Agrobacterium Mediated Transformation of Two Peanut (*Arachis hypogaea L.)* Explants using *Na$^{+/}$H^{+}* Antiporter Gene to Generate Transgenies to Resist Salinity Stress

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIRMENTS FOR THE MASTER OF SCIENCE IN BIOTECHNOLOGY

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Dedicated

To

My parents

DECLARATION

I hereby solemnly declare that the research work embodying the results reported in this thesis entitled "***Agrobacterium*** **mediated transformation of two peanut (*Arachis hypogaea L.*) explants using Na^{+}/H^{+} antiporter gene to generate trangenesies to resists salinity stress.** Submitted by - Noor Jahan has been carried out under supervision of Dr. Aparna Islam, Professor, Biotechnology Programme, Department of Mathematics and Natural Sciences, BRAC University, Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

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ABBREVIATIONS

A.	*Agrobacterium*
BAP	6-Benzylaminopurine
Conc.	Concentration
DNA	Deoxyribo nuclic acid
EDTA	Etylene diamine tetra acetic acid
HCl	Hydrochloric Acid
IBA	Indole-3 Butyric Acid
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
Npt	Neomycin phosphotransferase
OD	Optical Density
pH	-log [H+]
T-DNA	Transfer DNA
Ti-Plasmid	Tumor inducing plasmid
YMB	Yeast Manitol Broth
µl	Micro litre
µM	Micro Mole

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Abstract

Peanut is one of the most popular sources of healthy addible oil. Its susceptance to various biotic and abiotic stresses is limiting crop production worldwide. Salinity is prior to them. Moreover, it is strictly monocious and geocarpic in nature. For these reason traits development through conventional breeding is almost impossible. So, Transformation is the only potential way to introduce stress tolerance gene in the trait of peanut. Here transformation done with two vector *pK7WG2_AtNHX1_1.6* and *pK7WG2_OsNHX1_1.6* mediated by *Agrobacterium tumefaciencce.* Decapitated whole embryo and Decapitated half embryo were used as explants. Four local peanut varieties named BINA Chinabadam 2,BINA Chinabadam 3,BINA Chinabadam 4, and BINA Chinabadam 6 used in this study. Regeneration media with 2 mg/l BAP supplement was found best for maximum number of shoot formation. The parameters for transformation like optical density, infection time, co-cultivation period optimized for all the varieties. For decapitated whole embryo transformation with bacterial solution OD_{600}1.0-1.3 with infection time 15 hr without co-cultivation period showed higher regeneration response under selection for all verities. For decapitated half embryo transformation with bacterial solution OD_{600} 1.0-1.2 with infection time 90 min followed by 48 hr of co-cultivation period showed higher regeneration response under selection pressure. Regeneration frequency of putatively transformed plantlets appeared more than 65% under selection whereas non transformed plants gave no regeneration under selection pressure. Kanamycine concentration of 100 mg/l found to be efficient for this purpose. Transformant detection was carried out in three distinct way antibiotic selection, leaf disk senescence assay and rooting under salt stress. The putatively transformed plants obtained after transformation observed to tolerate up to 100mM of salt stress, which was 50mM higher than base line salinity stress level. This study offers further study to establish salt tolerant peanut variety.

Introduction

Peanut is a crop of high nutritional and economic value. Scientific name of peanut is *Arachis Hypogaea L.* derived from Greek word. *Arachis* means weed and *Hypogaea* means underground chamber i.e. in botanical terms a weed which produces fruit bellow the soil surface this feature known as geocarpy. Ground nut and peanut is the two most common name of this crop. Ground nut mostly used in Asia, Africa, Europe and Australia and Peanut mostly used in North and South America.

1.1 Origin and Distribution:

The cultivated groundnut or peanut originated in South America. Europeans explorers first discovered peanut in Brazil. As early as 1500 BC the Incans of Peru and Brazil used peanut for ritual purposes. Tasty and hardy, the plant quickly spread. It reached Mexico by the 1st century AD, and soon after European explorers reached the New World, sailors were transporting it to China and Africa, where it became popular by the 1500s. (Where Did Peanuts Come From_.html-16.1016)

Now a day's peanuts are cultivated around the world almost in 80 countries. In Bangladesh peanuts are cultivated in Noakhali, Faridpur, Kishoreganj, Patuakhaliu, Rangpur and Dhaka districts.(We love Our Bangladesh_ Peanut or Cheena Badam is popular outdoor leisure snack food in Bangladesh.html,15.1.16)

Dr. George Washington Carver considered as the father of the peanut industry. He started his research on peanut in 1903.Nowadays peanut cultivated all over the world and china india producing the most. (History of Peanuts & Peanut Butter.htm-15.1.16)

1.2 Taxonomy:

Peanut belongs to 2nd largest family Fabaceae. Its genus Arachis is morphologically well defind and distinguished from other genera by having peg and geocarpic reproductive growth.The genus Arachis has70 wild species among them only Arachis hypogaea L. is domesticated and cultivated. Scientific classification of peanut:

Kingdom: Plantae
Order: Fabales
Family: Fabaceae
Genus: Arachis

Species: Arachis hypogaea

Binomial name: Arachis hypogaea L.

https://en.wikipedia.org/wiki/Peanut 16.1.16

1.3 Growth stage and pollination:

Growth stage of peanut divided into two stages – Vegetative stage , Reproductive stage. Both stages are visually observable. The vegetative stage determined by counting the number of cotyledonary node on main stem, beginning with the 1st cotyledonary node as zero. A node is counted when its tetrafoliolate is unfolded and its leaflets are flat. With blooming of flower reproductive stage started and ends up with maturity of pod(Holbrook and Stalker ,2003).

Peanut flower is self pollinating in nature. It`s unique characteristics is strictly cleistogemous; which means pollination occur before flower open. This characteristic is responsible for their lower genetic variation. Flower stalks start to elongate right after pollination and bend towards ground. Continued stalk growth pushes the ovary underground for forming peg. Peg matured to pod and it becomes countable measurable after 60-70 days (H.E.Patee and H.T.Stalker, 1995).

1.4 Nutrition Facts of Peanut

100g of peanut contains 567 calories; 49g of Fat, in this 7g saturated, 16g Polyunsaturated fat, 24g Monounsaturated fat; no Cholesterol; 18mg of Sodium; 705 mg of Potassium; 16g Carbohydrate; It also contains Vitamin A, Vitamin B-6, Vitamin B-12, Vitamin C ,Vitamin D

(National Peanut Board, History of Peanuts & Peanut Butter.htm-19.1.16; Peanuts.htm-19.1.16)

1.5 Climatic requirements and soil requirement:

Maximum production of peanut occurs in semiarid tropics. It can be grown in a wide range of temperature. Optimal temperature for its growth ranges from 20º C to 30º C (Prasad *et al* ,2003). But it's productivity lowers below 16º C and above 32º C. Minimum rainfall requirement of it better production ranging from 200 mm to 1000 mm (Prasad *et al* ,1999). Peanut production is affected by day length and light intensity. Vegetative growth lengthens during 13 to 16 hour day length. Soil selection is very critical for peanut production (Reddy *et al* ,2003).

1.6 Nutritional facts and Health benefits

Peanut contains high levels of monounsaturated and polyunsaturated fats, which helps to keep to keep **heart healthy**. Olic acid, in particular helps to lower the LDL or 'bad' cholesterol and increase HDL or 'good' cholesterol level in blood. Thus it prevents Heart diseases.

A recent study said that taking peanut daily **lower the risk of Diabetes** by 21%. This can be attributed by the presence of manganese, a mineral that plays an important role in fat and carbohydrate metabolism, calcium absorption and blood sugar regulation.

Eating an ounce of peanut daily **lowers the risk of developing gallstone** by 25%.

Peanut contains high concentration of a phytosterol called beta-sitosterol. These phytosterol prevents against cancer by inhibiting tumor growth. Peanut taking at least twice a week **lowers the rate of colon cancer** up to 27%. (14 Amazing Benefits And Uses Of Peanuts.htm-29.1.16)

Peanut contains Vitamin B3 or Niacin which improves brain functioning and boosts memory. It also contains a flavonoid called resveratrol which improve blood flow into brain up to 30%,it is called brain food. Niacin rich food lowers **the risk of Alzhemer`s diseses** up to 70%.

Sretonin is a vital brain chemical for mood regulation. Inadequate secreation of serotonin in brain causes depression. Peanut contains an amino acid called tryptaphan which aidsthe release of serotonin thus **helps to fight depression**. (Amazing Health Benefits and Uses Of Peanuts (Mungfali) For Skin, Hair And Health.htm-29.1.16)

Peanut is a good source of folate. Consuming daily 400micrograms of folic acid before and during early pregnancy **lowers the risk of allergic diseases, like asthma** in children.

Peanut has high concentration of poly-phenolic antioxidants, namely P-coumaric acid and olic acid which protects the heart and inhibit the growth of free redicals. It also contains Vitamin C and Vitamin E. These chemicals **prevent the signs of aging**. (Information About Peanuts.htm-29.1.16)

1.7 Uses of Peanut

Peanut oil is sweet flovoured and light. It is low in saturated fats,free from cholesterol. Contains essential fatty acid,omega-6,good source of plant sterol, espiciallyβ-sitosterol made it healthiest cooking oils.peanut oil is high in calories and antioxidants. It is ideal

for deep frying for it`s higher smoke point (450ºF), resuite lower oil retention in the fried foods. (List of Things Dr. George Carver Invented With Peanuts _ The Classroom _ Synonym.htm-29.1.16)

Peanut used as **animal feed**. Peanut hearts are good feed for egg-lying hens. Hulls of peanut used to make bran and meal. Dried peanut plant used as hay.

Peanut is a potential source of **colorants**. It used in producing wood stain, paint and ink.

Peanut hull or shell,fibers used for producing **kraft paper**. It also used for producing white, colored and news print paper. (12 Unusual Uses for Peanuts _ Life Hackery.htm-29.1.16)

Rudlof Diesel, inventor of engines, his first creation of engine was ran by various vegetable oils. Peanut oil is a potential source of **biodiesel**. Demand of biodiesel is increasing day by day for its cost effectiveness, environment friendly nature and renewable source of energy. (Peanuts.htm-19.1.16)

1.8 An overview of Peanut production: Worldwide and Bangladesh

The world peanut production in 2015 is 29 million metric tons. (Information About Peanuts.htm-19.1.16). Peanut originated from South America but now it is predominantly growing in developing countries (Asia and Africa). About 90% of the total world production comes from these regions and about 60% of production comes from semiarid tropic area. China is the largest producer of peanut and accounts fot 37% of world production, followed by India with 22%. In the year of 2014 China produced 2,720 thousand Mt of peanut, whereas India produced 1,150thouand Mt peanut. Average productivity of peanut in asia is 1739 kg/ha. (Peanut Oil Production by Country in 1000 MT - Country Rankings.html-29.1.16)

Bangladesh ranks 29 at the world peanut production scale. In the year of 2015, produced 33 thousand Mt, which is constant for 7 years. Whereas Bangladesh produced 48 thousand Mt peanut produced in the year of 1987. (Bangladesh Peanut Oilseed Production Annual Growth Rate.htm-29.1.16) There are two season for peanut production in Bangladesh Robi and Kharip season. Average production 51944 ton with shell and 4054 ton seed produced.

1.9 Peanut production constrains

Approximately 70% of the world`s peanut production comes from semiarid tropic regions. But productivity of peanut of this region is very low compared to worlds average production. This is dure to various abiotic and biotic constrains.

Abiotic factors of prime importance includes salinity, drought, high temperature, soil alkalinity, water logging. The influence of abiotic stress is complex, it is often confounded and associated with one another. Accumulation of salt in soil of pod zone affects peanuts yield. Salinity decreases the rate of photosynthesis, nitrogen fixation as a result vegetative growth rate gets lower (J.S.Boyer, 1982). It leads to increase of percentage of immature ods which lowers the yield. Flower and peg production is affected by high temperature during late stage of vegetative development decrease flower production (L. Sun et al,2013). Decrease in flower and peg number reduce the number of pod formation ultimately loss of yield. Drought stress inhibits leaf expansion and stems elongation as it alters both leaf and stem morphology. A 30 days drought stress delays the nodule formation, thus depriving nutrition's and continuous drought stress affects pod development seriously which lowers the yield as a result (D.Rains and I.S.Goya ,2003).

Biotic stress includes pests, diseases and weeds (Anuradha *et al,* 2006) . The most serious pest of peanut of this subcontinent is *Spodtera litura*,*Aproaerema modicella* and *Heliocoverpa armiger*. Intracellular feeder such as aphids(*Aphis craceivora*),thrips (*Frankliniella sp*) and soil pest like termites(*Microtermes sp*.) are also important pest in some arid regions (Khaleque et al, 1985). Peanuts are often attacked by fungi. The most common fungal pathogens are leaf spots (*Cercospora sp.),* rust (*Paccinia arachidis*) (Janila *et al* ,2013). Weeds are also important biotic factors. They complete with the crop for space nutrients ,light and water and act as alternative hosts for various other pests and diseases (Prasad *et al* ,1999).

Among various production constrains cited above environmental constrains are major targets of research in semiarid topic region. These environment constrains are of more concern because of the likely effects of global warming and climate change n agriculture.

1.10 Soil salinity problem in crop production and Bangladesh perspective

Minimal level of salt in soil is normal. But when, exchangeable sodium percentage is of 15 or more and electrical conductivity of saturation extract in the root zone exceeds

$4ds^{-1}$ at 25ºc is called saline soil.(Bahmani etal,2015). Salinity is a major problem for agriculture in arid and semi arid region f the world. This environment adversary has caused crop yield loses and hence very low crop productivity,33% of irrigated agricultural lands and 20% of cultivated lands worldwide are badly affected by high salinity(Rhodes and loveday,1990),(Flowers and Yeo ,1995) , (Szabolcs 1992). Its increasing up to 10% annually (Rains and Goyal ,2003).

