture

(iv) Root induction

(v) Transplantation

(vi) Maintenance of transplanted plants

3.6.1 Axenic culture

As a first step, seeds were washed under running tap water for 3-5 minutes. At that time, floating seeds were discarded, as they had abnormality. Later the seeds were

stirred gently with 70% ethanol for 1 minutes followed by thorough washing with double distilled water inside laminar air flow cabinet.

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

3.6.2 Seedling development

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

3.6.3 Explant designing and explant culture

Germinated seeds of the mentioned varieties were the source of the two different explants, leaflet and half decapitated embryo.

Leaflet explants were collected from 7, 9 and 11 days old germinated seeds. On the other hand, immature embryos were collected from overnight and 2 days long emerged seeds. These explants were cultured in MS media with various combinations of hormonal supplements to achieve regeneration.

3.6.4 Subculture

All the *in-vitro* cultures were transferred into fresh media at an interval of 2 weeks. Morphogenic changes were under regular observation for proper data maintenance.

3.6.5 Root induction

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

3.6.6 Transplantation

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

3.7 *Agrobacterium tumefacience* culture media preparation

YEP medium was prepared to culture *Agrobacterium tumefaciens* strain LBA4404. After mixing all the components of YEP medium (Table 3.3), the pH was set at 7.0-7.2 and the volume was made up to 1 litre. Then agar 0.6% (w/v) was added to prepare solid media. After cooling down the autoclaved media, antibiotics were added. For *Agrobacterium tumefaciens* containing *pK7WG2_OsNHX1_1.6* and *pK7WG2_AtNHX1_1.6* 100 mg/l streptomycin and 200 mg/l spectinomycin were added to each 100 ml medium. Kanamycin was added at 200 mg/l to the medium as selective agent for the *Agrobacterium tumefaciens* containing any of the three vectors, named, *pBI121, pK7WG2_OsNHX1_1.6 &pK7WG2_AtNHX1_1.6*.

Table 3.3 Components required for the preparation of 1L YEP medium

Components	Amount (grams)
Yeast extract	10
Bacto peptone	10
Nacl	5

3.8 Co-cultivation medium preparation

MS medium with growth hormones was used as co-cultivation medium. Hormonal concentration that was found to be the best for tissue culture of peanut was added to this medium. No antibiotics were added here.

3.9 Methodology of kanamycin sensitivity test

Different concentrations (0, 20, 30, 40, 50, 100, 150, and 200 mg/l) of kanamycin were added to regeneration media. The explants were placed in those media to check their regeneration response. The result was recorded after 20 days of inoculation of decapitated explants of BINA Chinabadam 2, BINA Chinabadam 4 and BINA Chinabadam 6.

3.10 Selection media preparation

For transformed shoot selection, cefotaxime along with kanamycin were used with regeneration media. These media contain best hormonal concentration found in plant tissue culture experiment.

3.11 Preparation for performing histochemical GUS assay

3.11.1 Preparation of histochemical reagent (X gluc) solution

10 mg of X-Gluc (β- glucuronide, cyclohexylaminonium salt, $C_{14}H_{13}BrClNO_7$ $C_6H_{13}N$), (1mg/ml) was dissolve in 100 µl of dimethyl formamide (DMF) in a pyrex tube.

Volume was made upto 10 ml with 50 mM phosphate buffer, pH 7.0. X-Gluc solution was stored in dark container at -20°C.

3.11.2 Histochemical GUS assay

Tissue segments were immersed in fixation solution in sterile eppendorf tubes and incubated for overnight. Then the solution was discarded and washed the tissue three times with 50 mM phosphate buffer, pH 7.0. Enough X-Gluc solution was added to cover the tissue pieces in eppendorf tubes. Incubated at 37°C overnight and allow the blue color to develop. X-Gluc solution was discarded and ice cold 70% ethanol was added and again incubated at 37°C for 48 hours for degreening. Slides of transformed explants were prepared for observing under microscope.

3.12 Peanut tissue culture dependent method of transformation

Day 1: YEP solid media was prepared with required antibiotics (kanamycin for *Agrobacterium* strain with *pBI121, pK7WG2_OsNHX1_1.6 & pK7WG2_AtNHX1_1.6* and both streptomycin and spectinomycin for *Agrobacterium* strain with *pK7WG2_OsNHX1_1.6 & pK7WG2_AtNHX1_1.6*) for *Agrobacterium* stock maintenance.

Day 2: A single colony of *Agrobacterium tumefaciens* (containing the desired construct) was streaked on an antibiotic containing YEP media plate with a sterilized loop. The Petri-dish was sealed with Para-film and kept upside down at 28°C for 72 hours and after that stored at 4°C to control overgrowth of bacteria. The subculture was done in fresh media in every week to maintain the stock.

Day 3: Media were prepared with required antibiotics which are needed for the maintaining *Agrobacterium* stock and for the infection of explants. Liquid YEP medium was prepared for liquid culture of bacteria. MS media was prepared for transferring explants after infection.

Day 4: Seeds were sterilized and preserved in dark chamber for 1 to 3 days in case of transformation with decapitated half embryo. Single colony was picked from *Agrobacterium* culture to inoculate with an inoculation loop in 100 ml of antibiotic containing liquid YEP media and the liquid culture was kept in a shaker (180 rpm) at 28°C for overnight.

Day 5: Optical Density at 600 nm (OD_{600}) of the overnight grown culture was measured while comparing with autoclaved fresh liquid YEP media by using spectrophotometer. The Petri-dish with filter paper is soaked with liquid MS media and then the Petri-dish was used to cut explants. Explants were dipped in bacterial suspension for 30, 60, 90 and 120 minutes for infection and then placed on co-cultivation medium and kept there for next 1 to 3 days (co-cultivation period).

Day 6: The Petri-plates were checked for bacterial overgrowth. Some of the explants having different transformation treatments (different $OD_{600,}$ infection and cocultivation duration, age of the embryo) were taken for GUS histochemical assay.

Day 7: Explants were transferred to cefotaxime containing regeneration media. If there is any bacterial overgrowth shown on explants, then those explants were washed with cefotaxime before transferring to cefotaxime containing MS media. Otherwise explants were directly transferred.

After a week, explants were placed on kanamycin or containing regeneration media to allow the transformed explants to grow. The selected healthy shoots were transferred to the rooting media. Non-infected explants were placed on regeneration media for comparative studies of regeneration between transformed and non transformed plants.

3.14 Determination of baseline saline tolerance level of peanut seedlings

In the present study, the effect of salinity on germination of peanut seeds was investigated. Therefore, three locally grown peanut varieties, such as, BINA Chinabadam 2, BINA Chinabadam 4 and BINA Chinabadam 6 were selected for the

study. To represent various salinity conditions, different NaCl concentrations ranging from 5-200 mM, which in turn stands for 0.5-20 dS/m were chosen for the study. MS media was prepared with different amount (0mM, 5mM, 20mM, 50mM, 100mM, 150 mM, and 200mM) of NaCl in each 100 ml of media.

Appendix-1

Different components for preparation of 1000ml stock solutions in MS media

Macronutrients (10X)	**mg/l**
KNO_3	1900
NH_4NO_3	1650
$MgSO_4 \cdot 2H_2O$	370
$CaCl_2 \cdot 2H_2O$	440
KH_2PO_4	170
Inorganic micronutrients (100X)	**mg/l**
KI	0.83
H_3BO_3	6.20
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.60
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
Fe-EDTA (100X)	**mg/l**
$FeSO_4 \cdot 7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
Organic (100X)	**mg/l**
Nicotinic acid	0.5
Pyridoxin HCI	0.5
Thiamin HCl	0.1
Glycin	2.0

Chapter 4

Result

Present investigation is divided into steps, such as, evaluation of the established tissue culture protocol using two different explants and *Agrobacterium*-mediated transformation using best responding explants to determine factors influencing transformation and regeneration potential when transformed with *pBI121, AtNHX1* and *OsNHX1*.

4.1 Analysis of shoot formation and rhizogenesis response of three peanut varieties following previously reported protocol

Chowdhury (2014) reported a genotype independent regeneration protocol for peanut was established using two types of explants, named, decapitated half embryo and embryonic leaflet. In the current experiment, regeneration potential for three peanut varieties was evaluated following that protocol.

4.1.1 Regeneration potential of decapitated half embryo of peanut varieties

Decapitated half embryo was observed to give good response in 3.0 mg/l BAP in combination with 1.0 mg/l kinetin for shoot regeneration. Among the three varieties, maximum regeneration was observed in BINA Chinabadam 2 which is relatively higher than BINA Chinabadam 4 and BINA Chinabadam 6 (Table 4.1). Shoots were developed within 15-19 days after inoculation (Figure 4.1).

During this study, spontaneous *in vitro* root induction did not take place. Roots were developed within 41-54 days after transferring the shoots into root induction media containing half strength MS media supplemented with 0.2 mg/l IBA (Table 4.2). However, rooting response was low in all the varieties (Figure 4.2).

4.1.2 Regeneration potential of embryonic leaflet of peanut varieties

For shooting from embryonic leaflet, MS media supplemented with 2 mg/l BAP and 0.5 mg/l kinetin was used. Leaflet explants responded well into callus (Figure 4.3) where

BINA Chinabadam 6 gave 84% calli which was the maximum response compared to BINA Chinabadam 4 and BINA Chinabadam 2. However, shoot regeneration percentage was only 10-14% among the varieties (Table 4.3).

Spontaneous *in vitro* root induction did not occur during previous study. Therefore, 2.5-4.2 cm long shoots were excised and then cultured on half strength MS media supplemented with IBA, and the effect was investigated. However, root formation from embryonic leaflet explants did not occur. Low percentage of shoot regeneration without any root development was seen in the present experiment with leaflet explants (Table 4.3). Therefore, no further study could be conducted with this explants.

4.1.3 Transplantation of regenerated plantlets and survivability in the natural environment

Mature rooted plantlets regenerated from decapitated half embryo were transferred to the soil in small pots and covered with perforated polybags for hardening (Figure 4.4). During hardening, maximum success (72.7%) was obtained in BINA Chinabadam 4 and BINA Chinabadam 6. After successful hardening, plantlets were acclimatized in natural environment where >80% of plants survived having proper growth (Table 4.4).

Table 4.1 Shoot regeneration from peanut decapitated half embryo on media supplemented with 3 mg/l BAP and 1mg/l kinetin

Peanut varieties	Time required for regeneration initiation (days)	Percentage of shoot regeneration± SD	Time required for shoot development (days)	Mean no. of shoots ± SD
BINA Chinabadam 2	11	96.67± 5.77	19	2.43± 0.51
BINA Chinabadam 4	6	86.67± 5.77	15	2.39± 0.20
BINA Chinabadam 6	6	83.33± 20.81	15	2.42± 0.15

Table 4.2 Rhizogenesis of shoots developed from decapitated half embryo explants of peanut varieties on media supplemented with 0.2 mg/l IBA

Peanut varieties	Percentage of Root induction ± SD	Time required for root induction (days)
BINA Chinabadam 2	35±15.00	54
BINA Chinabadam 4	30±12.49	41
BINA Chinabadam 6	26±10.58	43

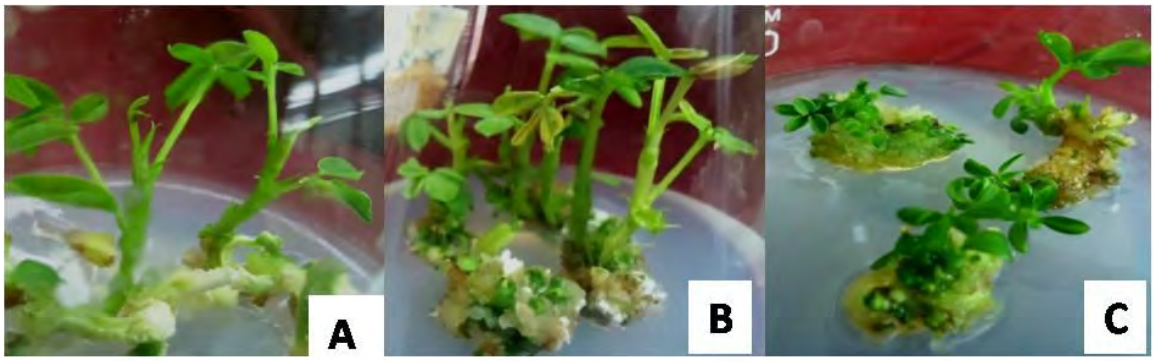

Figure 4.1 Shoot regeneration from decapitated half embryo of three peanut varieties, A. BINA Chinabadam 2, B. BINA Chinabadam 4 and C. BINA Chinabadam 6 in MS media containing 3mg/l BAP and 1mg/l kinetin after 15-19 days of their inoculation