In Bangladesh, costal area covers more than 30% of cultivable lands of country. Among them 53% is affected with salinity. The factors which plys vital role in development of saline soil are tidal flooding during wet season(June-October), direct inundation by saline water and upward or lateral movement of saline ground water during season (November-May)(Karim et al-1990). Soil salinity increasing with the desiccation of soil. Soil p^H level in coastal region range from6.0-8.4, which is strongly alkaline. In high alkaline soil micronutrients deficiencies are expected. Soil of this area are also very poor in organic content (1.0-1.5%)(Singarraval R,1996)

Moreover, Bangladesh is the most vulnerable country of the climate change and sea level rise. Inter governmental panel on climate change (IPCC) predicts that the climate change of Bangladesh may result in rising sea level up to 14 cm by 2025, 32cm by 2050 and 88 cm by 2100. which will create more saline area.

Salinity affects crops depending on degree of salinity at the critical stage of growth. Salinity has a number of deleterious effects on plants, some of them ae osmotic stress, ion toxicity, mineral deficiency, ion imbalance, oxidative stress, stomatal blockade, immature senescence, photosynthesis prohibition, increase of sensitivity to diseases and combination of these. These problems reduces yield and in severe case total yield loss (Rahman and Ahsan ,2001).

1.11 Plant mechanism against salinity

Every creature has its own defense mechanism against adverse environment. Plant has also some natural defense system of its own. Some plants can grow extreme saline condition naturally. Based on growing in saline condition plants divided in to two classes-(1) glycophyte, (2) halophytes. Glycophytes are those plant spices which grown in normal soil condition. Halophytes are grown in extreme saline soil condition(Zhang et al,2001)plant response against salinity are two types-(1)salt exclusion and (2) sequestering salt in cells vacuole (Greenway and Munns, 1981). Salt exclusion is done by controlling ion uptake in root and shoot level or synthesis of them into cellural

organic solutes. But this system not works in high saline condition. Sequestering salt mainly found in halophytes plants. In this mechanism osmotic adjustment is mostly attained by inorganic ion uptake from the soil and sequestering them in the cell vacuoles of leaves or other plant organ (Flowers *et al*, 1980).

Whereas, most of the edible plants are glycophytes. They are vulnerable to saline condition. Salinity problem is rising with time. Hybridization of trait development of plant is not a good alternative. Genetic engineering against this problem is the most suitable alternative, through which salinity resistant plant can get in short period of time.

1.12 Genetic engineering for salinity tolerance in plants

Crop tolerant to salinity stress is very important to run into the growing food demand through sustainable agriculture. This could be achieved either by selection and breeding or through modern molecular biology approaches. Success rate in conventional breeding is very poor because of very low genetic variation in gene pool of important crop. So that, transgenic approach actively perused these days by plant scientists all over the world for improving crop salt tolerance. Different genes are related to salt tolerant mechanism. Some gene which used in producing salt tolerant plants are :

- Signaling molecule
- Regulatory genes
- Osmolytes
- Antioxidant enzymes
- Glyoxalase pathway
- Transporter genes

Signaling molecule

Environmental signals are first received by signaling molecule. Thus over expression of them showed better stress tolerance in plants. CBL, AtNDPK2,SAPK4,MAPK are mostly reported signaling molecule. CBL sense the Calcium signal and participate in salt stress signal transduction pathway. It works at germination and seedlings stages (Wang *et al* ,2007)). SAPK4 is a serine threonine type of kinase. Its over expression resulted improved germination, growth and development under salt stress and also improved photosynthesis (Die´dhiou *et al* ,2008)).besides salt stress MAPK also shows cold stress tolerance (Xiong and yang ,2003; Jeong 2006).

Regulatory gene

Most regulatory genes, which work for stress tolerance are transcription factors (TFs). They interact with different cis-elements in the promoter regions of various downstream genes and modulate their expression. Some regulatory genes are – DREB, NAC, Myb etc. DREB2A from *Salocornia brachiata* showed enhanced salt tolerance in E. coli indicating that SbDREB@A is interacting with transcriptional network in bacterial cells (Gupta *et al*, 2010). Heu *et al* (2006) reported that rice SNAC1 showed significantly better salinity stress tolerance in transgenic line. Liao *et al* (2006) has studied expression of large number of soyabean Myb genes under salt and drought stress.

Osmolytes

Osmolytes are organic metabolites of low molecular weight known as compatible solutes and do not interfere the cellular function. The osmolytes such as glycine betaine, fructanns, trehalose, manitol, sorbitol, onanitol and pinnitol play vital role as osmoprotectants. Higher accumulation of osmopratectants enhanced slat and drought tolerance (Iturriaga *et al*,2009;Molinari *et al*, 2004; Rhodes *et al*, 1993)

Antioxidative Enzyme

Salinity stress generates reactive oxygen species (ROS) including singlet oxygen, super oxide, anion radicals, hydroxyl ions and hydrogen peroxide are considered as marker for stress activation in the plants (97). Transgenic plants developed by over expression of several enzymes such as glutathione peroxidase (GPX), SOD, APX showed improved stress tolerance (Wang *et al* ,2005; Roxas *et al* 1997; Wang *et al* 2004).

Glyoxalase Pathway

Glyoxalase pathway has emerged as a prospective candidate for the genetic engineering of salt tolerant (Espartero *et al*, 1995; Jain *et al*, 2002) 138,140). This pathway involves to enzyme Glyoxalase-1 and Glyoxalase-2. Both gene of this pathway used for producing transgenic line (Singla-Pareek et al, 2008).

Transporter Gene

Plants apply both ionic and osmotic homeostasis to reestablish themselves in saline environmental conditions. Transporter proteins are important candidates for genetic engineering to develop salt tolerant plants. AtNHX1, SOS 1-5, OsNHX1 genes are of this class. Of late, several efforts have been made to develop salt tolerant plants by up regulating of transporter genes as compared to other classes of gene (Fukuda *et al*,2004;

Wu *et al*, 2004; Lu *et al*, 2005; Martı´nez-Atienza *et al* 2007). Single or combination of these gene are used in developing transgenic line.

1.13 In vitro regeneration of peanut

Peanut is economically the most important species of legume family. So that, further improvement of its trait subjected to research from mid 18s century (Harvey and Schulz,1943; Neclus,1955). Clistogamous pollination (Rao *et al*,2003), ploidy difference (Moss and Stalker,1987) made conventional hybridization near to impossible for the improvement of its trait. Besides hybridization takes a long time most cases several years involving crossing and selection of desirable traits (Bhatia *et al*, 2004). In Bangladesh scientist are improving peanut production via gamma irradiation. But it has some adverse effect on crop reproducibility. Tissue culture technique helps to provide unique possibilities for overcoming the barrier of incompatibility between remote species and it facilitates rapid introduction of new varieties (Parveen,2011)

Various parts of peanut used as explants in tissue culture, based on their regeneration competence. Among explants, leaflets are most used explants. But regeneration of leaflet explants is highly dependent on genotypic variation, leaf age and location of cutting. It is very complex to work with these explants. Besides from leaflet callus formation to plantlet development took a long time. Other explants that studied in peanut are petiol, cotyledons (Bhatnagar *et al*, 2010; Lacorte,1999) cotyledonary node(Srinivasan *et al* ,2010), epicotyle and hypocotyls (Marion *et al*,2008), axulary meristems (Singh and Hazra 2009) Embryo. Even different stages of micropropagenesis from anther culture of A. hypogeae were examined (Saxena *et al*,1999).

A number of combinations of plant growth regulator have been used with various concentrations. In general, compounds with cytokinine activity such as benzylaminipurine(BAP), kinetin(Kn) and thidiazuran(TDZ) promot shoot initiation and development; Auxin such as 2,4-D induce smatic embryogenesis. IBA (Indole Butaric Acid), NAA (Napthaline acetic acid),IAA (Indole Acetic Acid) auxin like compounds used in root development.

Transformation efficiency frequently and directly related to tissue culture response. High regenerative cultures often more transformation competent. Susceptibility to Agrobacterium infection also varies greatly among genotype of peanut (Nuchowicz,1988). Researchers of BRAC University are working to establish a

reproducible regeneration protocol of locally grown peanut with decapitate half embryo and decapitate whole embryo explants.

1.14 Transformation of peanut

Desired characteristics can be interested into plants directly through genetic engineering. By this method long time of selection period of conventional breeding can be avoided. Gene can be introduced into plants in two methods. Indirect method based on Agrobacterium infection and direct gene delivery method of electroporation or microprojectile bombardment. Electroporation requires the DNA direct contact with plasma membrane of competent cells and is most effective with protoplast (Li *et al*, 1955). Least genotype dependent method for DNA delivery in peanut is microprojectile bombardment, where virtually any tissue can serve as target. Higher rate of transformation (45%) achieved using gene gun in zygotic embryo of peanut compared to only 1.8% with agrobacterium mediated gene transfer (Haggis and Dietzgen,2000)(Schnall *et al*,1993). But this method is laborious, costly and high professional requires for gene transfer. Compared to this Agrobacterium mediated gene transfer is cost effective and easy process. Agrobacterium mediated gene transfer reported mostly in peanut tansformation (Ozias-Akins, *et al,* 1993, Cheng *et al*, 1996; Eapen *et al*, 1994; Livingstone *et al*, 1995; McKently *et al*,1995; Banjara *et al*,2011).

1.15 Objectives

Development of salinity tolerant local peanut varity is the main objective of this work. To gain the aim, this study was designed to establish following division:

- Determination of effect of kanamycine concentration in the selection medium.
- Effect f salinity in peanut seed germination.
- Determination of factors influencing transformation efficiency.
- Regenerate putative transgenic peanut plant using pK7WG2_AtNHX1_1.6 and pK7WG2_OsNHX1_1.6 by Agrobacterium mediated gene transfer.
- Bioassay of putative transgenic peanut plant.

MATERIALS

2.1.1 Plant materials

Seeds of four local varieties of peanut *(Arachis hypogaea L.)* were used in this study. They are BINA Chinabadam 2, Bina Chinabadam 3, Bina Chinabadam 4, and BINA Chinabadam 6. All varieties were collected from Bangladesh Institute of Nuclear Agriculture (BINA). A brief description of the mentioned peanut varieties are represented in Table 2.1**.** Decapitated whole embryo and decapitated half embryo collected from 2 days old embryo of these four varieties were used as explants source to perform transformation.

2.1.2 *Agrobacterium* strain and plasmid vectors

Agrobacterium tumefaciense strain LBA4404 with two plasmids constructs, *pK7WG2_AtNHX1_1.6* and *pK7WG2_OsNHX1_1.6* were used for transformation.

pK7WG2_OsNHX1_1.6: The Na^{+}/H^{+} antiporter gene (*OsNHX1_1.6*) cloned from rice was immobilized to gateway vector, *pK7WG2_OsNHX1_1.6* under the control of promoter p35S and terminator T35S. This final construct *pK7WG2_OsNHX1_1.6* (Fig. 2.1 B) was transformed into *A.tumefaciense* LBA4404 to be used in peanut transformation. It contains spectinomycine and streptomycin resistance for selection in bacteria and kanamicin resistance for selection in plant.

***pK7WG2_AtNHX1_1.6*:** The Na^{+}/H^{+} antiporter gene (*AtNHX1_1.6*) cloned from Arabidopsis was immobilized to gateway vector, *pK7WG2_AtNHX1_1.6* under the control of promoter p35S and terminator T35S. This final construct *pK7WG2_AtNHX1_1.6* (Fig. 2.1 A) was transformed into *A. tumefaciense* LBA4404 to be used in peanut transformation. It contains spectinomycin and streptomycine resistance for selection in bacteria and kanamycin resistance for selection in plant.

Peanut varity	Year of release	Yield	Crop duration	Cultivating season	Characteristics
BINA Chinabadam 2	2000	3.2 t/ha in winter and 1.7t/ha in summer	160 days in winter and 125 days in summer	Summer and winter	• Dwarf plant. • Leaves are small,dark,ovate shape. • Pods are shiny without constriction and absence of prominanent break.
BINA Chinabadam 3	2000	3t/ha in winter and 1.6 t/ha in summer	150 days in winter and 135 days in summer	Summer and winter	• Intermediate dwarf plant. • Leaves are mostly upright, lonceolate, dark green. • Moderately resistant to coller rot, cercospora, leaf spot and rust disease.
BINA Chinabadam 4	2008	Maximum 3.5 t/ha average 2.6 t/ha	140 days in winter and 120 days in summer	Summer and winter	• High yielding • Pods are kernel and medium bold. • Resistant to leaf spot, coller root and rust diseases.
BINA Chinabadam 6	2011	2.9 t/ha maximum and 2.4 t/ha average.	Winter season	140-150 days	• Dwarf and erect • Pods are medium • Resistant to coller rot and rust diseases.

Source: www.bina.gov.bd.

2.1.3 Antibiotics used in transformation of this study:

Four antibiotics were used in this study. For *Agrobacterium* culture, three antibiotics (streptomycin and spectinomycin (Duchefa Bioc) for *Agrobacterium* strain LBA4404 and kanamycin for plasmid *pK7WG2_AtNHX1_1.6* and *pK7WG2_OsNHX1_1.6*) were used. Kanamycin (Duchefa Bioc) was also used as selectable agent to screen out the non-transformed explants. Cefotaxime (Duchefa Bioc) was used after co-cultivation as bacteriostatic against *Agrobacterium.*

2.1.4 Different media used in different phases of the study:

Media used for seed germination and embryo development

Autoclaved cotton soaked media with distil water used for seed germination and embryo development.

Media used for shoot regeneration

For shoot regeneration, full strength solid MS medium supplemented with BAP (2mg/l) and kinetin (1mg/l) was used.

Media used for root induction

The basal medium for rooting was half strength MS medium. The medium was supplemented by 0.1 mg/l IBA. For solidification, 0.6% (w/v) phytagel (Sigma) was used in root formation media.

2.1.5 Media used in transformation

Media used for *Agrobacterium* culture

YEP (Yeast Peptone Media) media with appropriate concentrations of antibiotics were used for bacterial culture. Liquid YEP medium was used for growing *Agrobacterium tumefaciense* strain LBA4404. This bacterial suspension was used as working culture for infection. YEP medium solidified with agar was used to maintain bacterial pure culture

Co-culture media

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

Selection media

Appropriate concentration of antibiotics Cefotexim as bacteriostatic against agrobacterium different concentrations of kanamycin was used in the best regeneration media as the selection media for screen out nontransformed plant.

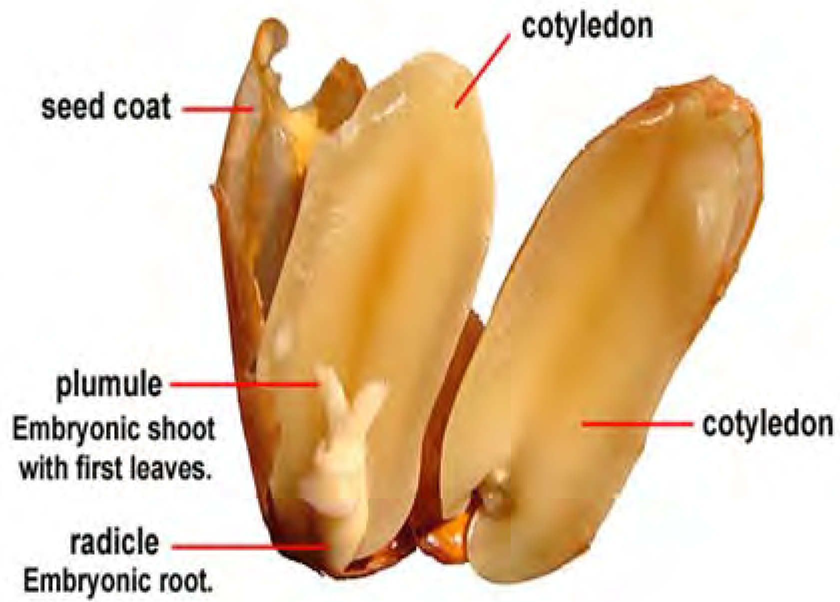

Figure 2.1 Peanut seed

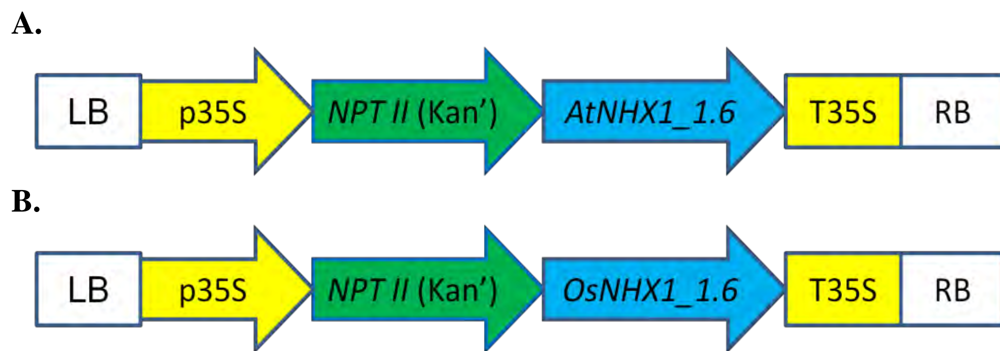

Figure 2.2 A. ConstructedvectorpK7WG2_AtNHX1_1.6
B. Constructed vector pK7WG2_OsNHX1_1.6.

METHODS

In the present study, Murashige and Skoog (MS) medium (1962) was used as a basal medium with different kinds of hormonal supplement. Various constituents of the medium were prepared into stock solutions for ready use during the preparation of medium. Different components were required for the preparation of stock solution in MS media (Table 2.2).

2.2.1 Stock solution A (Macro-nutrients) for MS medium

This stock solution was made in such-a-way that its strength was 10 times more than the final strength of working medium. For this purpose, the weight of different salts required for 1000ml of stock solution were weighted correctly. Then the salts were sequentially dissolved one after another in a 1000 ml volumetric flask with 500 ml of distilled water. The final volume of the solution was made up to 1000 ml by further addition of distilled water. The solution was poured into a clean container and tagged. Finally the solution was autoclaved (Model: WAC-47, Korea) and stored in a refrigerator at 4ºC for several weeks.

2.2.2 Stock solution B (Micro-nutrients) for MS medium

For this constituent of the medium two separate stock solutions were prepared:

Stock solution Bl (all micro-nutrients except iron) for MS medium

The solution was made 100 times of the full strength of the media. The components of micronutrients (Table 2.2) were mixed in a flask with distilled water by using a magnetic stirrer. Then the total 1000 ml of the solution was autoclaved. Once cooled down, stored it at 4ºC for some weeks.

Stock solution B2 (Iron chelate solution) for MS medium

The second solution was also made 100 times the final strength of $FeSO_4.7H_20$ and Na_2-EDTA in 500 ml distilled water in a conical flask and heated slowly at low temperature until the salts were dissolved completely.

Finally the solution was volume to1000 ml stored in refrigerator at 4°C for several weeks.

2.2.3 Stock solution C (Organic constituents) for MS medium

It was also made 100 times the final strength of the medium in 500 ml of distilled water. Mixed properly then final volume up to 1000ml. Tag properly and stored at 4°C future use.

2.2.4 Stock solutions for growth regulators

The following different growth regulators were used in the present investigation:

Auxins

Indole-3-butyric acid (IBA)

Cytokinins

6-benzylaminopurine(BAP)

6-furfurylaminopurine(Kn)

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. The final concentration of the stock was (1mg/100ml). The stock solution was then filtered, labeled and stored at 4ºC for up to 2 months.

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 (1mg/10ml). The stock solution was then filtered, labeled and stored at 4ºC for up to 2 months.

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.

Table 2.2 Composition of stock solutions in MS media

Macronutrients(A)	mg/l	Micronutrients(B1)	mg/l
KNO_3	1900	KI	0.83
NH_4NO_3	1650	H_3BO_3	6.2
$MgSO_4 \cdot 2HO$	370	$MnSO_4 \cdot 4H_2O$	22.3
$CaCl_2 \cdot 2H_2O$	440	$ZnSO_4 \cdot 7H_2O$	8.6
KH_2PO_4	170	$Na_2MoO_4 \cdot 2HO$	0.25
		$CuSO_4 \cdot 5H_2O$	0.025
		$CoCl_2 \cdot 6H_2O$	0.025
Fe-EDTA(B2)	**mg/l**	**Organic(C)**	**mg/l**
$FeSO_4 \cdot 7H_2O$	27.8	Nicotinic acid	0.5
$Na_2EDTA.2H_2O$	37.3	Pyridoxin HCI	0.5
		Thaimin HCl	0.1
		Glycin	2.0

Table 2.3 Different components for preparation 1 litre of MS media

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

2.2.5 Preparation of stock solution-antibiotics (25mg/ml)

Stock solutions of kanamycin sulfate, streptomycin, spectinomycin and cefotaxime were prepared. 1 g of each was dissolved in 35 ml of ddH_2O and the volume was made up to 40 ml with ddH_2O. The solutions were sterilized by syringe filtration, poured in autoclaved eppendorf and finally stored at -20ºC.

2.2.6 Preparation of one liter of MS medium

To prepare one liter of medium, 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 each stock 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. Hormone(s) the stock solutions were added sequentially according to its required concentration and volume to this solution and mixed thoroughly.

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 (at0.3%) 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).

2.2.7 Preparation of seed germination media

To obtain appropriate 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.

2.2.8 Preparation of shoot regeneration media

For shoot regeneration, solid MS medium supplemented with BAP (2mg/l) and kinetin (1mg/l) was used.

2.2.9 Preparation of media for subculture

For maintaining better growth rate regenerated explants needed to be subculture in every 4-6 weeks. Media of shoot regeneration used as media for subculture with same hormonal supplementation.

2.2.10 Preparation of root induction media

The basal medium for rooting was half strength MS medium. The medium was supplemented by 0.1 mg/l IBA. For solidification, 0.6% (w/v) phytagel (Sigma) was used in root formation media.

2.2.11 Preparation of culture media for *Agrobacterium tumefaciense*

Yeast Extract Peptone (YEP) medium was prepared to culture *Agrobacterium tumefaciense* strain LBA4404 (Table 2.4). The pH of the medium 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, antibiotic were added. *Agrobacterium tumefaciens* containing each of the vectors, kanamycin was added at 200 mg/l, 100 mg/l streptomycin and 200 mg/l spectinomycin were added to each 100 ml YEP media.

2.2.12 Maintainance of agrobacterium culture:

Agrabacterium culture maintained in two way - 1. subculture and 2.glycerine stosk YEP solid media was prepared with required antibiotics (kanamycin, streptomycin and spectinomycin, for *Agrobacterium* strains *pK7WG2_OsNHX1_1.6* & *pK7WG2_AtNHX1_1.6* for *Agrobacterium* stock maintenance. 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.

Liquid YEP medium was prepared for liquid culture of bacteria which is needed for glycerin stock. 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. 60 % glycerin stock prepared separately. Then in ratio 6:4 glycerin and *Agrobacterium* culture taken. Vortex this mixture to mix them well and preserved in -20°C. This stock maintained once in a month.

2.2.13 Preparation of co-cultivation media

MS medium with BAP was used as co-cultivation media. Hormonal concentration that was found best for tissue culture of tomato varieties was added to this media. No antibiotics were added here.

2.2.14 Preparation of selection media

For transformed shoot selection, cefotaxime along with kanamycin was used with regeneration media. The regeneration media contained best hormonal concentration found in plant tissue culture experiment. Regeneration medium autoclaved and cool down at 50-55°C and added Kanamycin at different concentration to determine the selection concentration. It was done inside laminar flow and separated into Petri dishes and wait till it becomes solid.

Table 2.4 Components for YEP medium preparation

Components	Amount (g) for 1 liter media
Yeast extract	10.0
Peptone	10.0
Sodium Chloride (NaCl)	5.0

Table 2.5 NaCl concentrations and amount in 100 ml media used in salinity test.

NaCl concentration in MS media		Amount of NaCl added in MS media
5 mM	0.5 dS/m	0.02925 g
10 mM	1 dS/m	0.0585 g
20 mM	2 dS/m	0.117 g
50 mM	5 dS/m	0.2925 g
100 mM	10 dS/m	0.5852 g
200 mM	20 dS/m	1.1704 g

2.2.15. Media sterilization

Fixed volume of melted medium was dispensed into culture vessels. Culture vessels were covered with aluminum foil and tagged to indicate specific hormonal supplements. The culture vessels were then autoclaved (EYELA AUTOCLAVE MAC_ 501, Japan) at 15 lb/sq. inch pressure at 121°c temperature for 20 minutes

2.2.16. Precautions to ensure aseptic conditions

All inoculation and aseptic manipulation were carried out in a Laminar Flow cabinet (ESCO; class II, Type R/83 Biohazard safety cabinet, Singapore). The cabinet was switched on for at least half an hour before use and cleaned with 70% alcohol to overcome the surface contaminations. The instruments like scalpels, forceps, inoculation loop, Petri dishes, micro-pipette tip, eppendrof tubes etc. were sterilized by steam steriliz.ation methods. At the time of inoculation these were again sterilized by flaming method inside the cabinet. Both the hands were sterilized with antimicrobial hand wash (Hexisol,ACI Ltd) . All measures were taken to obtain maximum contamination free condition during the surgical operation of the explants. After autoclaving bacterial media were poured inside the laminar air flow cabinet to avoid contamination. Antibiotics used for various media were filter sterilized and stored in sterile eppendrof tubes. Inside the laminar air flow cabinet antibiotics were added to the medium. After each transformation experiment, Agrobacterial suspension used and contaminated Petri dishes, cotton, filter papers, instruments; glass cuvettes were autoclaved to destroy genetically engineered *Agrobacterium* as a part of biosafety procedure. Any contaminants and old bacterial cultures were also autoclaved before discarding them.

2.17 Culture techniques

Sterilization of seed

At first, seeds were washed 3 times with double distil 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 whole work done under laminar hood cabinate.

Explants collection

Seeds germination medium were kept in the dark chamber to imitate the darker environment under soil. Germinated seeds of the mentioned varieties were the source of the two different explants decapitated half embryo and decapitated whole embryo. Embryos were collected from overnight emerged seeds.

Transformation of decapitated half embryo

Day 1: Seeds were sterilized and preserved in dark chamber overnight for 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 2: Optical Density at 600 nm (OD_{600}) of the overnight grown culture was measured while comparing with autoclaved fresh liquid YEP media as zero 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 60 and 90 minutes for infection and then placed on co-cultivation medium and kept there for next 24 to 72 hr (co-cultivation period).

Day 3: The Petri-plates were checked for bacterial overgrowth. If there is any bacterial overgrowth shown on explants, then those explants were washed with cefotaxime.

Day 4: Explants were transferred to cefotaxime containing regeneration media.

After a week, explants were placed on kanamycin 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.

Transformation of decapitate whole embryo

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

Day 2: Optical density (OD_{600}) of the overnight grown culture was measured by spectrophotometer, where autoclaved fresh liquid YEP media was used as blank. The zero time absorbance of culture density was obtained from the blank.The Petri-dish with filter paper is soaked with liquid MS media and then the Petri-dish was used to cut explants. The explants along with the *Agrobacterium* solution were transferred into a conical flask and kept in a shaker for infection duration of 16 hr hours.