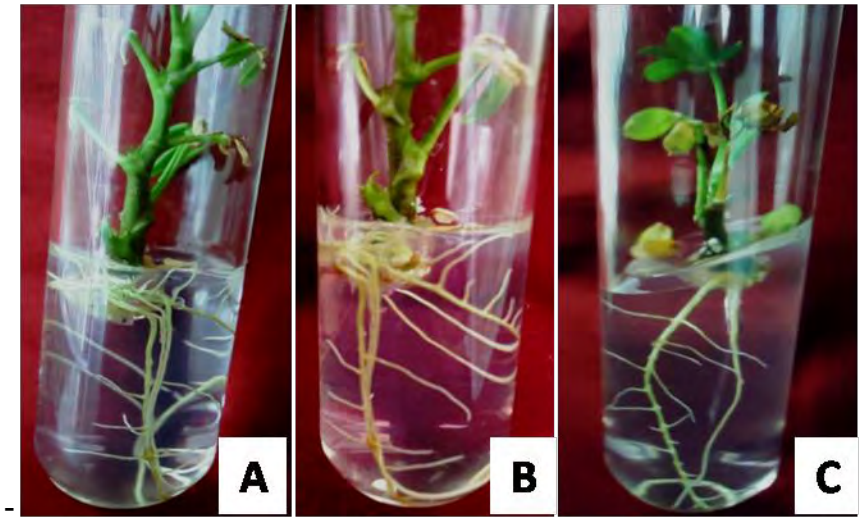

Figure 4.2 Rhizogenesis at the base of shoot regenerated from decapitated half embryo of three peanut varieties A. BINA Chinabadam 2, B. BINA Chinabadam 4 and C. BINA Chinabadam 6 in half MS media supplemented with 0.2 mg/l IBA after 55 days of inoculation

Table 4.3 Regeneration from embryonic leaflet explants of peanut varieties on media supplemented with 2 mg/l BAP and 0.5 mg/l kinetin

Peanut varieties	Time required for shoot regeneration (days)	Percentage of responsive explants ± SD	Percentage of shoot regeneration ± SD	Root formation (after 3 months)
BINA Chinabadam 2	32	77.78±11.11	10.19±3.20	0
BINA Chinabadam 4	21	72.20±12.73	12.03±8.90	0
BINA Chinabadam 6	36	84.26±5.78	14.25±6.94	0

0= Died, no shoot regeneration

Table 4.4 Transplantation of regenerated plantlets and survivability in the natural environment of three peanut varieties

Peanut varieties	Successfully hardened plantlets	Percentage of success (%)	Percentage of survived plants in natural environment
BINA Chinabadam 2	7	63.63	85.71
BINA Chinabadam 4	8	72.70	87.50
BINA Chinabadam 6	8	72.70	87.50

11 plantlets were taken for hardening in each trial

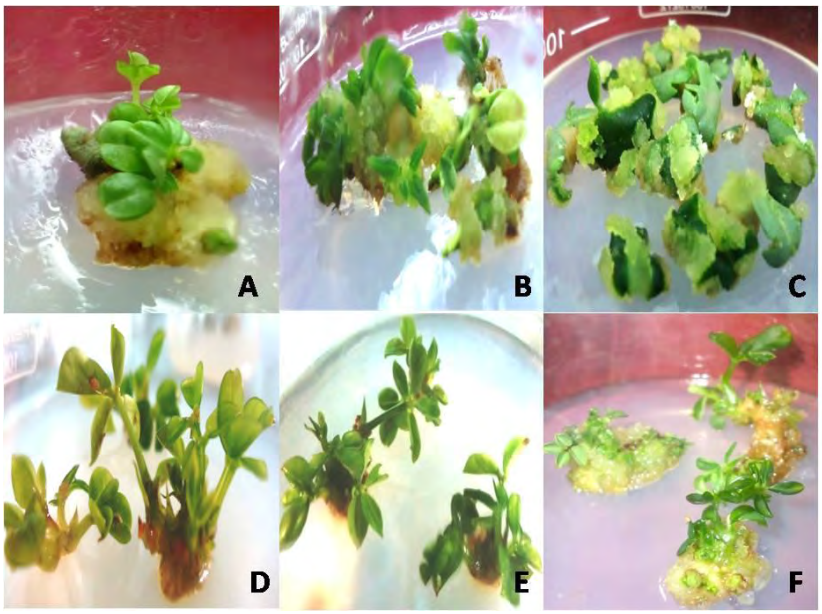

Figure 4.3 Callus formation and further regeneration of shoots from embryonic leaflet explants of three peanut varieties, namely, BINA Chinabadam 2 (A and D), BINA Chinabadam 4 (B and E) and BINA Chinabadam 6 (C and F) in the MS media supplemented with 2 mg/l BAP and 0.5 mg/l kinetin

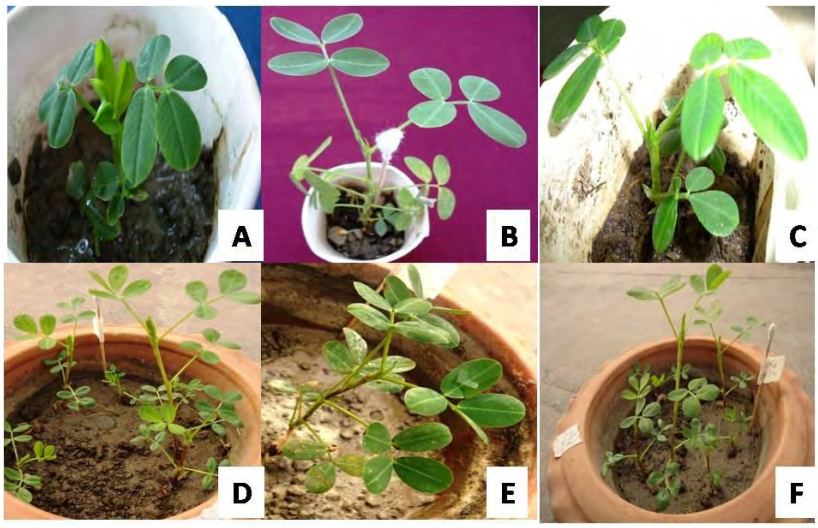

Figure 4.4 Hardening and acclimatization of regenerated plantlets of three peanut varieties, namely, BINA Chinabadam 2 (A and D), BINA Chinabadam 4 (B and E) and BINA Chinabadam 6 (C and F)

4.2 Analysis of the role of different transformation factors on transformation efficiency using decapitated half embryo as explant

In the present study, LBA4404 *Agrobacterium* strain harboring *pBI121* binary vector was used for the infection of decapitated embryo explants of three peanut varieties. Following infection, explants were analyzed by GUS histochemical assay to understand various factors on transformation (Figure 4.5).

4.2.1 Effect of bacterial density on transformation efficiency

Maximum percentages of GUS positive explants were observed at OD_{600} of 1.5 and at OD_{600} of 1.2 in the varieties BINA Chinabadam 6 (Table 4.5) and BINA Chinabadam 4 (Table 4.6), respectively. It was seen that at lower OD_{600} (<1.0), the percentage of GUS positive explants obtained was low. Whereas, it was also observed that the percentage of GUS positive explants fell drastically when explants were infected for longer time with optimum bacterial density.

4.2.2 Effect of infection duration on transformation efficiency

For both the test varieties, BINA chinabadam 4 and BINA chinabadam 6, maximum percentage of GUS positive explants was observed after 60 minutes of infection. Further increase of infection duration caused decrease in the percentage of GUS positive explants for both the varieties. It was also seen that with the increase of infection duration from 90 to 120 minutes the percentage of GUS positive explants remained constant at 50% for BINA Chinabadam 6 (Table 4.5) while the percentage dropped to 33% for BINA Chinabadam 4 (Table 4.6).

4.2.3 Effect of co-cultivation duration on transformation efficiency

In the present study, the explants were allowed to co-cultivate for 1-3 days where 3 days of co-cultivation duration gave the highest percentage of GUS positive explants in both, BINA Chinabadam 6 (Table 4.5) and BINA Chinabadam 4 (Table 4.6). The result of positive transformation from 1 day of co-cultivation was not satisfactory at all.

Table 4.5 Analysis of various transformation factors on transformation of decapitated half embryo of BINA Chinabadam 6

Optical density (OD_{600})	Infection time (mins)	Co-cultivation duration (days)	No. of explants used in GUS assay	Percentage of GUS positive explants
1.0	30	1	20	0
	60		20	0
	90		20	0
	120		20	0
1.5	30		20	0
	60		20	20
	90		20	10
	120		20	0
1.0	30	2	20	0
	60		20	10
	90		20	30
	120		20	10
1.5	30		20	25
	60		20	75
	90		20	50
	120		20	10
1.0	30	3	20	20
	60		20	80
	90		20	78
	120		20	42
1.5	30		20	100
	60		20	100
	90		20	33
	120		20	52

Table 4.6 Analysis of various transformation factors on transformation of decapitated half embryo of BINA Chinabadam 4

Optical density (OD_{600})	Infection time (mins)	Co-cultivation duration (days)	No. of explants used in GUS assay	Percentage of GUS positive explants
0.7	30	1	6	0
	60		6	0
	90		6	0
	120		6	0
1.0	30		6	0
	60		6	0
	90		6	50
	120		6	0
1.0	30	2	6	0
	60		6	50
	90		6	50
	120		6	0
1.2	30		6	66.66
	60		6	83.33
	90		6	66.66
	120		6	33.33
1.0	30	3	8	12.5
	60		8	12.5
	90		8	33.33
	120		8	50
1.3	30		6	33.33
	60		6	83.33
	90		6	16.67
	120		6	33.33

4.2.4 Effect of age of the embryo on transformation efficiency

Decapitated half embryo taken from 1 day old seed of BINA Chinabadam 6 and BINA Chinabadam 4 gave 75% and 25% of GUS positive explants respectively which were also the highest percentage obtained when compared to 2 days and 3 days old explants for both the varieties (Table 4.7).

Table 4.7 Effect of age of embryo on transformation efficiency of decapitated half embryo analyzed by transient GUS histochemical assay for BINA Chinabadam 4 and BINA Chinabadam 6

Peanut varieties	Age of embryo	No. of explants used in GUS assay	Percentage of GUS positive explants
BINA Chinabadam 6	**1 day**	**8**	**75**
	2day	13	38
	3 day	12	58
BINA Chinabadam 4	**1 day**	**12**	**25**
	2day	14	14.28
	3 day	12	16.66

OD_{600} of 1.0, 60 minutes of infection duration and 3 days of co-cultivation duration were maintained in all the experiments.

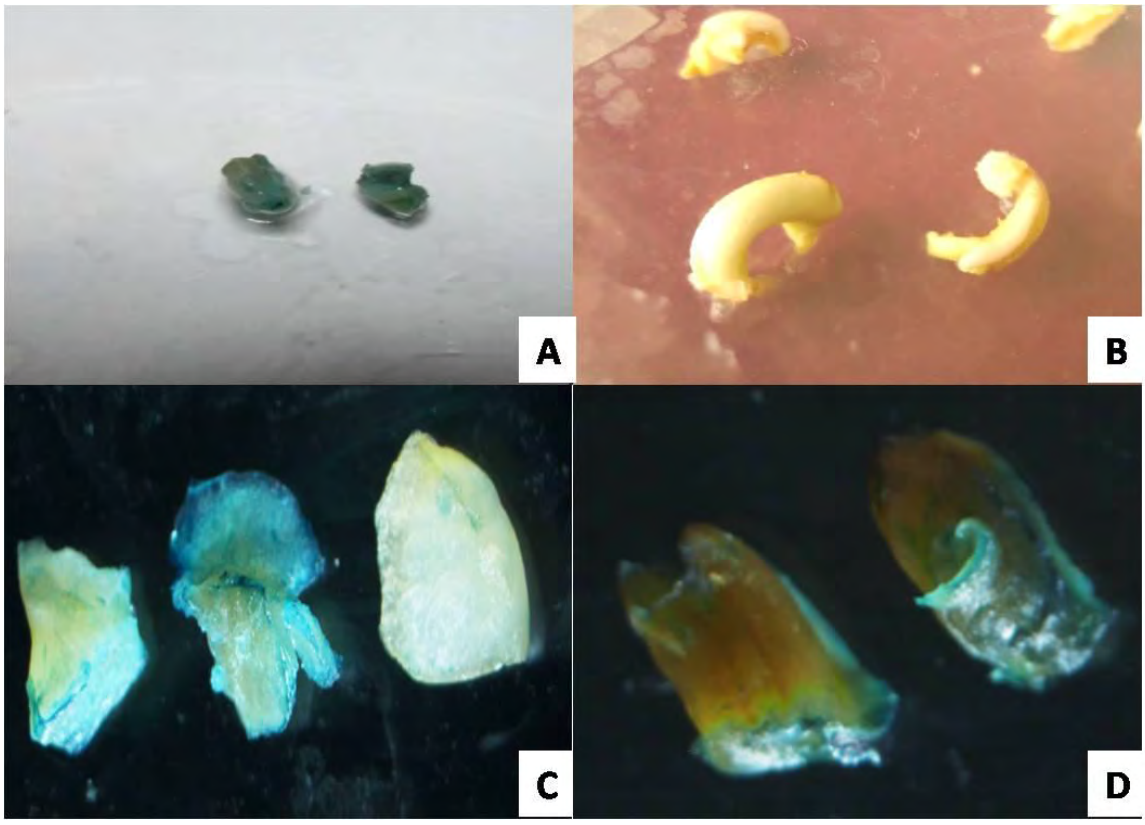

Figure 4.5 Observation of transformant through GUS assay. A. Blue coloration due to GUS histochemical assay observed in the decapitated half embryo, B. bacterial overgrowth due to high bacterial density in the co-cultivation medium and GUS positive explants under stereomicroscope (Olympus, Japan) C. BINA Chinabadam 4 and D. BINA Chinabadam 6.

4.3 Kanamycin sensitivity test

For this study, selection media containing kanamycin at eight different concentrations (0, 20, 30, 40, 50, 100, 150, and 200 mg/l) were used for sensitivity test (Figure 4.6). It was found that explants were able to resist upto 20mg/l of kanamycin. None of the explants regenerated in selection media containing 100mg/l or above while 100% regeneration percentage was found in control experiment (Table 4.8). Only 10% explants regenerated to shoots in presence of 50mg/l kanamycin which is very low regeneration frequency. Therefore, 100mg/l and above was optimum for selection.

Table 4.8 Effect of various concentrations of kanamycin on the regeneration of decapitated half embryo explants

Kanamycin concentration (mg/l)	Percentage of shoot formation	Percentage of survival
0	100	100
20	100	100
30	70	70
40	40	20
50	10	0
100	0	0
150	0	0
200	0	0

(Data was collected after 20 days of inoculation)

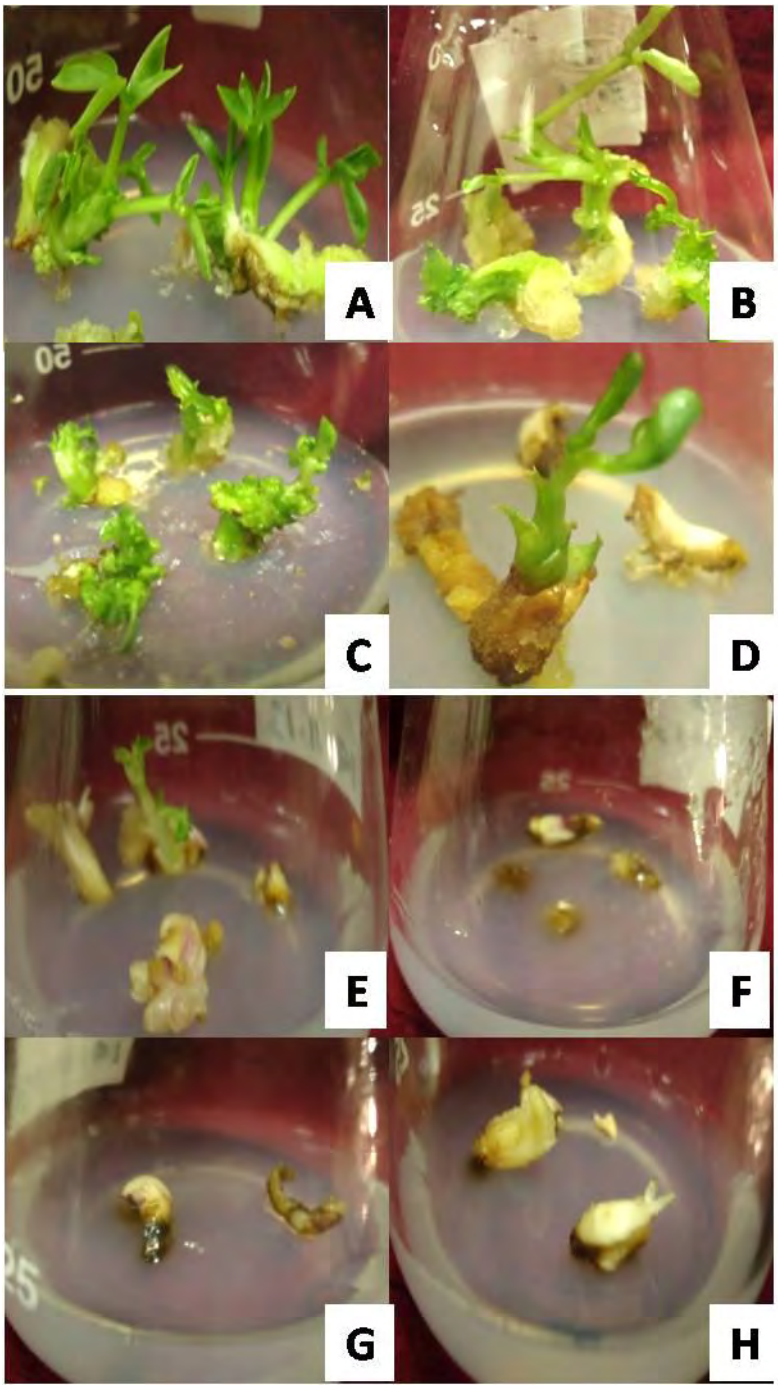

Figure 4.6 Effect of various concentrations of kanamycin on decapitated half embryo of BINA Chinabadam 6, (A) Control, 0mg/l (B) 20mg/l (C) 30mg/l (D) 40mg/l (E) 50mg/l (F) 100mg/l (G) 150mg/l (H) 200mg/l of kanamycin (Photos were taken 14 days of inoculation)

4.4 Analysis of regeneration efficiency after transformation with *pBI121, AtNHX1* and *OsNHX1* from decapitated half embryo

Transformation factors were determined solely on the basis of the results received after GUS histochemical assay. However, these optimized values may not be suitable for shoot regeneration. Hence, it is necessary that these factors are again evaluated based on the maximum percentage of regeneration of putative transformants in selection media containing 50 mg/ml kanamycin. Three peanut varieties namely BINA Chinabadam 2, BINA Chinabadam 4 and BINA Chinabadam 6 were taken under the present experiment after transformation with *pBI121, AtNHX1 and OsNHX1* to get utmost regeneration efficiency.

4.4.1 Regeneration frequency of three peanut varieties following transformation with *pBI121*

Higher *Agrobacterium* density (OD_{600} 1.0 to 1.5) was found to be efficient for better transformation frequency through GUS assay. Regeneration efficiency of putative transformed plants also varied due to different bacterial density. Regeneration percentage was higher with the bacterial density 1.0 than OD_{600} 1.5 in almost all three varieties in 60 minutes incubation and 3 days of co-cultivation period which were found to be optimum for high percentage of transient transformation (Table 4.9).

Regeneration efficiency was also checked against different incubation duration (60 minutes and 90 minutes) with an OD_{600} of 1.0 for 3 days of co-cultivation duration. 60 minutes infection duration gave better regeneration efficiency in BINA Chinabadam 4 and BINA Chinabadam 6 than 90 minutes of infection duration. Furthermore higher regeneration percentage has been consistently found in 60 minutes of infection duration when OD_{600} was increased into 1.5 in BINA Chinabadam 4 and BINA Chinabadam 2 (Table 4.9).

Higher co-cultivation time leads to devastating change of the rate of regeneration whereas higher co-cultivation duration of 3 days was found as optimum to ensure highest transformation. Regeneration frequency was greatly hampered when co-cultivation duration was increased from 2 days to 3 days in case of BINA Chinabadam

6 and BINA Chinabadam 2. Hence, high co-cultivation duration had negative effect on the regeneration potential of the explants of those two varieties. On the other hand, infected explants of BINA Chinabadam 4 could regenerate better in 3 days co-cultivation duration where the regeneration percentage was 97 % (Table 4.9).

4.4.2 Regeneration frequency of three peanut varieties following transformation with *pK7WG2_AtNHX1_1.6*

Regeneration percentage was higher with the bacterial density OD_{600} of 1.0 than OD_{600} 1.3 in almost all three varieties in 60 minutes of infection and 3 days of co-cultivation duration which were found to be the most suitable conditions for transient transformation. Moreover, extension of infection duration from 60 minutes led to reduce the regeneration efficiency in case of BINA Chinabadam 2 and BINA Chinabadam 6 when bacterial density was optimum as OD_{600} 1.0 (Table 4.10).

4.4.3 Regeneration frequency of three peanut varieties following transformation with *pK7WG2_OsNHX1_1.6*

In case of, transformation with *pK7WG2_OsNHX1_1.6,* maximum regeneration efficiency (≥90%) was found from BINA Chinabadam 6 and BINA Chinabadam 4 whereas BINA Chinabadam 2 showed only 60% regeneration which was the lowest regeneration efficiency under optimum transformation condition (Table 4.11).

Table 4.9 Putative transformed shoot regeneration efficiency under various transformation conditions (*pBI121*)

Peanut varieties	OD_{600}	Co-cultivation duration (days)	Infection duration (mins)	No of infected explants	Regeneration efficiency of putative transformed plants (50 mg/l kan)
BINA Chinabadam 6	1.0	2	60	21	80.95
			90	20	75
		3	60	31	64.51
			90	25	44
	1.5	2	60	26	73.077
			90	20	65
		3	60	26	53.84
			90	11	63.64
BINA Chinabadam 4	1.0	2	60	16	75
			90	23	60.87
		3	60	15	97.78
			90	24	75
	1.5	2	60	10	50
			90	10	70
		3	60	5	80
			90	10	70
BINA Chinabadam 2	1.0	2	60	12	83.33
			90	12	66.67
		3	60	12	58.33
			90	12	75
	1.5	2	60	12	66.67
			90	12	91.67
		3	60	12	91.67
			90	12	58.33

Table 4.10 Putative transformed shoot regeneration efficiency under various transformation conditions (*pK7WG2_AtNHX1_1.6*)

Peanut varieties	OD_{600}	Infection duration (mins)	No. of infected explants	Regeneration efficiency(%) on selection media
BINA Chinabadam 6	1.0	60	12	91.67
		90	12	66.67
	1.3	60	12	66.67
		90	12	75
BINA Chinabadam 4	1.0	60	22	77.27
		90	22	90
	1.3	60	22	68
		90	22	36
BINA Chinabadam 2	1.0	60	15	86.67
		90	15	46.67
	1.3	60	15	73.33
		90	15	86.67

Table 4.11 Putative transformed shoot regeneration efficiency under various transformation conditions (*pK7WG2_OsNHX1_1.6)*

Peanut varieties	OD_{600}	Infection duration (mins)	No. of infected explants	Regeneration efficiency on selection media ((%)
BINA Chinabadam 6	1.0	60	10	90
		90	10	90
	1.3	60	10	80
		90	10	90
BINA Chinabadam 4	1.0	60	25	72
		90	25	92
	1.3	60	25	84
		90	25	92
BINA Chinabadam 2	1.0	60	10	60
		90	10	60
	1.3	60	10	60
		90	10	30

4.4.4 Comparison of regeneration efficiency of peanut among three transformation vectors *pBI121, pK7WG2_AtNHX1_1.6* and *pK7WG2_OsNHX1_1.6*

Three different vectors were used in the current study, namely, *pBI121, pK7WG2_AtNHX1_1.6*, *pK7WG2_OsNHX1_1.6*. Regeneration capability was investigated with these three vectors (Table 4.12).