Day 3: Explants were washed with cefotaxime and transferred to cefotaxime containing germination (MS) media.

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

Transferring of explants to fresh media with seletion pressure

Regenerated explants were subcultured into fresh media containing the same hormonal supplement for further proliferation, development and screening of transformants

with two antibiotics cefotaxim and kanamycine. Subculture was performed regularly at an interval of 2-4 weeks for maintenance. Cultures were routinely examined for different morphogenic development and data were recorded soon after 10-12 days of inoculation.

Transferring of explants to fresh media for root induction

Well developed shoots around 3-4 cm long, were placed individually in the root induction medium to obtain sufficient root formation with cefotaxime for avoiding bacterial overgrowth. Data were recorded after 13-20 days of placement in the root induction media.

Procedure of plant acclimatization in natural environment

Acclimatization is required to achieve adaptation of the regenerated plantlets to the natural environment. Following steps were taken in the process,

a. The regenerated plantlets were carefully removed from the rooting media using forceps when the roots were 3 to 5 cm long. The phytagel attached to their root part was gently washed with running tap water. It was done to make sure that the entire phytagel was removed completely to avoid any contamination and to ensure nutrients uptake by roots from soil.

b. The soil was autoclaved before pouring into pots where the plants were transferred. Perforated plastic bags were taken to cover the potted plantlets. The inside of the bags were sprayed with water to maintain the humidity and to prevent moisture shock. Plantlets were kept inside the culture room for 15 to 20 days. During these days the moisture inside the bags were maintained constantly. Liquid MS medium was given to the plants as water supplement.

c. After 20 days the bags were removed and the plantlets were kept for next 15 days inside culture room.

d. About four weeks after transplantation, plants were then kept in a shade place outside the culture room each day for 2 hours for 1 week.

e. On the eighth week, the plants were exposed to direct sunlight for 2 hours a day. This treatment was continued for 2 more weeks. Lastly the plants were placed in natural environment.

f. At this stage leaves were dark green than it was before and stem had secondary thickness. Finally the plants were transferred to pots containing mixture of ground soil, sand and cow dung in the ratio of 1:2:1 in net house.

2.2.18 Analysis of regeneration expression of the transformed plantlets

The putatively transformed shoots were selected through antibiotic selection; these seedlings were allowed to grow on natural environment. The plant material as leaves, shoots, shoot-tips were also needed for molecular analysis of the putative transformants.

2.2.19 Methodology of kanamycin sensitivity test

To study the effect of kanamycin on the growth of peanut embryonic explants, regeneration media containing various concentrations (0, 5, 10, 20, 50, 100, 150 and 200 mg/l) of kanamycin were added to autoclaved regeneration media after cooling down inside laminar air flow hood and then it was divided into Petri-dishes. Ten explants were subjected to each concentration of kanamycin per trail. The decapitate embryo were placed in those media to check their regeneration response. The result was recorded after 25-30 days of inoculation for all four varities

2.2.20 Determination of baseline saline tolerance level of peanut seedlings

In the present study, the effect of salinity on germination of peanut seeds was investigated as it is the first stage towards salinity tolerant transgenic peanut production. Therefore, all four varieties were used 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 (Table 2.5). MS media was prepared with different amount (5 mM, 10 mM, 20 mM, 50 mM ,100mM and 200 mM) of NaCl in each 100 ml of media. Seeds were placed in these media and the result was recorded after a week to get their germination response in different salt concentrations.

2.2.21 Bioassay

Leaf disc assay for tolerance against salinity stress

Leaf disk assay was carried out to evaluate the sensitivity of the transformed and untransformed peanut plants to sodium chloride (NaCl) stress as described by Fan *et al.,*(1996). Fully developed healthy leaves of wild-type and transgenic plants (of similar age, about 50 days old) were washed with double distilled water. Leaf disks (~1 cm diameter) were excised and floated on 100 mL of NaCl solution (5 mM, 10 mM, 20 mM, 50 mM and 100 mM) and 0 mM NaCl (sterile distilled water) was used as an experimental control for 14 days (Table. 2.4). The same were kept under the standard photoperiod at 25°C. The effect of salt treatment on leaf disks was observed by monitoring phenotypic changes, as browning, bleaching and freshness of leaves.

Salinity stress tolerance assay on transformed shoots:

This assay done with putatively transformed shoots described by S.l. single-pareek et al 2003. Fully developed wild type and transgenic shoots used in this assay. Shoots were excised and planted on rooting media containing different concentration of NaCl (5mM, 10mM, 20mM, 50mM, 100mM) and 0mM NaCl(without any salt) was used as an experiental control for 14 day. The effect of salt treatment on putative shoots was observed by phenotypic change, growth and rooting

Results

The objective of the present study was to generate peanut with salinity tolerance gene. For achieving the goal salt tolerant gene was successfully transferred into peanut genome. This work started with optimization of gene transfer parameters following putative transformed plant selection in antibiotic.

3.1 Baseline salinity stress tolerance test

Salinity stress tolerance level of natural germplasm had been assessed in this study. Effect of salinity on peanut seed germination was observed. Seed of all four varieties were subjected to different concentrations of NaCl (0.5 dS/m - 20 ds/m). Maximum number of seed germinated in control experiment. The germination rate decreased with the increase of concentration of NaCl (Table 3.1). Germination rate was below 50% in 50mM NaCl. The germination totally stopped at 100mM NaCl. Time requirement for seed germination was also increased from 20mM (Figure 3.1).

3.2 Kanamycin tolerance test for selection of transformed plant

For this test natural decapitate embryonic explants were tested with different concentrations of kanamycin (0mg/l - 200mg/l). In control trial all the explants survived but survival percentage dropped gradually with the rise of kanamycin concentration in the regeneration media. The explants start to become albino at 50mg/l kanamycin which was followed by death of explants at 100 mg/l kanamycine.

Therefore, in the following transformation experiments 100mg/l kanamycin concentration was considered as optimum for transformant selection. Explants which stayed alive at this concentration for at least one month would be considered as putative transformed (Table 3.2); (Figure 3.2).

3.1 Effect of salinity on peanut seed germination

NaCl concentrations (mM)	Germination %				Visual appeerence
	BN 2	BN3	BN4	BN6*	
0	100	100	100	100	Normal germination
5	93	91	93	92	Normal germinatio
10	78	75	60	60	Normal germination
20	53	40	60	40	Late germination
50	42	33	46	33	Late germination
100	0	0	0	0	No germination
200	0	0	0	0	No germination

*BN2-BINA Chinabadam 2,BN3-BINA Chinabadam 3,BN4-BINA Chinabadam 4,BN6-BINA Chinabadam 6. Thirty five seeds were subjected in each trail. Data were taken 14 days after inoculation. Values were obtained from three independent trails.

3.2 Effect of various kanamycine concentrations on the regeneration of nontransformed Decapitate embryonic explants

Kanamycine concentration (mg/l)	Regeneration response (%)	Percentage of survival	Visual appearance
0	100	100	Green, healthy
5	100	83	Green, healthy
10	66	50	Green
20	33	33	Slightly albino
25	33	10	Albino
50	1.6	0.13	Albino
100	0	0	Brown
200	0	0	Necrosis

Forty seeds were subjected in each trail. Data were taken 30 days after inoculation. Values were obtained from three independent trails.

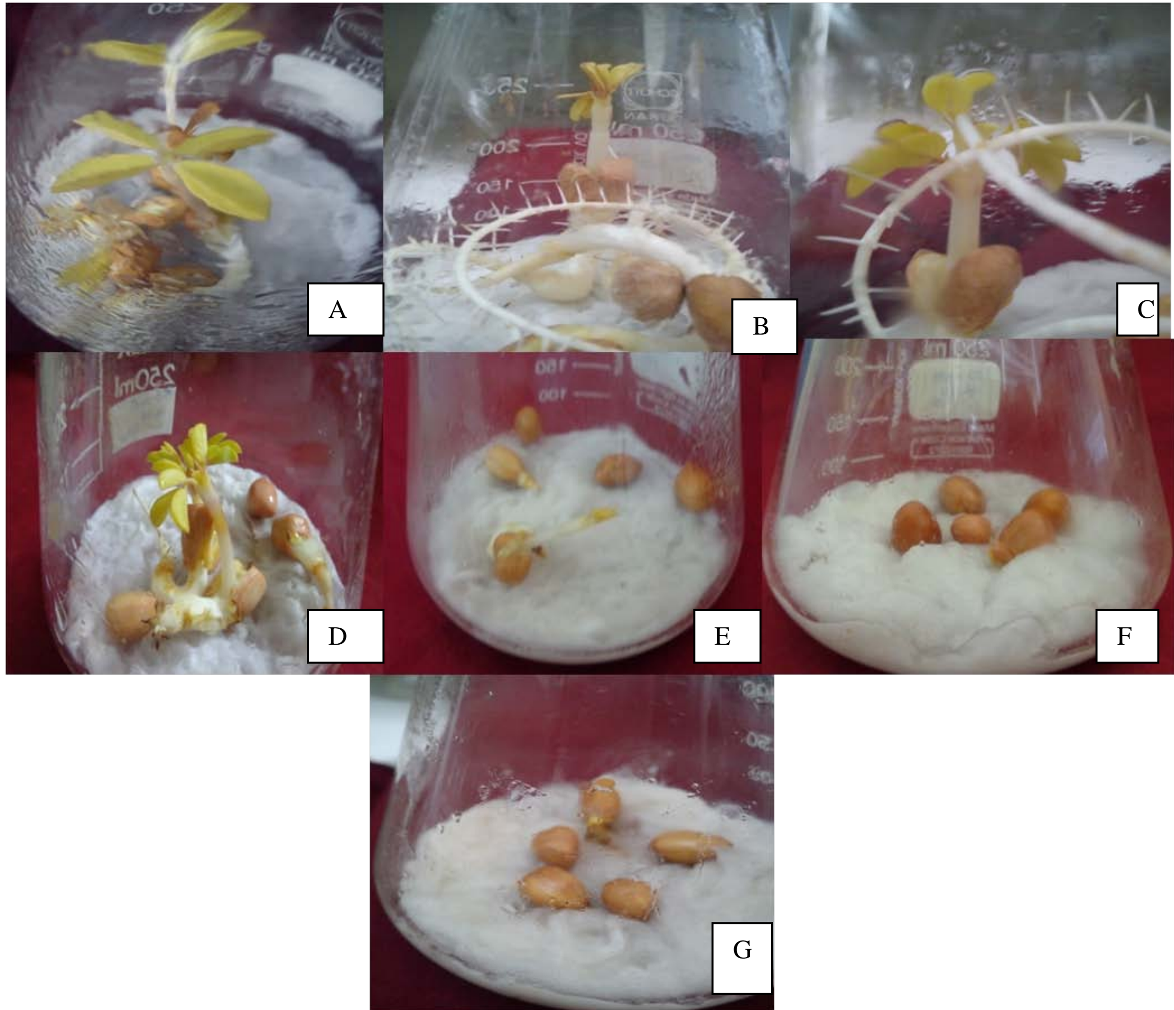

Figure 3.1 Seed germination of BINA Chinabadam 3 in presence different amount of NaCl A.0mM, B.5mM, C.10mM, D.20mM, E.50mM, F.100mM, G. 200mM NaCl. Photographs were taken 21 days after inoculation.

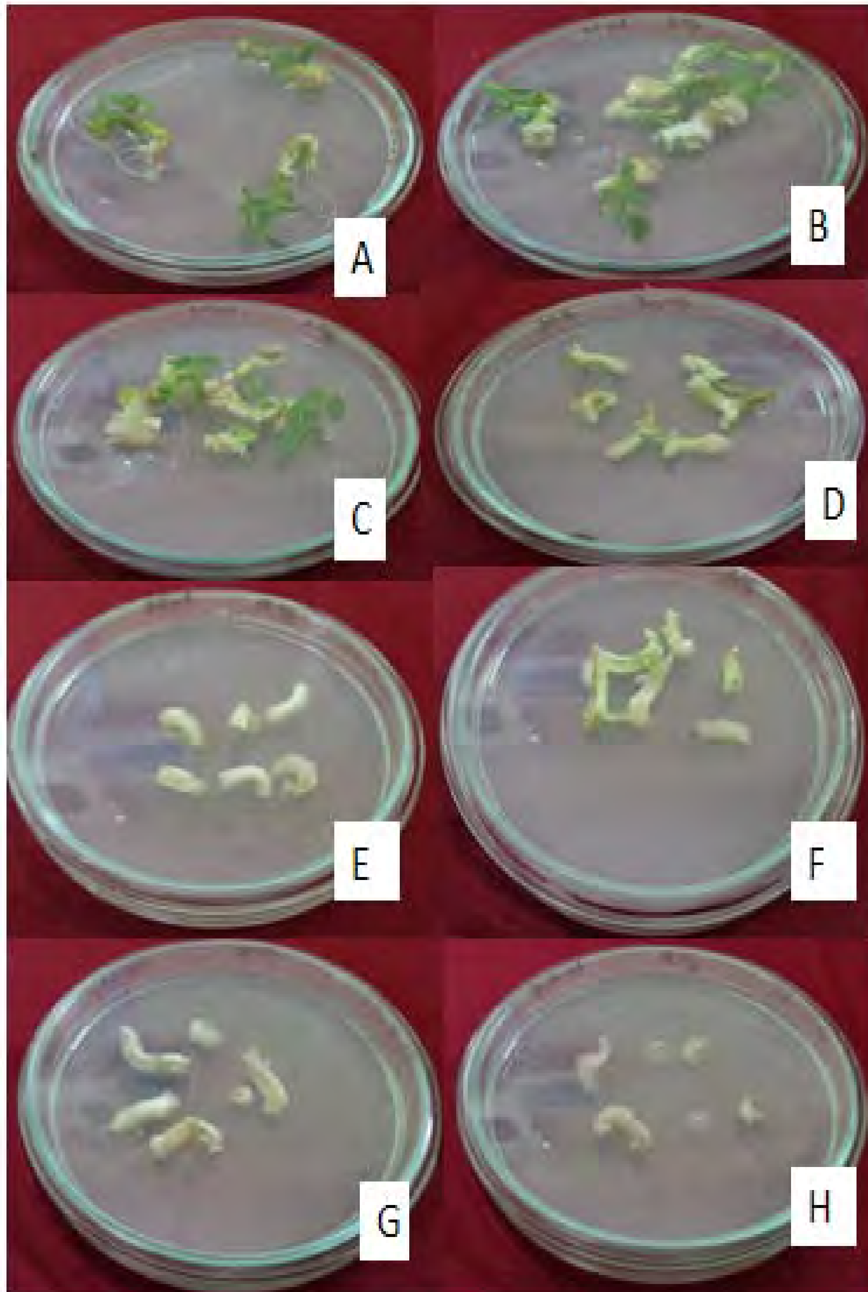

Figure 3.2 Effect of various concentrations of kanamycine on decapitated embryonic explants of BINA Chinabadam 4 A.0mg/l, B.5mg/l, C.10mg/l, D.20mg/l, E.25mg/l, F.50mg/l, G.100mg/l, H.200mg/l of kanamycin. Photographs taken 45 days after inoculation.