In case of BINA Chinabadam 6, high regeneration competence (90%) was seen with *pK7WG2_AtNHX1_1.6* and *pK7WG2_OsNHX1_1.6* whereas low level of regeneration of only 44% was showed by the transformation with *pBI121*.

In case of BINA Chinabadam 4, maximum 97.78% regeneration was seen from transformation with *pBI121*. About 84-92% of regeneration was found from the interaction of *pK7WG2_OsNHX1_1.6*. However, less numbers (36%) of plant explants were regenerated after the transformation with *pK7WG2_AtNHX1_1.6*.

Just like the BINA Chinabadam 4, BINA Chinabadam 2 also showed satisfactory regeneration with pBI121. This capability was absent during the transformation with *pK7WG2_OsNHX1_1.6* where only 30% regeneration was achieved. Regeneration efficiency (46.67%-86.67%) was varied with different transformation factors in case of transformation with *pK7WG2_AtNHX1_1.6* maintaining optimum transformation is compulsory for the better regeneration from this vector.

4.4.5 Regeneration and transformation competence of putative transformed shoots on different selection media

Following transformation, explants were transferred into selection media containing 200 mg/l Cefotaxime and 50 mg/l kanamycin to control *Agrobacterium* overgrowth and to obtain selectable regeneration from transformed explants, respectively. Regeneration competence was calculated by average number of regenerated shoots on selection media. Along with this experiment, control experiment was set up where no regeneration found under selection pressure (Figure 4.8). In selection media with 50mg/l kanamycin, regenerated shoot tips were healthy and green whereas green appearance turned into brown and further white with the gradual increase of kanamycin

concentration in media. Higher numbers of putative transformed shoots were seen after transformation with *pBI121* in selection media having 50mg/l of kanamycin than the selection media containing higher concentration of kanamycin in almost all the varieties of peanut (Figure 4.7). Equivalent drift was seen when explants were transformed with *pK7WG2_AtNHX1_1.6* and *pK7WG2_OsNHX1_1.6* as well (Table 4.13).

Shoots which survived upto 150mg/l kanamycin were transferred into rooting media for root induction (Figure 4.9). Albino shoot parts were separated from green parts for better survival. The highest percentage of roots (50%) was found in BINA Chinabadam 6 whereas lower numbers of roots was developed from BINA Chinabadam 2. Finally transformation efficiency was evaluated by calculating the percentage of GUS positive plant parts among the putatively transformed plantlets. Three different parts of the plants, leaf, stem and roots were taken for GUS histochemical assay (Figure 4.10). Maximum transformation frequency (14.28%) was observed in BINA Chinabadam 4. Whereas BINA Chinabadam 2 gave less than 7% transformation in GUS assay which was the lowest transformation frequency (Table 4.14).

Table 4.12 Regeneration efficiency on selection media among three transformation vectors namely *pBI121, pK7WG2_AtNHX1_1.6 and pK7WG2_OsNHX1_1.6*

Peanut varieties	OD_{600}	Infection duration (mins)	Regeneration efficiency (%) on selection media		
			pBI121	*pK7WG2_AtNHX1_1.6*	*pK7WG2_OsNHX1_1.6*
BINA Chinabadam 6	1.0	60	64.51	91.67	90
		90	44	66.67	90
	1.3	60	53.84	66.67	80
		90	63.64	75	90
BINA Chinabadam 4	1.0	60	97.78	77.27	72
		90	75	90	92
	1.3	60	80	68	84
		90	70	36	92
BINA Chinabadam 2	1.0	60	58.33	86.67	60
		90	75	46.67	60
	1.3	60	91.67	73.33	60
		90	58.33	86.67	30

Table 4.13 Regeneration competence of transformed shoots on different selection media

Vectors	Peanut varieties	No. of infected explants per trial	Average no. of putative transformed shoots found in 50mg/l of kan ± SD	Average no. of putative transformed shoots found in 100mg/l of kan ± SD
pBI121	BINA Chinabadam 2	12	7±5.19	3.33±2.08
	BINA Chinabadam 4	15	14.67±0.58	10.67±2.30
	BINA Chinabadam 6	31	20±5	12.66±1.15
pK7WG2_AtNHX1_1.6	BINA Chinabadam 2	15	13±2.0	7.66±2.08
	BINA Chinabadam 4	22	17±4.0	12±5.29
	BINA Chinabadam 6	12	11±1.73	7.66±0.58
pK7WG2_OsNHX1_1.6	BINA Chinabadam 2	10	8.66±0.57	6.66±1.52
	BINA Chinabadam 4	25	18±6.0	14.33±5.50
	BINA Chinabadam 6	10	9±1.73	6.33±0.47

Data taken from transformation condition of OD_{600} 1.0,60 minutes of infection duration and 3 days co-cultivation period
Average no of shoots has been calculated from the shoots obtained from three different trials

Table 4.14 Transformation frequency of three peanut varieties (transformed with *pBI121*) based on regeneration on selection media using decapitated half embryo as explants

Peanut varieties	No. of putatively transformed shoots found in 150mg/l kan	No. of putative transformed shoots giving roots	% of root induction	No. of plantlet parts taken for GUS assay	No of GUS^+ plantlet parts	Percentage of GUS^+ (transformation frequency)
BINA Chinabadam 2	33	7	23.33	15	1	6.66
BINA Chinabadam 4	46	22	47.83	21	3	14.28
BINA Chinabadam 6	36	16	50	18	2	11.11

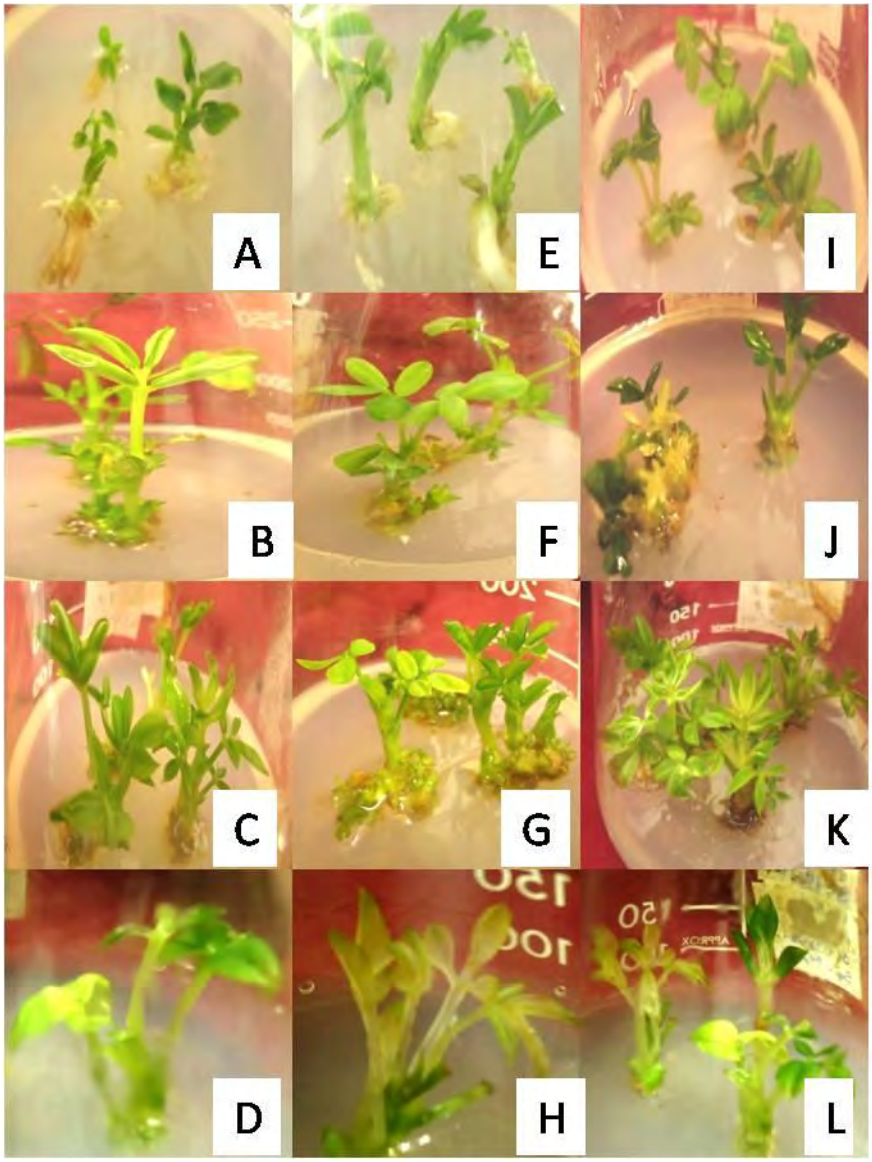

Figure 4.7 Regeneration response of putative transformed (with *pBI121*) shoots of BINA chinabadam 2 has shown under selection on kanamycin selection media in presence of A. 50mg/l B. 100mg/l C. 150mg/l D. 200mg/l of kanamycin, respectively.

Regeneration response of putative transformed (with *pBI121*) shoots of BINA chinabadam 4 has shown under selection on kanamycin selection media in presence of E. 50mg/l F. 100mg/l G. 150mg/l H. 200mg/l of kanamycin, respectively.

Regeneration response of putative transformed (with *pBI121)* shoots of BINA chinabadam 6 has shown under selection on kanamycin selection media in presence of I. 50mg/l J. 100mg/l K. 150mg/l L. 200mg/l of kanamycin, respectively.

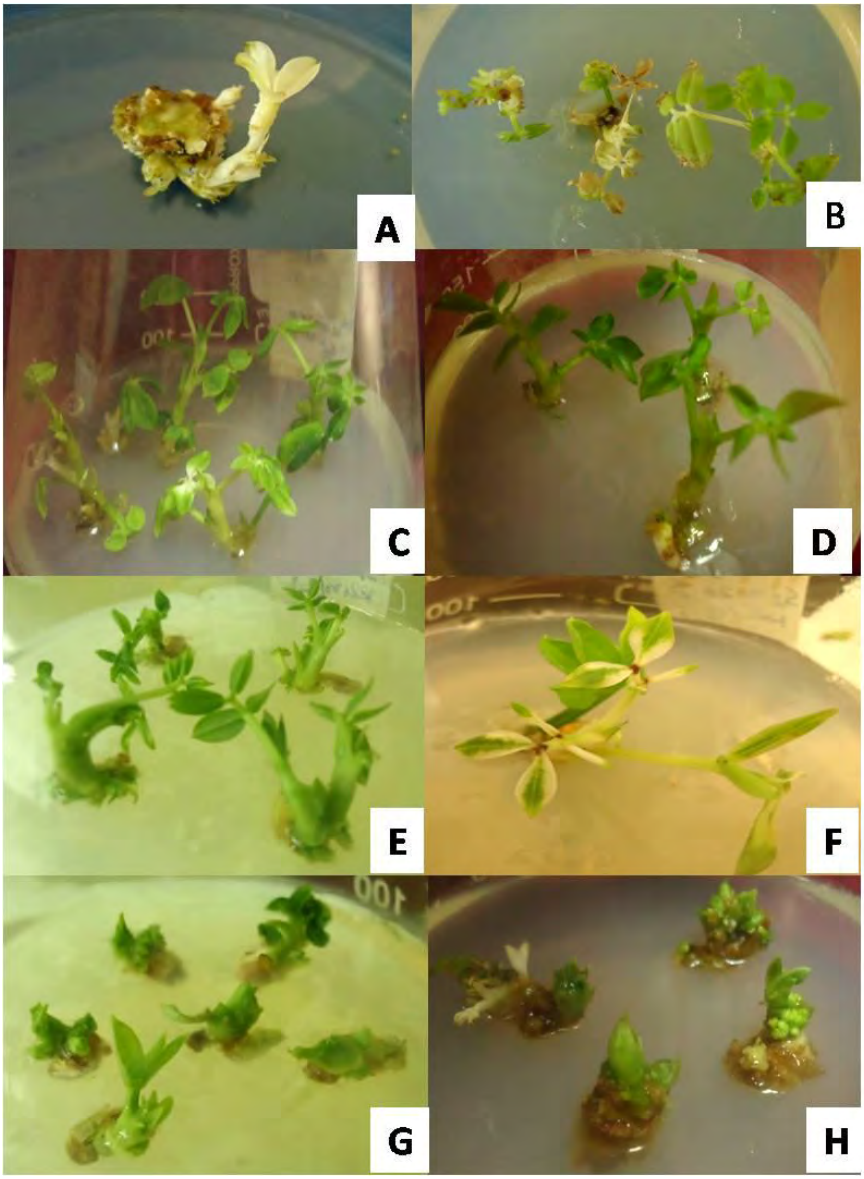

Figure 4.8 Regeneration response on selection media after transformation with *pK7WG2_AtNHX1_1.6*. A. Albino formation in control experiment (explants without infection but cultured on 50mg/l kanamycin), B. albino parts found in infected shoots, C-D. Putative transformed shoots of BINA Chinabadam 2 on selection media containing kanamycin C. 50mg/l and D. 100mg/l, E-F. Putative transformed shoots of BINA Chinabadam 4 on selection media containing kanamycin E. 50mg/l and F. 100mg/l, G-H. Putative transformed shoots of BINA Chinabadam 6 on selection media containing kanamycin G. 50mg/l and H. 100mg/l

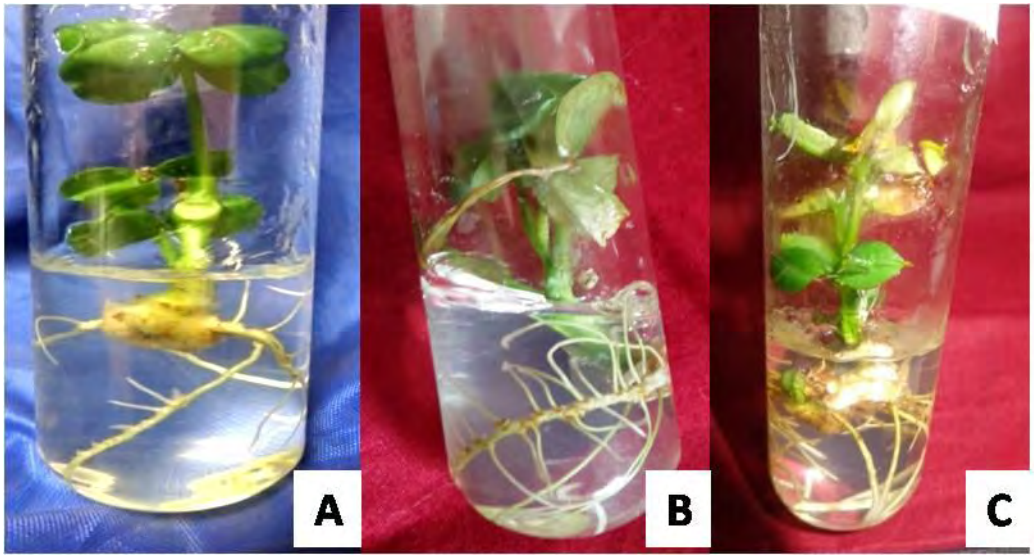

Figure 4.9 Rhizogenesis at the base of putative transformed shoots of BINA Chinabadam 2 (A), BINA Chinabadam 4 (B), BINA Chinabadam 6 (C).

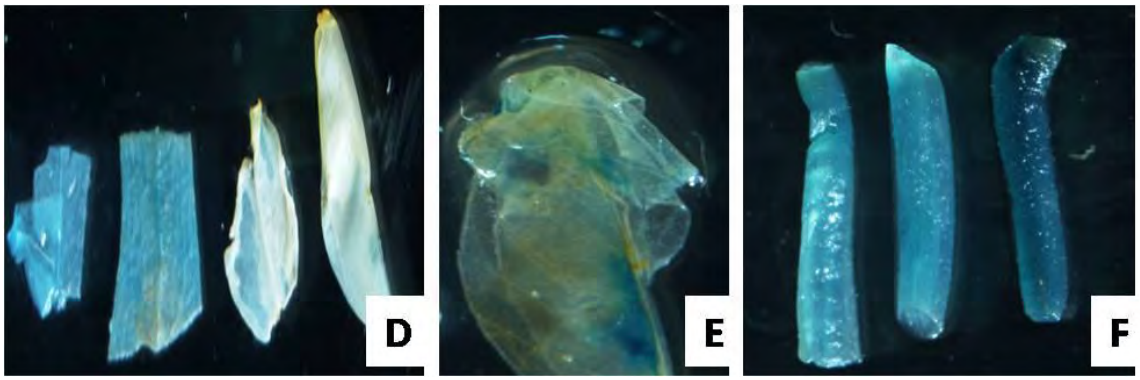

Figure 4.10 GUS positive expression on different putative transformed plants of BINA Chinabadam 6 (D) leaf, (E) stem, (F) root.

4.5 Salinity stress tolerance test of peanut seedlings of different peanut varieties

The assessment of salinity stress tolerance level of these peanut varieties is needed before transformation to get salt tolerant transgenic peanut. In this experiment, effect of salinity on peanut seed germination was observed. Seeds of BINA Chinabadam 2, 4 and 6 were placed on MS media with various conc. (0mM, 5mM, 20mM, 50mM, 100mM, 150 mM, and 200mM) of NaCl and data were collected after 14 days (Table 4.15). The highest number of seed germination was seen upto 10 mM of NaCl. The germination rate decreased gradually with the increase of NaCl in media and finally no germination was observed in MS media supplemented with 150mM of NaCl and above (Figure 4.11).

Table 4.15 Effect of salinity on seed germination of peanut

NaCl concentrations in germination media	No. of seeds inoculated	Percentage of germinated seeds
0 mM	12	100
5 mM	12	100
10 mM	12	100
20 mM	12	66
50 mM	12	33.33
100 mM	12	33.33
150mM	12	0
200mM	12	0

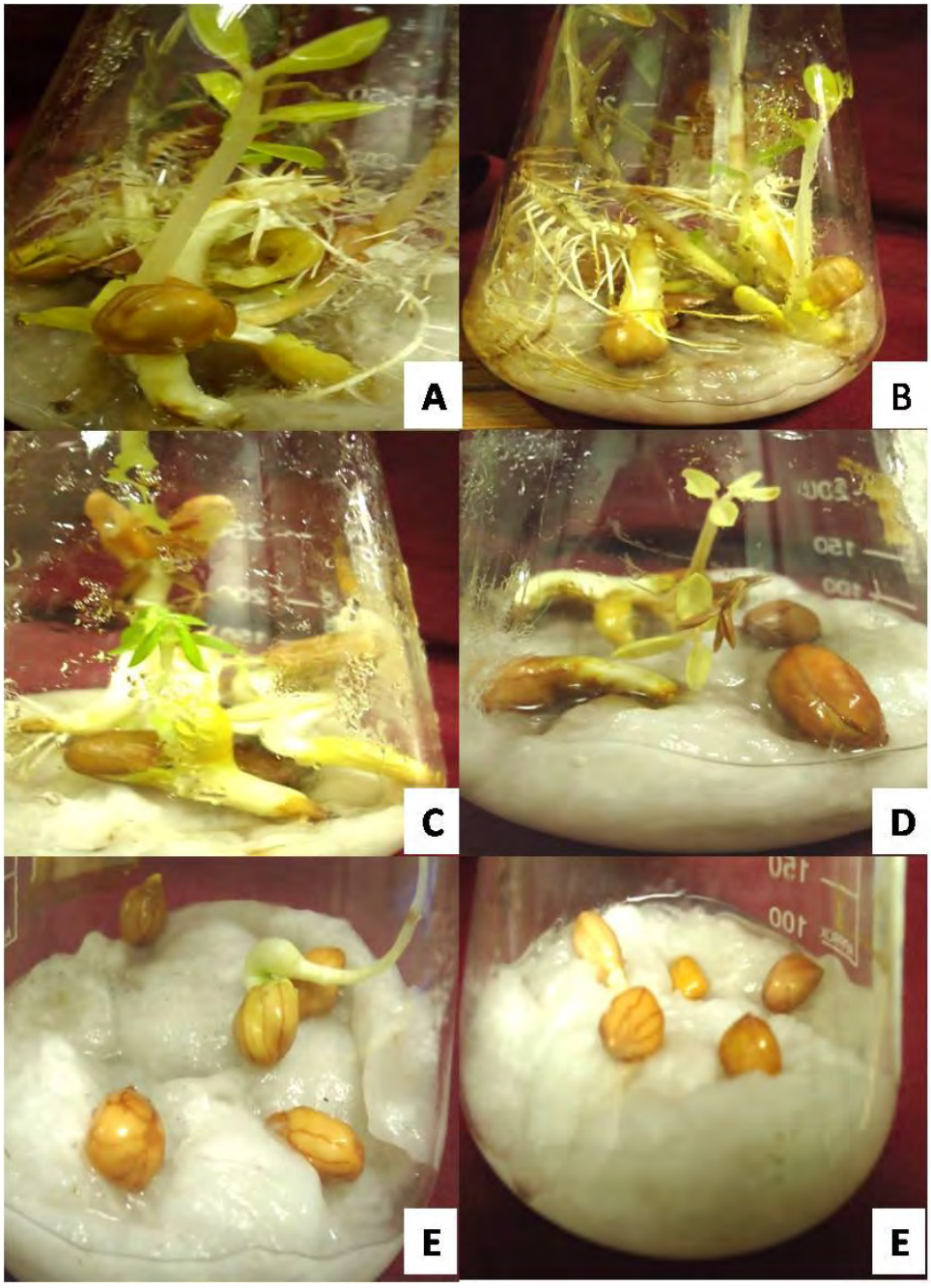

Figure 4.11 Seed germination in presence of different concentrations of NaCl (A) control (B) 10 mM, (C) 20 mM, (D) 50 mM, (E) 100 mM, (F) 150 mM (Photographs have been taken after 12 days of inoculation)

Chapter 5

Discussion

In the current experiment, appropriate protocols for regeneration and *Agrobacterium*-mediated transformation were examined for three peanut cultivars, namely, BINA Chinabadam 2, BINA Chinabadam 4 and BINA Chinabadam 6.

Tissue culture is the prerequisite of genetic engineering. Peanut tissue culture protocol establishment work had been going from the early 18th century (Harvey and Schulz, 1943; Nuchowicz, 1955). Established tissue culture protocols have been used for the transformation of peanut which has found in many previous reports (Higgins and Dietzgen, 2000; Sarker *et al*, 2003; Sharma and Anjaiah, 2000; Anuradha *et al*, 2006; Tiwari and Tuli, 2009). Furthermore, there are a lot of works on Bangladeshi varieties using various explants comparable to the present work (Sarker and Islam, 1999, Sarker *et al*, 2000). Peanut is still considered as recalcitrant crop due to inconsistency of regeneration response and variation of totipotency (Chowdhury, 2014a). Leaflet and embryo are prominent plant parts for regeneration of peanut (Sarker and Islam, 1999, Chowdhury, 2014a).

In the current experiment, previously determined optimum hormonal supplementation of Chowdhury (2014 a) was used to evaluate the shoot and root formation potential for three peanut varieties.

In case of leaflet explant, 2mg/l BAP with 0.5mg/l Kn was found best for direct regeneration (Chowdhury, 2014a). Current experiment resulted callus formation occurred from 70-80% explants whereas shoot regeneration percentage was only 10-14%. Sufficient numbers of elongated shoot regeneration were reported earlier from the same explant using MS media supplementing with BAP and Kn whereas, other previous reports with Bangladeshi varieties show better response in a media containing high concentration of BAP (Sarker and Islam, 1999, Sarker *et al*, 2000).