3.3 Transformation of Decapitate Half embryo

3.3.1 Effect of Optical density (OD_{600}) and Incubation period on regeneration efficiency

Different Optical Density ($OD_{600)}$ and two different incubation periods (60mins, 90mins) were used to find out the best regeneration response following transformation avoiding bacterial overgrowth. All four verities gave better regeneration with incubation period of 90 min at OD_{600} of 1.0-1.3 with the vector pK7WG2_*AtNHX1_1.6.* BINA Chinabadam 3 gave highest regeneration in this condition (Table 3.3).

For the vector *pK7WG2_OsNHX1_1.6* incubation period of 90 mins showed comparatively better result than 60 min at OD_{600} 1.0-1.3. In this condition BINA Chinabadam 6 and BINA Chinabadam 2 gave better regeneration. Whereas, BINA Chinabadam 3 gave better response for incubation period of 60 min (Table 3.4).

Therefore, OD_{600} 1.0-1.3 and incubation period of 90 min was used as optimum condition for better regeneration efficiency following transformation for further study.

3.3.2 Effect of co-cultivation period on regeneration efficiency

Co-cultivation period plays a vital role in transformation efficiency and regeneration capability as it allows growth of both plant and bacteria at the same time as no antibiotic is used at this stage. In this study, 48 hr co-cultivation gave better regeneration for all the varieties with two constructs under selection. Longer co-cultivation period (72 hr) lead to overgrowth of bacteria. Moreover, shorter co-cultivation period (24hr) gave poor regeneration rate under selection pressure indicating lesser transformation events. So, co-cultivation period of 48 hr was preferred as best for transformation for both *Agrobacterial* vectors (Table 3.5).

3.3 Effect of optical Density (OD_{600}) and incubation period on regeneration efficiency following transformation with *Agrobacterium* vector *pK7WG2_At NHX1_1.6*

Peanut Varieties	Optical Density (OD_{600})	Incubation Period (min)	Regeneration response on selection media (%)
BINA Chinabadam 2	0.6-0.8	60	42
		90	61
	1.0-1.3	60	55
		90	66
BINA Chinabadam 3	0.6-0.8	60	58
		90	63
	1.0-1.3	60	62
		90	67
BINA Chinabadam 4	0.6-0.8	60	47
		90	57
	1.0-1.3	60	53
		90	65
BINA Chinabadam 6	0.6-0.8	60	58
		90	62
	1.0-1.3	60	61
		90	66

Values were obtained from triplicate trials. Data were taken 30 days after infection. Regeneration occurred in presence of selection pressure of 100 mg/l kanamycin along with 200 mg/l cefotaxime.

3.4 Effect of optical density and incubation period on regeneration efficiency following transformation with *Agrobacterium* vector *pK7WG2_OsNHX1_1.6*

Peanut Varieties	Optical Density (OD_{600})	Incubation Period (min)	Regeneration response on selection media (%)
BINA Chinabadam 2	0.6-0.8	60	50
		90	58
	1.0-1.3	60	61
		90	65
BINA Chinabadam 3	0.6-0.8	60	48
		90	58
	1.0-1.3	60	60
		90	63
BINA Chinabadam 4	0.6-0.8	60	43
		90	55
	1.0-1.3	60	46
		90	58
BINA Chinabadam 6	0.6-0.8	60	52
		90	60
	1.0-1.3	60	58
		90	62

Values were obtained from triplicate trials. Data were taken 30 days after infection. Regeneration occurred in presence of selection pressure of 100 mg/l kanamycin along with 200 mg/l cefotaxime.

3.5 Effect of co-cultivation period on transformation efficiency of four peanut varieties

Peanut Varieties	Co-cultivation period (hr)	Regeneration percentage on 100 mg/l kanamycin selection media (%)	
		pK7WG2_AtNHX1	*pK7WG2_OsNHX1*
BINA Chinabadam 2	24	64	62
	48	66	65
	72	61	58
BINA Chinabadam 3	24	63	53
	48	67	63
	72	56	58
BINA Chinabadam 4	24	50	55
	48	65	58
	72	56	52
BINA cChinabadam 6	24	62	60
	48	66	62
	72	60	51

Values were obtained from three independent trials of the four peanut verieties with the specified vector. In each trail ten explants were inoculated. Experiment conditions were optical density of 1.0-1.3, incubation time 90 mins. Data were taken after 30 days after inoculation.

3.3.3 Transformation frequency and plantlet regeneration from decapitated half embryo on selection media

After transformation, explants were placed on media containing 200 mg/l cefotexime and 50 mg/l kanamycine to control overgrowth and screening transformed shoots. Survived plants were then transferred to 100mg/l kanamycin containing media within 3 to 4 weeks. Control nontransformed experiment gave no regeneration in this media.

During selection BINA Chinabadam 6 gave highest number of putative shoots, whereas BINA Chanabadam 4 gave lowest number of putative shoots with Agrobacterium vector *pK7WG2_AtNHX1* (Table 3.6). Rhizogenesis percentage with this vector was higher in BINA Chinabadam 3 (68%).

For *pK7WG2_OsNHX1* vector BINA Chinabadam 6 gave highest putative shoots compare to other three varieties. Best rhizogenesis (68%) with this vector gave by BINA Chinabadam 2 (Table 3.6).

After proper root development plantlets were transferred to plastic pots for hardening.

3.6 Regeneration of putative transformed plantlets from decapitated half embryo under selection

Peanut varieties	Mean number of shoots under selection of 100 mg/l of kanamycine ± SD		Percentage of root induction (%)		Survival rate of plants in natural environment.(%)
	pK7WG2_AtNHX1_1.6	*pK7WG2_OsNHX1_1.6*	*pK7WG2_AtNHX1_1.6*	*pK7WG2_OsNHX1_1.6*	
BINA Chinabadam 2	2.5±1.25	2.2±1.4	63	68	34
BINA Chinabadam 3	2.2±0.5	2.4±0.95	68	60	41
BINA Chinabadam 4	1.92±0.95	2.1±0.57	52	50	28
BINA Chinabadam 6	2.7±0.81	2.6±0.82	65	64	34

Mean number of putative transformed shoots came from the average number of multiple shoots on selection. Mean value in each experiments average of three sets of experiment with 50 decapitate half embryo explants in each set. Data were collected after 50 days of inoculation of explants.

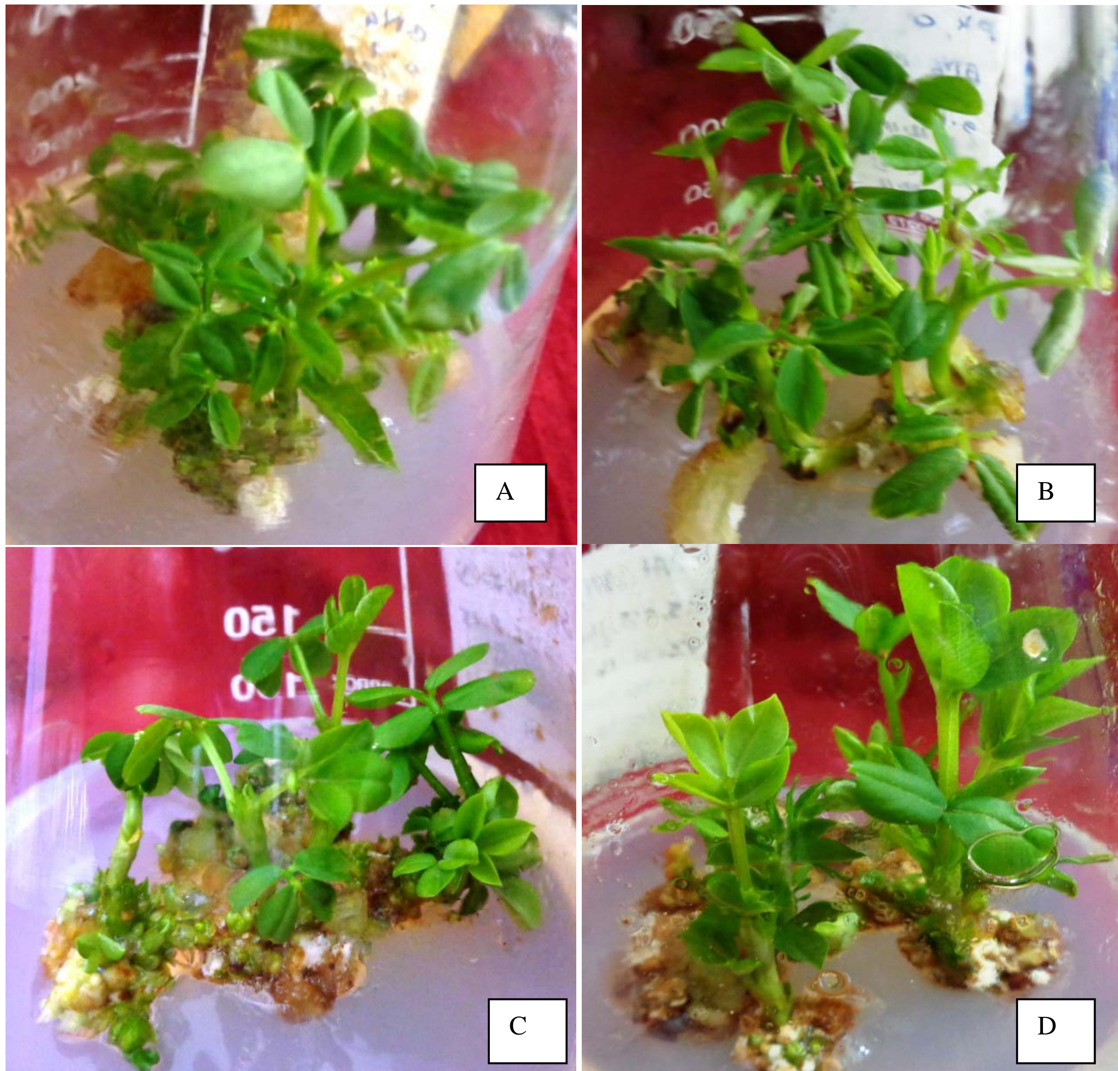

Figure 3.3 Putative transformed shoots from decapitated half embryo following transformation with *pK7WG2_AtNHX1_1.6* on kanamycin selection media (100mg/l) A. BINA Chinabadam 2, B. BINA Chinabadam 3, C. BINA Chinabadam 4, D. BINA Chinabadam 6. Photographs were taken 60 days after following transformation.