Decapitated half embryo explants was reported as viable explants for several Bangladeshi varieties, such as, Dhaka Chinabadam 8, BINA Chinabadam 2 and BINA Chinabadam 3 (Honey *et al*, 2010). In another effort, presence of embryo axes showed better regeneration capability than cotyledon segments (Vasil, 1986).

In the present study, 3mg/l BAP with 1 mg/l Kn supplemented medium was used to get rapid regeneration from this explants. Shoots were developed from within 15-19 days among the three varieties, maximum regeneration was observed in BINA Chinabadam 2 (96%) which is relatively lower than BINA Chinabadam 4 (86%) and BINA Chinabadam 6 (83%). This hormonal supplementation was found suitable in the work of Chowdhury (2014 a). Similarly root induction percentage was satisfactory using the rooting hormone IBA in a concentration of 0.2 mg/l better rooting was observed. On the other hand, NAA was reported as the best auxin by Sarkar and Islam (1999).

For peanut, genetic transformation rates were stated to be influenced by the type of explants (Venkatachalam *et al*, 1998). Similar observation reported in alfalfa (Chabaud *et al*, 1988), cowpea (Muthukumar *et al*, 1996) and blackgram (Geetha *et al*, 1997). In the current study, two types of explants namely decapitated half embryo and leaflet were used to study the role of different transformation factors on transformation frequency of peanut (*Arachis hypogaea L.*). Decapitated half embryo was found better as explants. This is supported by Somers *et al* (2003). Conversely, transformation efficiency was found better when work was done by leaf tissue as explants (Cheng *et al*, 1996). Similarly when regeneration and transformation frequency were analyzed among three types of explants, namely, leaflet, hypocotyl and epicotyls, the leaflet found better by Sarker and Islam (1999).

Transient GUS expression has been successfully used to determine optimum transformation procedure and gives a good indication of the rate of stable transformation (Janssen and Gardner, 1993). According to previous literature review, transient GUS expression percentages varied in different plants during *Agrobacterium*-mediated transformation, such as, kiwifruit (50%) (Janssen and Gardner, 1993), pine (60%), and spruce (90%) (Wenck *et al*, 1999).

In the present study, peanut varieties, namely, BINA Chinabadam 4 and BINA Chinabadam 6 were with tested with *Agrobacterium* Strain LBA4404 containing *pBI121* (containing *nptII* marker gene and *uidA* gene) plasmid vector for determination of influencing factors of genetic transformation for peanut. However, previous studies showed that transformation rate was found to be proportional to the relationship between infected (transformed) explants and inoculation time, co-cultivation time, bacterial concentration and selection antibiotic concentration (Padmanabhanan and Sahi, 2009; Zia *et al*, 2010).

Maximum percentages of GUS positive explants were observed in the present study at OD_{600} of 1.5 and at OD_{600} of 1.2 in the varieties BINA Chinabadam 6 and BINA Chinabadam 4, respectively whereas, the percentage of GUS positive explants fell drastically when the bacterial density was increased above the optimum density. In the same way, transformation frequency increased with increase of bacterial density and thereafter, decreased with further increase in number of *Agrobacterium* cells (Saini and Jaiwal, 2007). According to them, higher concentration of *Agrobacterium* during transformation may cause hypersensitive response of explants as well as it will be a difficult work to kill them after co-cultivation due to excessive aggregation of *Agrobacterium* cells. Similar result found in *Nicotiana tabacum* and *Arabidopsis thaliana* (Lin *et al*, 1994) and in most of the grain legumes (Bean *et al*, 1997). Maximum transformation frequency had been found at OD_{600} of 0.7 when working with decapitated half embryo of peanut as the previously reported in Rafiq (2014). On the other hand, better transformation was found at OD_{600} of 1.0 working with another legume soybean (Zia *et al*, 2010). The percentage of transient GUS positive explants was gradually raised with high bacterial density (OD_{600} 1.1) in case of embryonic leaflets of BINA Chinabadam 4. This result is extremely comparable to the work of Chowdhury (2014)b with BINA varieties but same explants. Similar results were reported for tomato working with cotyledonary leaf by Islam (2007).

Infection duration plays a diverged role in transformation. It was seen for Blackgram that further increase of infection duration from the optimum value (20-30 minutes) couldn't help to increase the transformation frequency and caused problems in

eliminating the bacteria (Saini and Jaiwal, 2007). This view was also observed by the present study where better transient transformation was found in 60 minutes of infection period in decapitated half embryo as well as in embryonic leaflets. 120 minutes of infection duration was sufficient to transform high numbers of explants of peanut of BINA varieties without showing bacterial overgrowth after 3 days of co-cultivation (Chowdhury , 2014b). On the other hand, browning of the target tissue had been seen in gherkin due to extending infection time (De Clercq *et al*, 2002). 1 day for soybean (Zia *et al*, 2010) and 30-45 minutes for *Sesbania drummondii* (Padmanabhan and Sahi, 2009) was seen as optimum infection duration for similar types of explant which is half seed cotyledonary node. In case of Chickpea, different infection durations were studied for transformation (Akbulut *et al*, 2008)

Co-cultivation duration can be varied on the basis of genotype (Zhang *et al*, 1997). In the present experiment, 72 hours co-cultivation time showed the highest percentage of GUS positive explants as the result of transient transformation in case of decapitated half embryo of BINA Chinabadam 4 and BINA Chinabadam 6 as well as embryonic leaflets of BINA Chinabadam 4. Rafiq (2014) worked with another BINA variety and reported that 24 hours co-cultivation duration is sufficient for the highest percentage of transient transformation when decapitated half embryo was taken as explants. On the other hand, 72 hours was found as optimum duration in another two study where the working explants were cotyledonary node (Sharma and Anjaiah, 2000 ; Anuradha *et al*, 2006; Iqbal *et al*, 2012) and cotyledon (Sharma *et al*, 2009) . In case of embryonic leaflet, the percentage of GUS positive explants were increased in BINA Chinabadam 6 and decreased in BINA Chinabadam 2 with the increase of co-cultivation duration (Chowdhury, 2014b). Further extension of co-cultivation time from 2-3 days leads to lower the regeneration potential of the explants in Blackgram (Saini and Jaiwal, 2007). Lower co-cultivation duration than the present study was found as sufficient for better transformation in canola (Opabode, 2006) whereas higher co-cultivation duration such as 4 days is needed for better transformation efficiency in peanut (Venkatachalam *et al*, 1998) and in alfalfa (Chabaud *et al*,1988). According to Venkatachalam *et al* (1998), more than 4 days caused explant abortion due to bacterial contamination. Similar result

was observed in cowpea (Muthukumar *et al*,1996) and in pea (Decathen and Jacobsen, 1990, Lulsdorf *et al*,1991)

Decapitated half embryo taken from 1 day old seed of BINA Chinabadam 6 and BINA Chinabadam 4 gave 75% and 25% of GUS positive explants respectively which were also the highest percentage obtained when compared with 2 day and 3day old explants for both the varieties in the present study. These phenomena can be put side- by- side with the experiment of Anuradha *et al* (2006) where cotyledonary nodes of peanut were collected from 1-6 days old seedlings for significant result of genetic transformation via a promoterless *gus::nptII* fusion gene vector. In addition, 1 day old seeds of BINA Chinabadam 2 showed 75% positive results with GUS histochemical assay while explants retrieved from 2 and 3 days old seeds showed 50% and 57.3% transformation efficiency (Rafiq, 2014).

The most widely used selectable markers for transformation of crops are genes encoding neomycin phosphotransferase (*nptII*) (Nap *et al*, 1992; Anklam *et al*, 2002; Sundar and Sakthivel, 2008 and Suratman *et al*, 2013). In this experiment, *nptII* selectable marker gene in *pBI121, pK7WG2_AtNHX1_1.6* and *pK7WG2_OsNHX1_1.6* was used in transformation of peanut explants.Ttransformation frequencies are correlated with the plasmid of transformation containing various selection marker genes which have an effect on genetic transformation (Guo *et al*, 2012). It was found that upto 20 mg/l of kanamycin concentration could be resisted inherently by the decapitated half embryo of peanut. None of the explants regenerated in selection media containing kanamycin of 100 mg/l or above. So this range of kanamycin conc. used in media was optimum for selection of transformed plants. Similar work was reported for cotyledonary node explants in peanut where kanamycin resistant shoot induction percentage decreased with increasing kanamycin concentration and finally chlorotic shoots were seen at 175 mg/l kanamycin. Hence, this concentration was chosen for the selection of putative transformants (Anuradha *et al*,2006). This report also showed the dependency of antibiotic utilization by plants on plant species and plant explants just like the previous study of Saini *et al* (2003).

In the present study, regeneration percentage was calculated as follows:

Number of explants with regenerated shoots /number of total explant x 100

Growth was observed for 1 month and then scored for the regeneration percentage. Moreover, regeneration percentage was compared among different concentration of kanamycin in the present study. Same methodology was used to calculate regeneration efficiency of *Sesbania drummondii* (Padmanabhan and Sahi, 2009). Transformation will not be successful if transformation or regeneration is uncoupled (Venkatachalam *et al*, 1998). Apparently more research works were suggested to establish *in vitro* regeneration with combination of the transformation protocol by Rafiq (2014). The extension of different transformation factors may reduce regeneration efficiency of the explants such as extension of co-cultivation duration (Saini and Jaiwal, 2007) or high bacterial density (Opabode, 2006). In accordance with Venkatachalam *et al* (1998), explants abortion through bacterial contamination was seen due to extended co-cultivation duration. On the other hand, 3 days co-cultivation duration was found optimum resulting more number of kanamycin resistant shoots and higher frequency of GUS positive sectors in transient GUS analysis (Anuradha *et al*, 2006).

Progressing transformation frequency remains the most crucial factor in plant transformation (Gheysen *et al*,1998). In the present study, putative transformed plants were recovered through gradual amplification of kanamycin concentration on media. Among 54 putative transformants of three peanut varieties only 6 plants were found to be positive for GUS showing blue spots in at least one plant parts. GUS histochemical assay of peanut and blackgram were done in the same way by Anuradha *et al* (2006) and Saini and Jaiwal (2007) where 24 GUS positive plants out of 141 and 11 GUS positive plants out of 255 putative transformed plants were noticed respectively. Actually in both cases transformation frequency was evaluated by scoring the number of sectors showing GUS activity on different explants which was comparatively similar way of current experiment. Some other experiments were taken into consideration to observe and compare the stable transformation frequency where 4% for *Sesbania*

drummondii (Padmanabhan and Sahi, 2009), 1.2-3.9% for wheat (Khanna and Daggard, 2002), 2.7% for plum (Yancheva *et al*, 2002), 0.8-1.5% for marula (Mollel and Goyvaerts, 2004), 3.5%-5.8% for apricot (Petri *et al*, 2008) of transformation frequency were achieved. Maximum transformation frequency of 14.28% was observed in BINA Chinabadam 4 and 6.66% which the lowest transformation frequency was found in the present study by BINA Chinabadam 2. It was seen in another research work of peanut that shoot regeneration ceased under selection pressure of 50mg/l kanamycin. Furthermore, presence of GUS gene was monitored in the root part but not in the leaf tissue. Authors justified the reasons of the incident by rearrangement, loss or non-expression of GUS gene (Sarker *et al*, 2000). Similar result was also found in other peanut varieties (Eapen and George, 1994).

To dertrmine a baseline salinity tolerance level of non transformed peanut plants, decapitated half embryos were subjected to germinate on media composing different conc. of NaCl ranging from 5-200mM. After the observation of the result of present study, it was revealed that germination rate decreased gradually with the increase in amount of NaCl in media and finally no germination was observed in MS media supplemented with 150mM of NaCl and above. Similar reports have been found where salinity severely influenced the plant physiology (Munns, 1993; Murphy and Durako, 2003; Shahid *et al*, 2011).

Chapter 5
References

Akbulut, M., Yucel, M., & Oktem, H. A. (2008). Analysis and Optimizaion of DNA Delivery into Chickpea (Cicer arietinum L.) Seedlings by Agrobacterium tumefaciens. *African Journal of Biotechnology , 8* (7), 1011-1017.