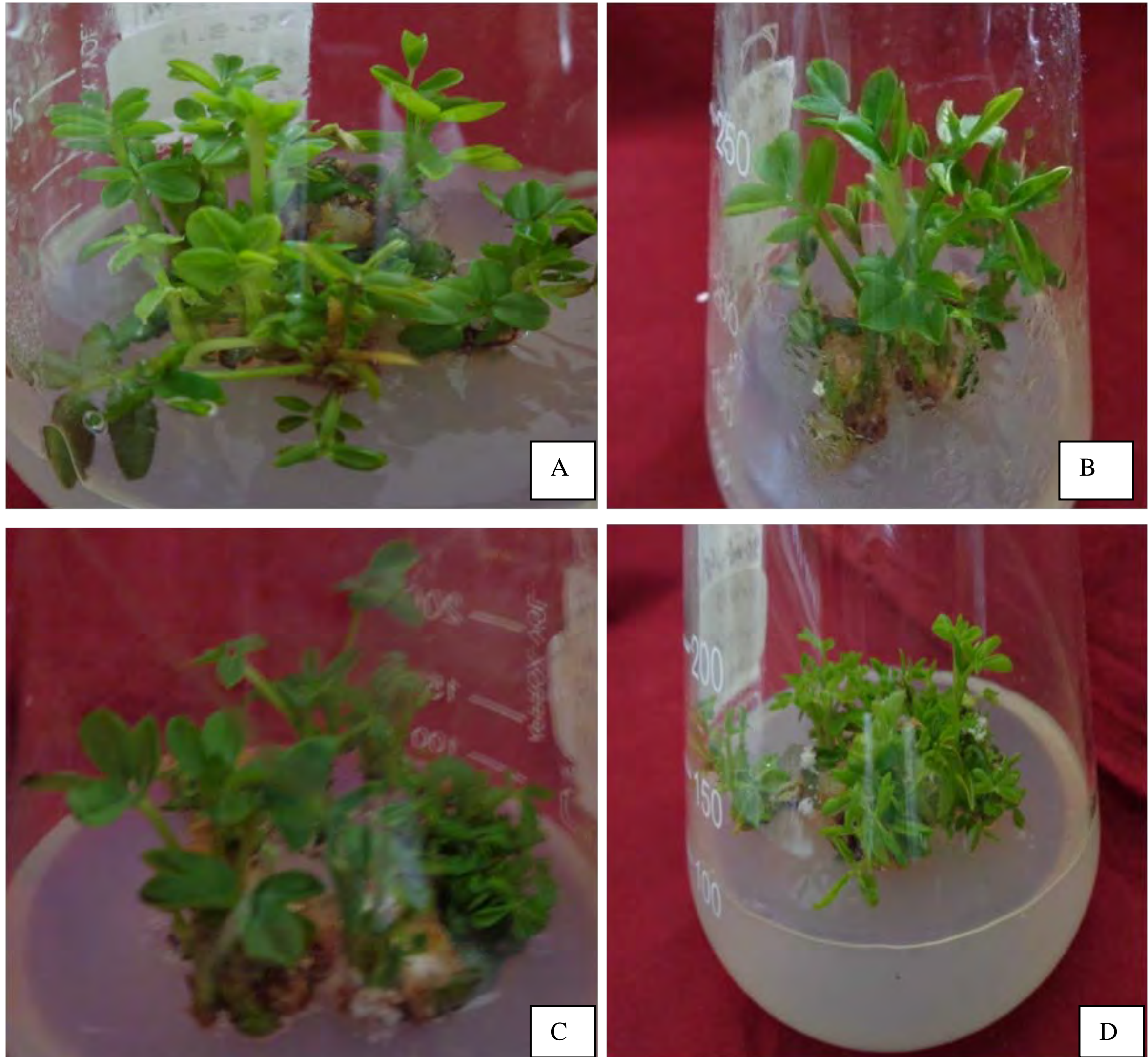

Figure 3.4 Putative transformed shoots from decapitated half embryo following transformation with *pK7WG2_OsNHX1_1.6* on kanamycin selection media (100mg/l) A. BINA Chinabadam 2, B. BINA Chinabadam 3, C. BINA Chinabadam 4, D. BINA Chinabadam 6. Photographs were taken 60 days after following transformation.

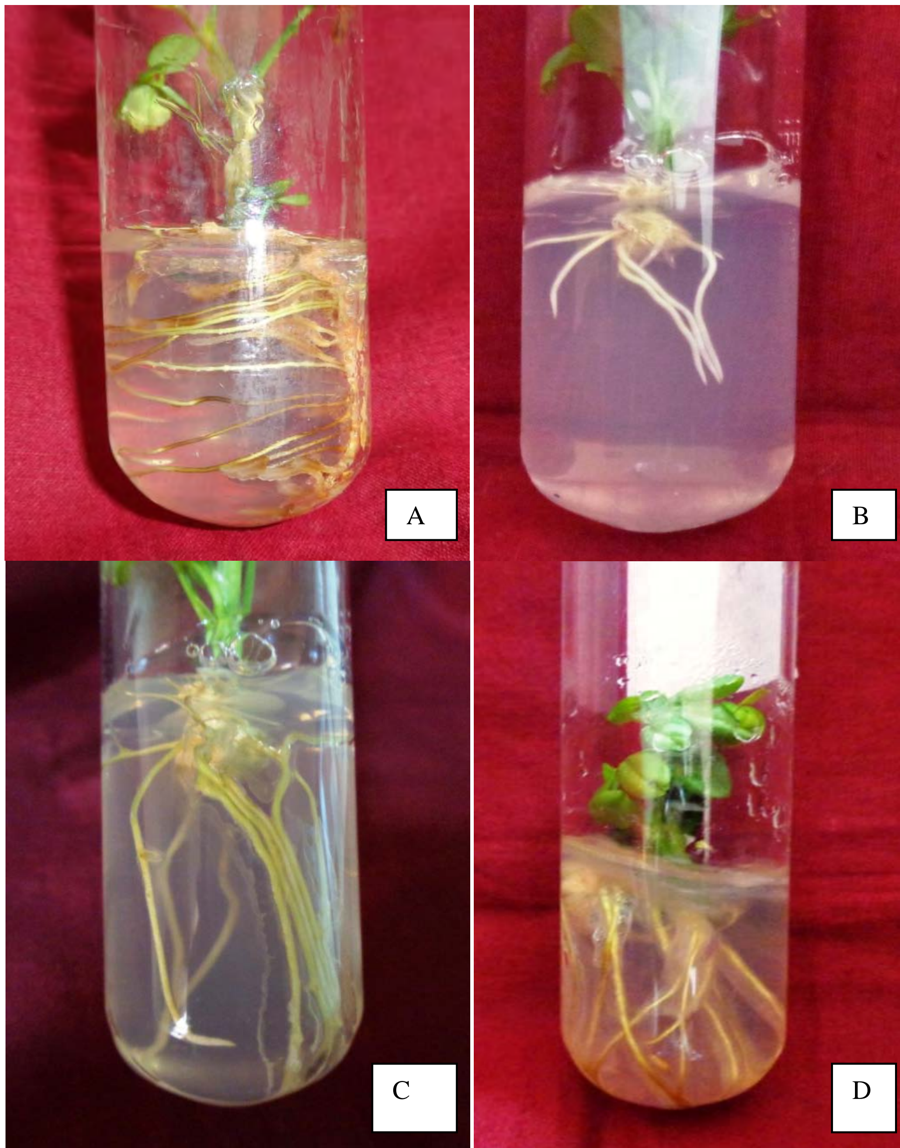

Figure 3.5 Rhizogenesis of shoot regenerated from decapitated half embryo following transformation after selection of peanut varieties A. BINA Chinabadam 2, B. BINA Chinabadam 3 with vector *pK7WG2_AtNHX1_1.6* ; C. BINA Chinabadam 4 and D. BINA Chinabadam 6 with vector *pK7WG2_OsNHX1_1.6*. Photographs were taken 30 days after root inoculation.

3.4 Transformation of Decapitate whole embryo

3.4.1 Effect of Optical Density (OD_{600}) on regeneration efficiency following transformation

With the *Agrobacterium* vector *pK7WG2_AtNHX1_1.6* four peanut varieties were subjected to infection under a range of optical density (OD_{600}) from 0.6 to 1.3 with 15 hours of incubation period. All the varieties gave good regeneration response at 1.0-1.3. Highest regeneration was found in BINA Chinabadam 3 (Table 3.7).

Similar to previous experiment when explants were infected with *pK7WG2_OsNHX1_1.6,* at the optical density (OD_{600}) 1.0-1.3 range gave better regeneration. Here maximum regeneration found in BINA Chinabadam 2 (Table 3.8).

For long duration of incubation period co-cultivation phase was omitted in decapitated whole embryonic explants to avoid overgrowth and to achieve better regeneration.

3.4.2 Transformation frequency of four peanut varieties from decapitated whole embryo on selection media

Following transformation, decapitated whole embryonic explants were placed on 200mg/l cefotexime and 50mg/l kanamycin containing media for primary screening. The survived explants then transferred to 100mg/l kanamycine containing media for selecting transformed shoot. The control nontransformed explants gave no regeneration under selection.

In presence of selection pressure following transformation with the vector *pK7WG2_At NHX1_1.6,* BINA Chinabadam 6 gave maximum number of shoots. But maximum rhizogenesis found in BINA Chinabadam (Table 3.9).

Alike previous vector, with *pK7WG2_Os NHX1_1.6* vector BINA Chinabadam 6 gave highest number of shoots among four varieties. BINA Chinabadam 3 and BINA Chinabadam 6 gave highest rhizogenesis with this vector (Table 3.9). After proper growth of root, plantlets were transferred to soil for acclimatization and hardening to be adjusted in the natural environment.

3.7 Effect of optical density (OD_{600}) and incubation period on regeneration efficiency following transformation with *Agrobacterium* vector *pK7WG2_AtNHX1_1.6*

Peanut Varieties	OpticalDensity (OD_{600})	Incubation Period (hrs)	Regeneration efficiency on selection media
BINA Chinabadam 2	0.6-0.8	15	51
	1.0-1.3		55
BINA Chinabadam 3	0.6-0.8	15	60
	1.0-1.3		66
BINA Chinabadam 4	0.6-0.8	15	53
	1.0-1.3		56
BINA Chinabadam 6	0.6-0.8	15	53
	1.0-1.3		61

Values were obtained from triplicate trials. Data were taken 30 days after infection. Regeneration occurred in presence of selection pressure of 100 mg/l kanamycin along with 200 mg/l cefotaxime.

3.8 Effect of optical density (OD_{600}) and incubation period on regeneration efficiency following transformation with *Agrobacterium* vector *pK7WG2_OsNHX1_1.6*

Peanut Varieties	Optical Density (OD_{600})	Incubation Period (hrs)	Regeneration efficiency on selection media
BINA Chinabadam 2	0.6-0.8	15	61
	1.0-1.3		64
BINA Chinabadam 3	0.6-0.8	15	55
	1.0-1.3		58
BINA Chinabadam 4	0.6-0.8	15	50
	1.0-1.3		53
BINA Chinabadam 6	0.6-0.8	15	58
	1.0-1.3		60

Values were obtained from triplicate trials. Data were taken 30 days after infection. Regeneration occurred in presence of selection pressure of 100 mg/l kanamycin along with 200 mg/l cefotaxime.

3.5 Development and acclimatization of regenerated plantlets from both explants in natural environment

Mature rooted plantlets were transplanted to soil in small pots and covered with perforated poly bags for adjustment procedure in natural condition. During this procedure maximum number of success was obtained in BINA Chinabadam 3 and minimum number attained by BINA Chinabadam 4.

Decapitated whole embryo took shorter period of time to develop into plantlet, on an average 79 days. On the other hand, plantlet regenerated from decapitated half embryo took approx 94 days for plantlet development (Table 3.10-3.13).

Then plantlets were transferred to natural environment in net house. But survival rate in natural condition was very poor. Plant growth was stunted and started to die after one and a half month of transplantation (Figure 9).

3.6 Bioassay

3.6.1 Leaf disk assay

Salinity stress tolerance level of these varieties was assessed to confirm transformation. Effect of salinity on peanut leaf disk has been analyzed within time period 14 days and bleaching of leaf disk was the indicator of change.

Comparison done between transformed plant and control plant leaf disk. It showed reciprocal relation of bleaching between transformed plant and control plant. Bleaching rate increased with the increase of NaCl concentration. At 100mM control disk started to bleach in two days. But in 5mM it took 11 days to bleach (Table 14) (Figure 10).

On the other hand, transformed plants remain green up to 14 days at the concentration of 50mM (0.5ds/lm). Bleaching of the disk started at the day 13 at 100mM. Leaf disk remain green within this duration, no sign of bleaching observed. Even bleaching rate not increased with the increases of NaCl concentration. This indicates successful transformation of salt tolerant gene in peanut genome.

3.6.2 Salinity stress test on transformed shoots

In this study shoot growth and root development was also observed in putative transformed shoots under salinity stress in root regeneration hormone. Shoot death and bleaching rate increased with the increase of NaCl concentration in nontransformed control shoots.

Whereas transformed shoots were full green up to 14 days and some of them gave normal root during this test. This confirms the transformation along with antibiotic selection (Table 15); (Figure 11).

3.7 Comparative analysis of facts observed in two explants transformation

In present study, *Agrobacterium* mediated transformation done with two explants, decapitate half embryo and decapitate whole embryo. A comparison to both peanut explants indicated variations in transformation efficiencies and regeneration of transformants.

Initially, variations were observed during optimization of different transformation parameters. Optical Density (OD_{600}) found same for both the explants. But co-cultivation and infection time varied among explants. Co-cultivation phase avoided and lengthen infection time used in decapitated whole embryo transformation (Table 16).

Analysis also done by measuring different parameters like, shoot regeneration, yield of transformed shoots and plantlet development etc. Transformation of decapitated half embryo found to be time consuming in plantlet development and required a large number of explants in transformation. In compare to that decapitate whole embryo found to give putative transformed plantlet in short time with high percentage of transformants.

To sum up, explants decapitate whole embryo stands over decapitate half embryo in *Agrobacterium* mediated transformation.

3.9 Regeneration of putative transformed plantlets from decapitated whole embryo under selection

Peanut varieties	Mean number of shoots under selection 100 mg/l of kanamycine ± SD		Percentage of root induction (%)		Survival rate of plants in natural environment (%).
	pK7WG2 _AtNHX1_1.6	*pK7WG2 _OhNHX1_1.6*	*pK7WG2 _AtNHX1_1.6*	*pK7WG2 _OhNHX1_1.6*	
BINA Chinabadam 2	2.3±0.57	2.3±0.57	62	59	30
BINA Chinabadam 3	2.4±1.0	2.2±0.81	63	65	40
BINA Chinabadam 4	2.1±1.15	2.0±0.5	66	62	33
BINA Chinabadam 6	2.6±0.57	2.5±0.816	57	65	36

Mean number of putative transformed shoots came from the average number of multiple shoots on selection. Mean value in each experiments average of three sets of experiment with 50 decapitate whole embryo explants in each set. Data were collected 50 days after inoculation of explants.

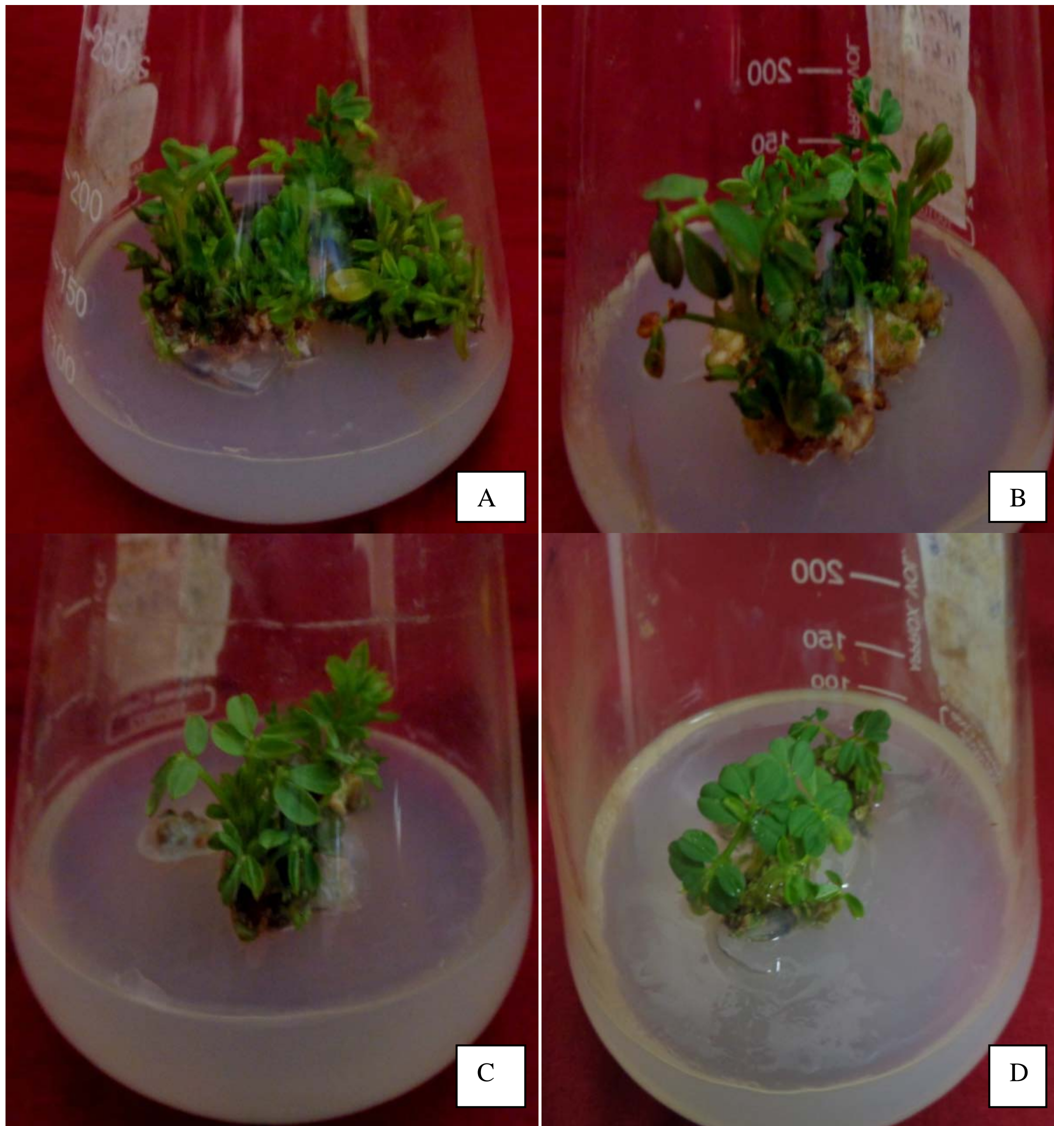

Figure 3.6 Regeneration response of decapitated whole embryonic explants putative transformed shoots under selection with *pK7WG2_AtNHX1_1.6* on kanamycin selection media (100mg/l) has shown in A. BINA Chinabadam 2, B. BINA Chinabadam 3, C. BINA Chinabadam 4, D. BINA Chinabadam 6 (Photographs were taken 60 days after following infection).

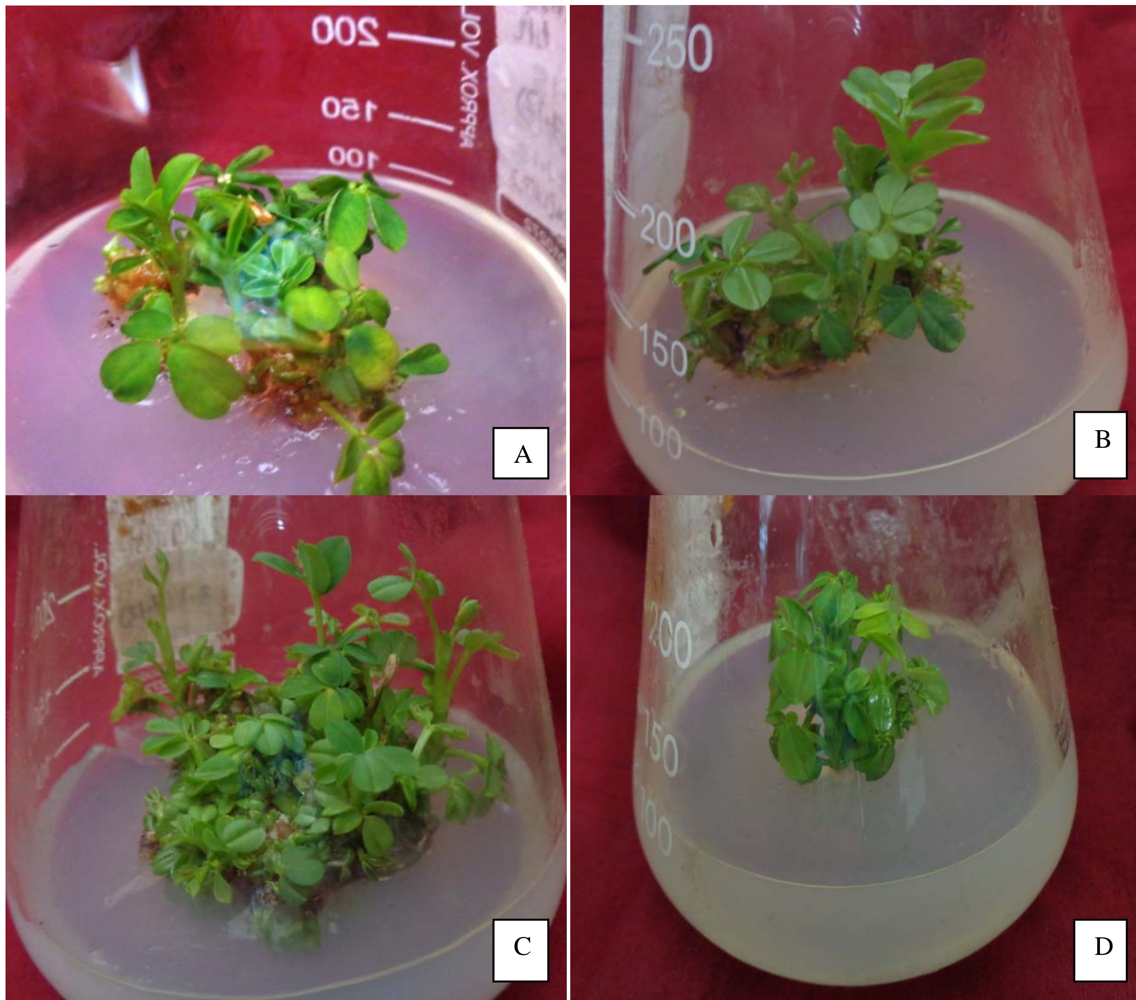

Figure 3.7 Regeneration response of decapitated whole embryonic explants putative transformed shoots under selection (with *pK7WG2_OsNHX1_1.6*) on kanamycin selection media (100mg/l) has shown in A. BINA Chinabadam 2, B. BINA Chinabadam 3, C. BINA Chinabadam 4, D. BINA Chinabadam 6 (Photographs were taken 60 days after following infection).

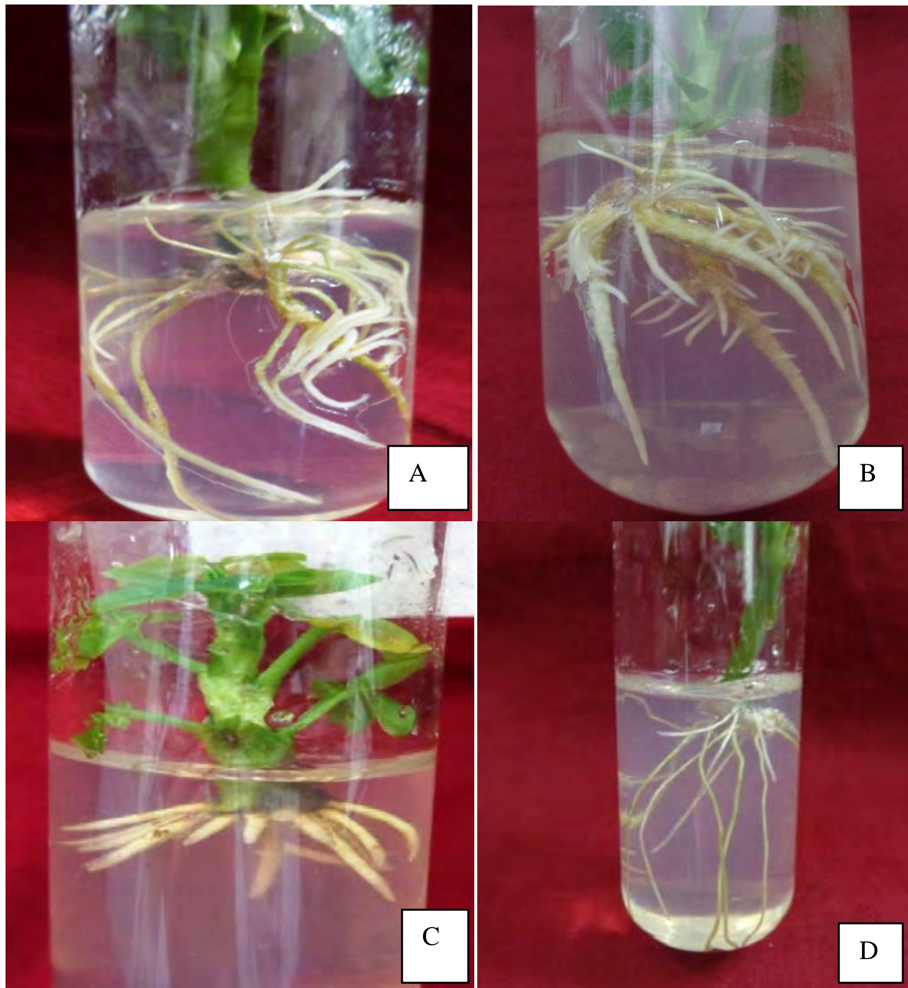

Figure 3.8 Rhizogenesis of shoot regenerated from decapitated whole embryo following transformation after selection of peanut varieties A. BINA Chinabadam 2, B. BINA Chinabadam 4 with *pK7WG2_AtNHX1_16*. C. BINA Chinabadam 4 and D. BINA Chinabadam 6 with *pK7WG2_OsNHX1_1*. Photographs were taken 30 days after root inoculation.

3.10 Time required for plantlet development with decapitated half embryo with vector *pK7WG2_AtNHX1_1.6*

Peanut varieties	Time required for regeneration initiation(d)	Time reqired for shoot development(d)	Time required for root development(d)	Total time requirement for plantlet development(d)
BINA chinabadam 2	20.75±0.95	39.5±0.55	31.25±0.95	91.5
BINA chinabadam 3	21±0.5	38.75±0.95	33±0.83	92
BINA chinabadam 4	23±0.81	40.5±0.53	29.75±0.9	93.5
BINA chinabadam 6	25±0.57	41.5±0.5	31.75±0.9	97.5

Values were obtained from three independent trials of the four peanut varieties with the specified vector. Experiment conditions were optical density of 1.0-1.3, incubation time 90 min and 48 hrs of co cultivation.

3.11 Time required for plantlet development with decapitated half embryo with *pK7WG2_OsNHX1_1.6*

Peanut varieties	Time required for regeneration initiation(d)	Time reqired for shoot development(d)	Time required for root development (d)	Total time requirement for plantlet development(d)
BINA chinabadam 2	18.5±0.95	40.25±0.95	30.5±0.57	89.5
BINA chinabadam 3	22±0.81	39.5±.57	33.5±0.58	95
BINA chinabadam 4	22.5±1.29	39±.81	30.5±0.55	92
BINA chinabadam 6	25.75±0.5	40.5±1.29	32.5±0.53	98.75

Values were obtained from three independent trials of the four peanut varieties with the specified vector. Experiment conditions were optical density of 1.0-1.3, incubation time 90 min and 48 hr co cultivation.

3.12 Time required for plantlet development with decapitated whole embryo with *pK7WG2_AtNHX1_1.6*

Peanut varieties	Time required for regeneration initiation(d)	Time reqired for shoot development(d)	Time required for root Development(d)	Total time requirement for plantlet development(d)
BINA chinabadam 2	14±0.81	36±0.82	33.5±0.57	83.5
BINA chinabadam 3	13±0.83	31.5±0.57	30.5±0.53	75
BINA chinabadam 4	15.5±0.57	33±0.8	28.5±0.58	77
BINA chinabadam 6	14.5±0.50	36±0.79	30.5±0.5	81

Values were obtained from three independent trials of the four peanut varieties with the specified vector. Experiment conditions were optical density of 1.0-1.3, incubation time 15hr.

3.13 Time required for plantlet development with decapitated whole embryo with *pK7WG2_OsNHX1_1.6*

Peanut varieties	Time required for regeneration initiation(d)	Time reqired for shoot development(d)	Time required for root development (d)	Total time requirement for plantlet development(d)
BINA chinabadam 2	12.5±0.57	34.25±0.95	34.5±0.57	81.5
BINA chinabadam 3	13.75±0.5	33.75±0.5	30.5±0.5	78
BINA chinabadam 4	17.5±0.53	34.5±0.53	27.5±0.8	79.5
BINA chinabadam 6	15.5±0.55	37.5±0.57	30.5±0.83	83.5

Values were obtained from three independent trials of the four peanut varieties with the specified vector. Experiment conditions were optical density of 1.0-1.3, incubation time 15hr.