Al-Joboury, K. R. (2012). In Vitro Propagation of Groundnut (Arachis hypogea L.). *Ibn Al-Haitham Journal for Pure and Applied Science* , 13-19.

Amoah, B., Wu, H., Sparks, C., & Jones, H. (2001). Factors influencing Agrobacterium-mediated transient expression of uidA in wheat inflorescence tissue. *J. Exp. Bot , 52*, 1135-1142.

Anuradha, T. S., Jami, S. K., Datla, R. S., & Kirti, P. B. (2006). Genetic transformation of peanut (Arachis hypogaea L.) using cotyledonary node as explant and a promoterless gus::nptII fusion gene vector. *Journal of Bioscience* , 235-246.

Apse, M., Aharon, G., Snedden, W., & Blumwald, E. (1999). Salt tolerance conferred by overexpression of a vacuolar Na+/H+ antiport in Arabidopsis. *Science* , 1256-1258.

Ashraf, M., & Harris, P. (2004). Potential biochemical indicators of salinity tolerance in plants. *Plant Science , 166*, 3-16.

Banjara, M. (2010). *EXPRESSION OF THE AtNHX1 GENE IN PEANUT TO INCREASE SALT TOLERANCE IN PEANUT PLANTS.* Thesis, Texas Tech University, Biology.

Bartels, D., & Sunkar, R. (2005). Drought and salt tolerance in plants. *Cri. Rev. Plant Sci., 24, 23-58. , 24*, 23-58.

Bean, S., Gooding, P., Mullineaux, P., & Davies, D. (1997). A Simple System for Pea Transformation. *Plant Cell Reports* (16), 513-519.

Bent, A. F. (2000). Arabidopsis in Planta Transformation. Uses,Mechanisms, and Prospects for Transformation of Other Species. *Plant physiology , 124*.

Bohnert, H., & Jensen, R. (1996). Strategies for engineering waterstress tolerance in plants. *Trends Biotechnol* , 86-97.

Boyer, J. S. (1982). Plant productivity and environment. *Science* , 443-448.

Burns, S. P. (2010). *STRATEGIES FOR ENHANCING LEAF SPOT (Cercospora arachidicola AND Cercosporidium personatum) TOLERANCE IN PEANUT (Arachis hypogaea L.).* thesis, University of Florida.

Chabaud, M., Passiatore, J. E., Cannon, F., & Buchanan-wollaston, V. (1988). Parameters Affecting the Frequency of kanamycin Resistant alfalfa Obtained by Agrobacterium tumefaciens Mediated Transformation. *Plant Cell Reports , 7*, 512-516.

Chakrabarty, R., Viswakarma, N., Bhat, S., Kirt, i. P., Singh, B., & Chopra, V. (2002). Agrobacterium-mediated transformation of cauliflower: optimization of protocol and development of Bt-transgenic cauliflower. *J. Biosci. , 27*, 495-502.

Chandra, A., & Pental, D. (2003). Regeneration and genetic transformation: An overview. *Current Science , 84*, 381-387.

Chee, P. P., Fober, K. A., & Slighton, J. L. (1986). Transformation of soybean (Glycine max) by infecting germinating seeds with Agrobacterium tumefaciens. *Plant physiology , 93*, 1212-1218.

Cheng, M., Jarret, R. L., Li, Z., & Xing, A. a. (1996). Production of fertile transgenic peanut (Arachis hypogaea L.) plants using Agrobacterium tumefaciens. *Plant Cell Reports , 15* (9), 653-7.

Chowdhury, I. M. (2014). *Analysis of factors influencing successful Agrobacterium mediated genetic transformation in two Bangladeshi peanut (Arachis hypogaea L.) varieties BINA Chinabadam-2 and BINA Chinabadam-6 using embryonic leaflet explant.* BRAC University, Department of Mathematics and Natural Sciences, Dhaka.

Chowdhury, S. (2014). *Determination of an Efficient Regeneration protocol for Bangladeshi Peanut Varieties (Arachis hypogaea L.).* BRAC University, Mathematics and Natural Science, Dhaka.

Cushman, J., & Bohnert, H. (2000). Genomic approaches to plant stress tolerance. *Curr. Opin. Plant Biol* , 117-124.

De Clercq, J., Zambre, M., Van Montagu, M., Dillen, W., & Angenon, G. (2002). An Optimized Agrobacterium- mediated Transformation Procedure for Phaseolus acutifolius A. Gray. *Plant Cell Reports* (22), 333-340.

Decathen, A., & Jacobsen, H. (1990). Agrobacterium tumefaciens mediated transformation of Pisum sativam L. using binary vector and cointegrated vectors. *Plant Cell Reports , 9*, 276-279.

Desfeux, C., Clough, S., & Bent, A. (2000). Female reproductive tissues are the primary target of Agrobacterium mediated by the Arabidopsis floral-dip method. *plant physiology , 123*, 895-904.

Eapen, S., & George, L. (1994). Agrobacterium tumefaciens mediated gene transfer in peanut (Arachs hypogaea L.). *Plant Cell Reports* , 582-586.

Egnin, M., Mora, A., & Prakash, C. (1998). Factors enhancing Agrobacterium tumefaciens-mediated gene transfer in peanut (Arachis hypogaea L.). *In Vitro Cell. Dev. Biol. Plant , 34*, 310-318.

FAO, F. a. (1997). *The state of Food and Agriculture* .

Feldmann, K. A., & Marks, M. (1987). Agrobacterium-mediated transformation of germinating seeds of Arabidopsis thaliana: a non-tissue culture approach. *Mol. Gen. Gene , 208*, 1-9.

Galgaro, L., Lopes, C., Gimenes, M., Valls, J., & Kochert, G. (1998). Genetic variation between several species of sections Extranervosae, Caulorrhizae, Heteranthae, and Triseminatae (genus Arachis) estimated by DNA polymorphism. *Genome* , 445-454.

Garcia, G., Stalker, H., & Kochert, G. (1995). Introgression analysis of an interspecific hybrid population in peanuts (Arachis hypogaea L.) using RFLP and RAPD markers. *Genome* , 166-176.

Geetha, N., Venkatachalam, P., & Rao, G. R. (1997). Factors Influencing Production of Agrobacterium Mediated Genetically Transformed Calli and Plant Regeneration in Blackgram (Vigna mungo L. Hepper). *Plant Tissue Culture , 7*, 149-152.

Gheysen, G., Angenon, G., & Van, M. M. (1998). Agrobacterium Mediated Plant Transformation: a scientifically intriguing story with significant applications in transgenic plant research. *K Lindsey (Amsterdam: Harwood Academic Publishers)* , 1-33.

Graves, A., & Goldman, S. (1986). The transformation of Zea mays seedlings with Agrobacterium tumefaciens. *Plant molecular biology* , 43-60.

Gregory, W., & Gregory, M. (1976). Groundnut,. *N. Simmonds (Ed.), Evolution of crop plants, Longman Group Ltd., London, UK.* , 151-154.

Grieshammer, U., & Wynne, J. (1990). Mendelian and non-mendelian inheritance of three isozymes in peanut Arachis hypogaea L. *Peanut Science* , 101-105.

Halward, T., Stalker, H., & Kochert, G. (1993). Development of an RFLP linkage map in diploid peanut species. *Theoretical and Applied Genetics* , 379-384.

Halward, T., Stalker, T., Larue, E., & Kochert, G. (315-325.). Use of single-primer DNA amplifications in genetic-studies of peanut (Arachis hypogaea L.). *Plant Molecular Biology* , :315-325.

Haque, S. (2006). Salinity Problems and Crop Production in Coastal Regions of Bangladesh. *Pak. J. Bot* , 1359-1365.

Harvey, R., & Schulz, E. F. (1943). Cultures of Arachis Embryos. *J. Amer. Soc. Agron , 631* (35).

Hassan, F., Hoque, M. I., Kiesecker, H., & Jacobsen, H.-J. (2007). Transient GUS Expression in Decapitated Lentil Embryos. *Plant Tissue Culture and Biotechnology* , 97-102.

Herselman, L. (2003). Genetic variation among Southern African cultivated peanut (Arachis hypogaea L.) genotypes as revealed by AFLP analysis. *Euphytica* , 319-327.

Higgins, C. M., & Dietzgen, R. G. (2000). Genetic Transformation, Regeneration and Analysis of Transgenic peanut. *Australian centre for International Agriculture Research* .

Islam, A., Chowdhury, J., & Seraj, Z. I. (2010). Establishment of optimal conditions for an Agrobacterium-mediated transformation in four tomato varieties grown in Bangladesh. *Journal of Bangladesh Academy of Science , 2* (34), 171-179.

Janila, P., Nigam, S., Pandey, M. K., Nagesh, P., & Varshney, R. K. (2013). Groundnut improvement: use of genetic and genomic tools. *Frontiers in Plant Science , 4*, 1-16.

Janssen, B., & Gardner, R. (1993). The use of transient GUS expression to develop an Agrobacterium-mediated gene transfer system for kiwifrui. *Plant Cell Rep , 13*, 28-31.

Keshamma, E., Rohini, S., Rao, K. S., Madhusudhan, B., & Kumar, M. U. (2008). Tissue Culture-independent In Planta Transformation Strategy:an Agrobacterium tumefaciens-Mediated Gene Transfer Method to Overcome Recalcitrance in Cotton (Gossypium hirsutum L.). *The journal of cotton science , 12*, 264-272.

Khanna, H., & Daggard, G. (2002). Agrobacterium tumefaciens mediated transformation of wheat using a superbinary vecto rand a polyamine-supplemented regeneration medium. *Plant Cell Rep.* (21), 429-436.

Knauft, D., & Gorbet, D. (1989). Genetic diversity among peanut cultivars. *Crop Science* , 1417-1422.

Kochert, G., Halward, T., W.D., B., & C.E., S. (1991). RFLP variability in peanut (Arachis hypogaea L.) cultivars and wild-species. *Theoretical and Applied Genetics* , 565-570.

Lacks, G., & Stalker, H. (1993). Isozyme analyses of Arachis species and interspecific hybrids. *Peanut Science* , 76-81.

Lanham, P., Fennel, l. S., Moss, J., & Powell, W. (1992). Detection of polymorphic loci in Arachis germplasm using random amplified polymorphic DNAs. *Genome* , 885-889.

Lin, J., Garcia-Assad, N., & Kuo, J. (1994). Effect of Agrobacterium cell concentration on the transformation efficiency of tobacco and Arabidopsis thaliana. *Focus* (16), 72-77.

Liu, M., Yang, J., Cheng, Y.-g., & An, L.-j. (2009). Optimization of soybean (Glycine max (L.) Merrill) in planta ovary transformation using a linear minimal gus gene cassette. *Journal of Zhejiang University Science , 10*, 870-876.

Livingstone, D. M., & Birch, R. G. (1999). efficient transformation and regeneration of diverse cultivars of peanut (Arachis hypogaea L.) by particle bombardment into embryogenic callus produced from mature seeds. *Molecular breeding , 5*, 43-51.

Lu, J., & Pickersgill, B. (1993). Isozyme variation and species relationships in peanut and its wild relatives (Arachis L. - Leguminosae). *Theoretical and Applied Genetics* , 550-560.

Lulsdorf, M., Rempel, H., Jackson, J., Baliski, D., & Hobbs, S. (1991). Optimizing the Production of Transformed pea (Pisum sativum L.) Callus Using Disarmed Agrobacterium tumefaciens strains. *Plant Cell Reports , 9*, 479-483.

Mannan, A., Syed, T. N., & Mirza, B. (2009). Factors Affecting Agrobacterium tumefaciens Mediated Transformation of Artemisia absinthium L. *Pakistan Journal of Botany* , 3239-3246.

Mayavan, S., Subramanium, k., Arun, M., Rajesh, M., Kapil Dev, G., Sivanandhan, G., et al. (2013). Agrobacterium tumefaciens-mediated in planta seed transformation strategy in sugarcane. *Plant Cell Rep* , 1557-74.

Mckently, A. H., Moore, g. A., Doostdar, H., & Niedz, R. P. (1995). Agrobacterium-mediated transformation of peanut (arachis hypogaea L.) embryo axes and the development of transgenic plants. *plant Cell Reports , 14*, 699-703.

Mollel, M. H., & Goyvaerts, E. M. (2004). Preliminary examination of factors affecting Agrobacterium tumefaciens-mediated transformation of marula,Sclerocarya birrea subsp. caffra. *Plant Cell, Tissue and Organ Culture* (79), 321-328.

Mollel, M., & Goyvaerts, E. M. (2004). Preliminary examination of factors affecting Agrobacterium tumefaciens-mediated transformation of marulaSclerocarya birrea subsp. caffra (Anacardiaceae. *Plant cell, tissue and organ culture , 79*, 321-328.

Munns, R. (2002). Comparative physiology of salt and water stress. *Plant Cell Environ* , 239-250.

Munns, R. (1993). Physiological processes limiting plant growth in saline soils: some dogmas and hypotheses. *Plant Cell Environment , 16*, 15-24.

Munns, R., James, R., & Lauchli, A. (2006). Approaches to increasing the salt tolerance of wheat and other cereals. *Journal of Experimental Botany* , 1025-1043.

Murphy, K., & Durako, M. (2003). Physiological effects of short term salinity changes on Ruppia maritima. *Aquatic Botany , 75*, 293-309.

Muthukumar, B., Mariamma, M., Veluthambi, K., & and Gnanam, A. (1996). Genetic Transformation of Cotyledon Explants of Cowpea (Vigna unguiculata L. Walp) using Agrobacterium tumefaciens. *Plant Cell Reports , 15*, 980-985.

Narasimhulu, S., Deng, X., Sarria, R., & Gelvin, S. (1996). Early transcription of Agrobacterium T-DNA genes in tobacco and maize. *Plant cell , 8*, 873-886.

Nuchowicz, A. (1955). Studies on the Culturing of Embryos and of Embryo Fragments of Arachis hypogaea L. *Agricultura (Louvain) , 3* (3).

Opabode, T. J. (2006). Agrobacterium-mediatedTransformation of Plants: Emerging Factors that Influence Efficiency. *Biotechnology and Molecular Biology Review , 1* (1), 12-20.

Ozias-Akins, P., & Gills, R. (2001). Progress in the Development of Tissue Culture and Transformation Methods Applicable to the Production of Transgenic Peanut. *Peanut Science* , 123-131.

Ozias-Akins, P., Anderson, W., & Holbrook., C. (1992). Somatic embryogenesis in Arachis hypogaea L.: Genotype comparison. *Plant Science* , 103-111.

Padmanabhan, P., & Sahi, S. (2009). Genetic Transformation and Regeneration of Sesbania drummondii Using Cotyledonary Nodes. *Plant Cell Rep.* (28), 31-40.

Paik-ro, O., Smith, R., & Knauft, D. (1992). Restriction-fragment-length-polymorphism evaluation of 6 peanut species within the Arachis section. *Theoretical and Applied Genetics* , 201-208.

Pattee, H., & Young, C. (1982). Peanut science and technology American Peanut Research and Education Society, Yoakum, Tex. *American Peanut Research and Education Society* .

Pérez-Piñeiro, P., Gago, J., Landín, M., & Gallego, P. P. (2012). Agrobacterium-mediated Transformation of Wheat: General Overview and New Approaches to Model and Identify the Key Factors Involved. In Y. O. Çiftçi (Ed.), Agrobacterium-Mediated: Transformation of, Transgenic Plants - Advances and Limitations. Intech.

Pestana, M., Lacorte, C., Freitas, V. d., Oliveira, D. d., & Mansur, E. (1999). In vitro regeneration of peanut (Arachis hypogaea L.) through organogenesis: Effect of culture temperature and silver nitrate. *In Vitro Cell. Dev. BioI* , 214-216.

Peterson, L., & Shireen, S. (2001). Soil and water salinity in the coastal areas of Bangladesh. *SRDI* .

Petri, C., Lopez-Noguera, S., Alburquerque, N., Egea, J., & Burgos, L. (2008). An antibiotic-based selection strategy to regenerate transformed plants from apricot leaves with high efficiency. *Plant science , 175* (6), 777-783.

R, H., B, M. M., A, B., S, M., & M, N. (n.d.).

Rafiq, S. (2014). *Analysis of Factors Influencing Successful Agrobacterium Mediated Genetic Transformation in Two Different Explants of Peanut Variety BINA Chinabadam 2.* BRAC University, Department of Mathematics and Natural Sciences, Dhaka.

Rahman, M., & Ahsan, M. (2001). Salinity constraints and agricultural productivity in coastal saline area of Bangladesh. *Soil Resources in Bangladesh: Assessment and Utilization* .

Raina, S., Rani, V., Kojima, T., Ogihara, Y., Singh, K., & Devarumath, R. (2001). RAPD and ISSR fingerprints as useful genetic markers for analysis of genetic diversity, varietal identification, and phylogenetic relationships in peanut (Arachis hypogaea) cultivars and wild species. *Genome* , 763-772.

Rains, D., & Goya, l. S. (2003). Strategies for Managing Crop Production in Saline environments: An overview. *J Crop Prod. , 7*, 1-10.

Reddy, Y. S., Sridevi, O., Krishnaraj, P. U., & Salimath, P. M. (2007). In Planta strategy for gene transfer in chickpea (Cicer arietinum L.): Embryo transformation. *Indian journal crop science , 2* (2), 323-326.

Rohini, V. K., & Rao, S. K. (2000). Transformation of peanut:Arachis hypogaea L.): a non-tissue culture based approach for generating transgenic plants. *Plant science , 150*, 41-49.

Rohini, V., & Rao, S. (1999). Agrobacterium-mediated transformation of sunflower(Helianthus annuusL.): Asimple protocol. *Ann Bot , 53*, 347-354.

Rohini, V., & Rao, S. (2000a). Embryo transformation, a practical approach for realizing transgenic plants of safflower (Carthamus tinctorious L.). *Ann. Bot , 86*, 1043-1049.

Rohini, V., & Rao, S. (2000b). Transformation of peanut (Arachis hypogaea L.) : A non tissue culture based approach for generating transgenic plants. *Plant science , 150*, 41-49.

Saini, R., & Jaiwal, P. (2007). Agrobacterium tumefaciens - Mediated Transformation of Blackgram: An Assessment of Factors Influencing the Efficiency of uidA Gene Transfer. *BIOLOGIA PLANTARUM , 51* (1), 64-72.

Sanjaya, S., & Rai, V. (2005). Micropropagation of Pseudoxytenanthera stockssi. *In Vitro Cell. Dev Biol. Plant, , 41*, 333-337.

Sarker, R. H., & Islam, A. (1999). In Vitro Regeneration and Genetic Transformation of Peanut (Arachis hypogaea L). *Dhaka University Journal of Biological Science , 8* (2), 1-9.

Sarker, R. H., Islam, M. N., Islam, A., & Seraj, Z. I. (2000). agrobacterium Mediated Genetic Transformation of Peanut (Arachis hypogaea L.). *Plant Tissue Culture , 2*, 137-142.

Sarker, R. H., Islam, M. N., Islam, A., & Seraj, Z. I. (2000). Agrobacterium-mediated Genetic Transformation of Peanut (Arachis hypogaea L.'). *Plant Tissue Culture , 10* (2), 137-142.

Sarker, R. H., Mustafa, B. M., Biswas, A., Mahbub, S., Nahar, M., & Hashem, R. (2003). In vitro Regeneration in Lentil. *Plant Tissue Culture , 13* (3), 155-163.

Shahid, M., Pervez, M., Balal, R., Ahmad, R., Ayyub, C., Abbas, T., et al. (2011). Salt stress effects on some morphological and physiological characteristics of okra (Abelmoschus esculentus L.). *Soil environment , 30* (1), 66-73.

Sharma, K. K., & Anjaiah, V. (2000). An Efficient Method for the Production of Transgenic Plants of Peanut (Arachis hypogaea L.) Through Agrobacterium tumefaciens-mediated Genetic. *Plant Science , 159*, 7-19.

Sharma, M. K., Solanke, A. U., Jani, D., Singh, Y., & Sharma, A. K. (2009). A Simple and Efficient Agrobacterium-mediated Procedure for Transformation of Tomato. *Journal of Bioscience , 34* (3), 423-433.

Shi, H., Lee, B., Wu, S., & Zhu, J. (2003). Overexpression of a plasma membrane Na+/H+ antiporter gene improves salt tolerance in Arabidopsis thaliana. *Nat. Biotechnol.* , 81-85.

Siddique, M., & Jahan, A. (2006). Rapid multiplication of Nyctanthes arbor –tritis L. through in vitro axillary shoot proliferation. *World J. Agri. Sci. , 2*, 188-192.

Singh, A., Stalker, H., & Moss, J. (1991). Cytogenetics and use of alien genetic variation in groundnut improvement. *Chromosome engineering in plants: genetics, breeding, evolution.* , 65-77.

Smith, R., Gould, J., & Ulian, E. (1992). Method for transforming plants via the shoot apex. *US Pat. , 5*.

Soliman, M. H., Hussein, B. A., Osman, N. H., & Hussein, M. H. (2014). New approach for generating transgenic tomato plants expressing the cyanobacterium GRX-2 gene. *Arab J. Biotech. , 17*, 91-106.

Somers DA, S. D. (2003). Recent advances in legume transformation. *Plant Physiology* (131), 892-899.

Somers, D. A., Samac, D. A., & M, O. P. (2003). Recent Advances in Legume Transformation. *Plant Physiology , 131*, 892-899.

Subramanian, V., Gurtu, S., & Rao, R. S. (2000). Identification of DNA polymorphism in cultivated groundnut using random amplified polymorphic DNA (RAPD) assay. *Genome* , 656-660.

Sun, L., Hu, R., Shen, G., & Zhang, H. (2013). Genetic Engineering Peanut for Higher Drought and Salt-Tolerance . *Food and Nutrition Sciences* , 1-7.

Supertana, p., Shimizu, T., Nogawa, M., Shioiri, H., Nakajima, T., Haramoto, N., et al. (2006). Development of Simple and Efficient in planta Transformation Method for Wheat (Triticum aestivum l.) Using Agrobacterium tumefaciens. *Journal of bioscience and bioengineering , 102*, 162-170.

Tiwari, S., & Tuli, R. (2009). Multiple Shoot Regenerationin Seed Derived Immature Leaflet Explant In Peanut (Arachis hypogaea L). *Elsevier scientia Horticulturae , 121* (2), 223-227.

Vasil, I. (1986). *Plant regeneration and Genetic Variability* (Vol. 3). (I. K. Vasil, Ed.) Florida: Academic Press, Inc.

Venkatachalam, P., Geetha, N., Jayabalan, N., Saravanababu, & Lakshmi, S. (1998). Agrobacterium-Mediated Genetic Transformation of Groundnut (Arachis hypogaea L,) An Assessment of Factors Affecting Regeneration of Transgenic Plants. *Journal of Plant Research* (111), 565-572.

Wenck, A., Quinn, M., Whetten, R., Pullman, G., & Sederoff, R. (1999). High efficiency Agrobacterium-mediated transformation of Norway spruce (Picea abies) and Loblolly pine (Pinus. *Plant Mol. Biol , 39*, 407-416.

Yancheva, S., Druart, P., & Watillon, B. (2002). Agrobacterium mediated transformation of plum (Prunus domestica L.). *Acta Hortic* (577), 215-216.

Zhang, H., & Blumwald, E. (2001). Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. *Nat. Biotechnol* , 765-768.

Zhang, H., Hodson, J., Williams, J., & Blumwald, E. (2001). Engineering salt- tolerant Brassica plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. *Proc. Natl. Acad. Sci., 98, 12832-12836.* , 12832-12836.

Zhang, Z., Dermot, C. P., & Mitra, A. (1997). Factors Affecting Agrobacterium Mediated Transformation of Common Bean. *Journal of American Society of Horticultural Sciences , 122* (3), 300-305.

Zhu, J. K. (2003). Regulation of ion homeostasis under salt stress. *Curr. Opin. Plant Biol , 6*, 441-445.

Zia, M., Rizvi, Z. F., & Rehman, R. C. (2010). Agrobacterium Mediated Transformation of Soybean (Glycine max L.) Some Conditions Standardization. *Pakistan Journal of Botany , 4* (42), 2269-2279.