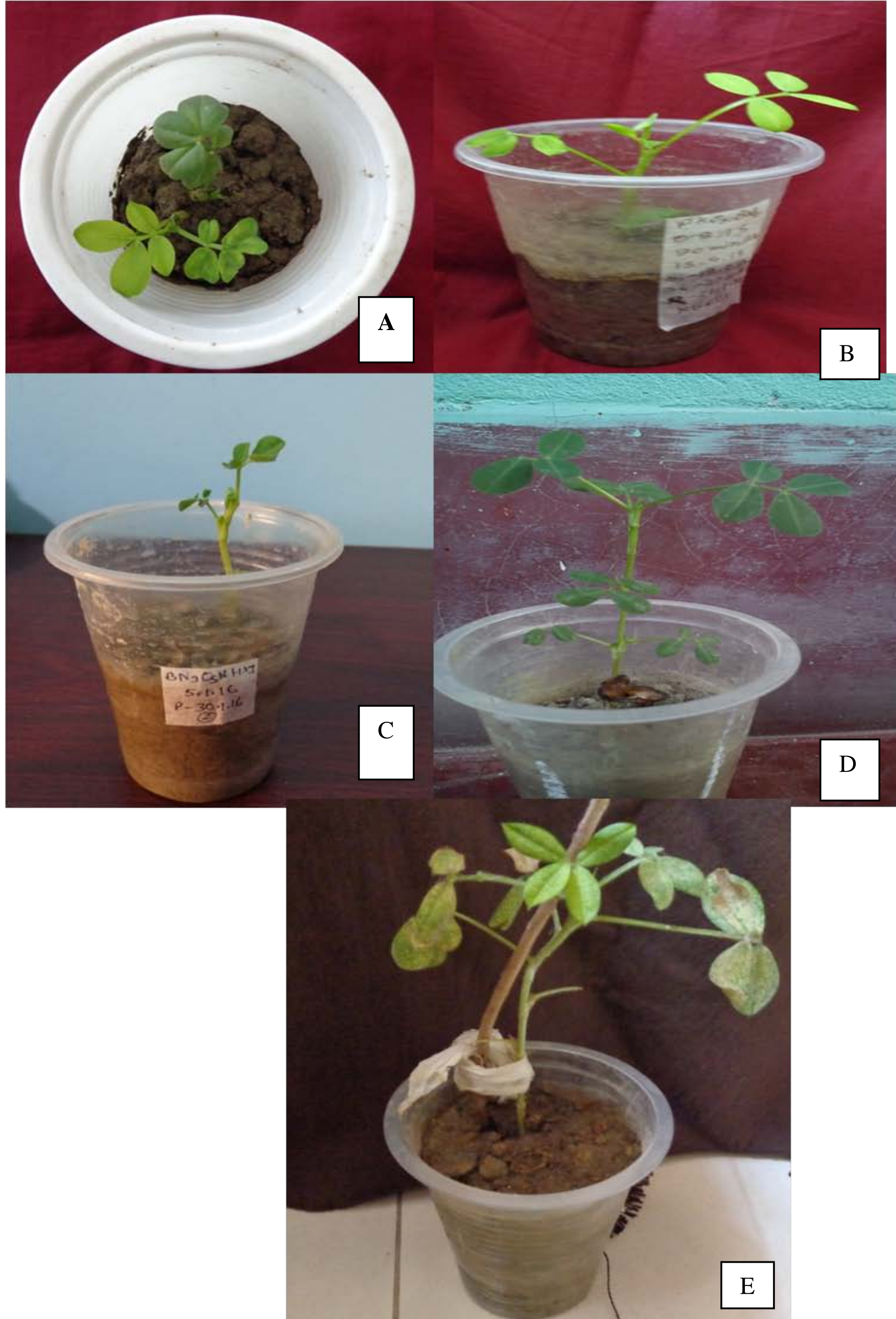

Figure 3.9 Acclimatization of plantlets to natural environment A.BINA Chinabadam 2 with *pK7WG2_AtNHX1_1.6*, B.BINA Chinabadam 6 with *pK7WG2_OsNHX*1_1.6, C.BINA Chinabadam 3 with *pK7WG2_OsNHX1_1.6*, D.BINA Chinabadam 4(control), BINA Chinabadam 4 with *pK7WG2_AtNHX1_1.6* .

Table 3.14 Effect of salinity on peanut leaf discs of putatively transformed seedlings

Salt concentrations (mM)	Visual appearance of the control plant	Time needed to change (d)	Visual appearance of the transformed plant	Time needed to change (d)
0mM	Normal	Not bleached	Normal, green	Not bleached
5mM	Bleached	11 days	Normal, green	14 days
10mM	Bleached	9 days	Normal, green	14 days
20mM	Bleached	7days	Normal, green	14 days
50mM	Bleached	5 days	Normal, green	14 days
100mM	Bleached	2 days	Started to bleach	13 days

Table 3.15 Effect of salinity on peanut shoots of putatively transformed seedlings

Salt concentrations (mM)	Visual appearance of the control plant	Time needed to change (d)	Visual appearance of the transformed plant	Time needed to change (d)
0mM	Normal	Not bleached	Normal, green	Not bleached
5mM	Bleached	14 days	Normal, green	14 days
10mM	Bleached	9 days	Normal, green	14 days
20mM	Bleached	7days	Normal, green	14 days
50mM	Bleached	5 days	Normal, green	14 days
100mM	Bleached	3 days	Started to bleach	14 days

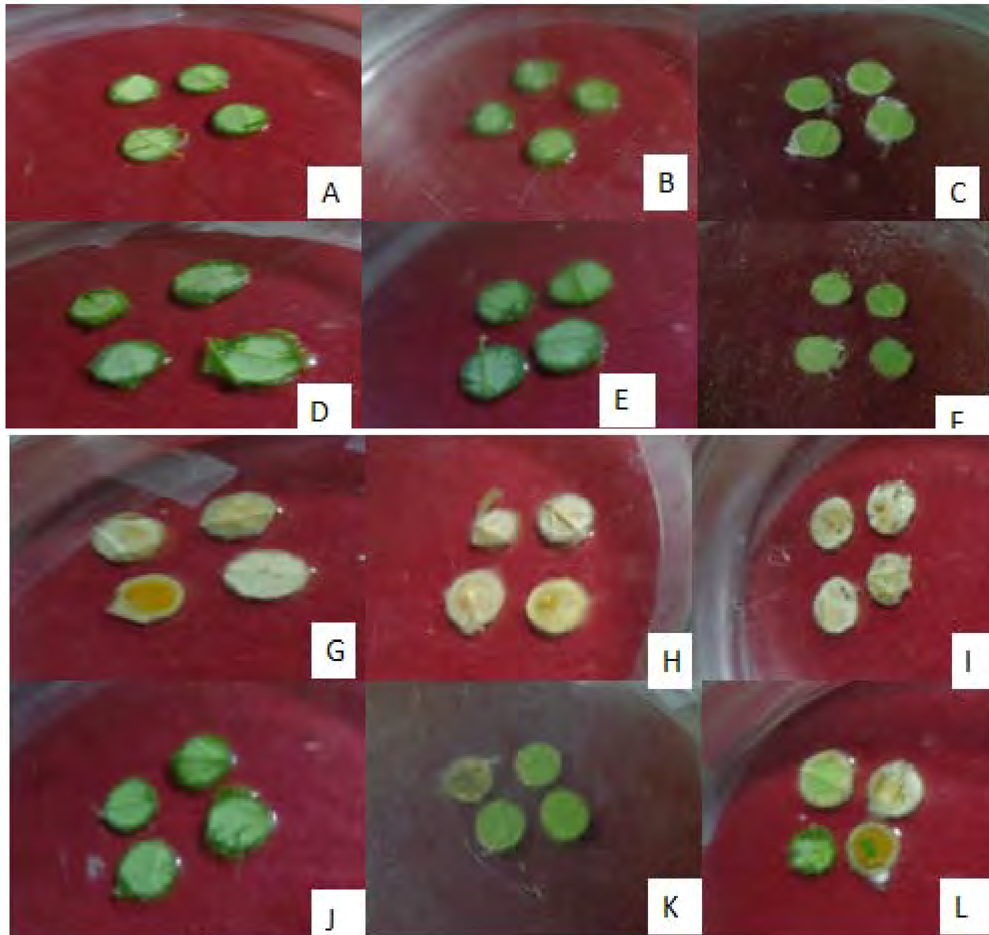

Figure 3.10 Effect of salinity on peanut leaf disk after 14 days; A. 0mM control, B. 5mM control, C. 10mM control, D.0mM transformed, E. 5mM transformed, F.10mM transformed, G. 20mM control, H.50mM control, I .100mM control, J. 20mM transformed, K. 50mM transformed, L. 100mM transformed.

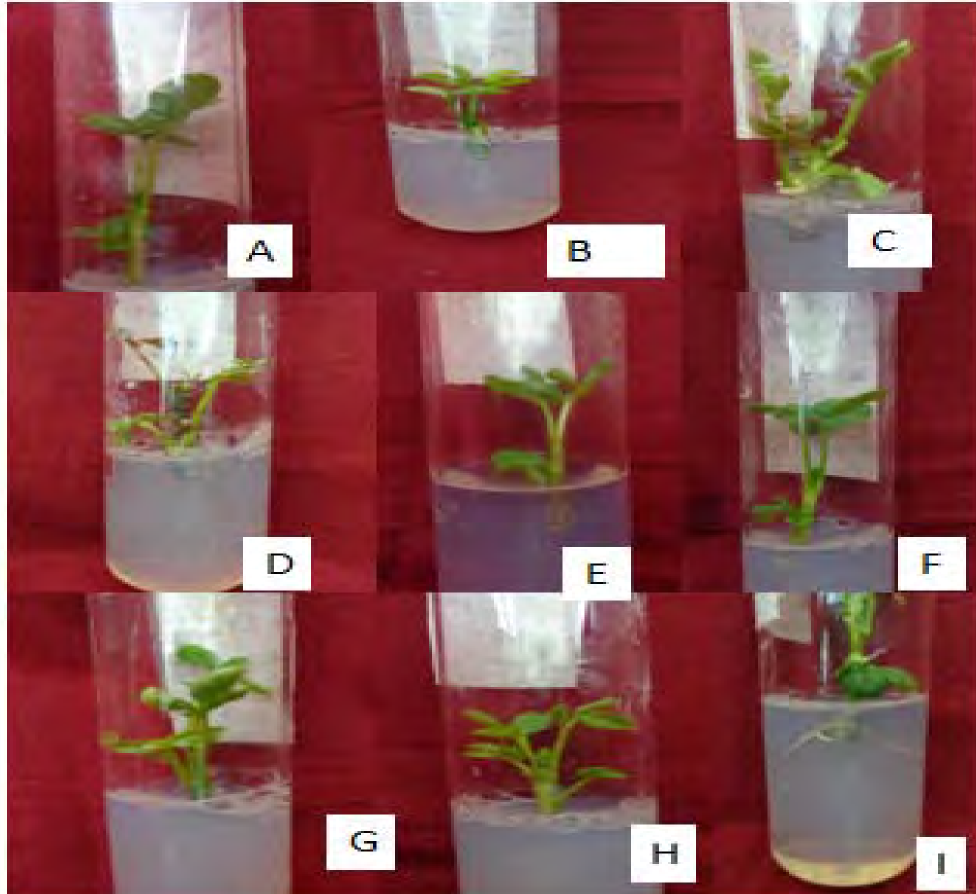

Figure 3.11 Effect of salinity on peanut shoots after 14 days; A.0mM control, B. 5mM control, C. 10mM control, D. 20mM control, E. 10mM transformed, F. 20mM transformed, G. 50 mM transformed, H. 100mM transformed, I . Rooting of transformed plant at 50mM.

3.16 Comparative analysis of facts observed in two different Explants transformation

Categories	Explant	Explant
Explant	Decapitate half of the embryo	Decapitate whole embryo
OD_{600}	1.0-1.2	1.0-1.3
Incubation time	90 min	15 hr
Co-cultivation period	48 hours	Nil
Number of regenerated shoot	2.50±0.8	2.6±0.57
Time required for shoot development	58days	55 days
Highest regeneration efficiency in selection (%)	67	66
Time required for plantlet development	93 days	79 days
Time required to get transformed plant	No plantlet regenerated	65-70 days
Bioassay	Antibiotic selection 50 mg/l kanamycin	GUS assay, antibiotic selection, leaf disc senescence assay
Salt tolerance	Not tested	100 mM NaCl
Responsive varieties	BINA tomato 2, BINA tomato 3	BINA tomato 2, BINA tomato 3

Discussion

Trait development of peanut, through tissue culture has a long history. Both organogenesis and embryogenesis applied in the developmental process.

A number of explants reported in peanut culture like leaf lets(Chang *et al*.,1996, Sarker and Islam 1999), petiol, cotyledons (Lacorte, 1999, Bhatnagar *et al*., 2010), epicotyls and hypocotyle (Marion *et al*., 2008), cotyledonary node (Srinivasan *et a.l*,2010), auxiliary meristem (Sing and Hazra, 2009), different stages of anther(Saxena *et al.,* 1999).Leaflet explant frequently reported explants in peanut culture. But result of culture with this explants adversely depend on leaf age, cut size, cutting place. In present study experiment has been carried out with two explants - decapitated half embryo and decapitated whole embryo. These explants were also reported in Honey *et al.,*(2010), Vasil, (1986), Somer *et al.,* (2003). These explants also used in pea Schroeder *et al.,* (1993), Chick pea and lentil (Jayanand *et al*., 2003, Tewari sing *et al*., 2004).

4.1 Optimization of transformation parameters (Optical Density, Infection Time, Co Cultivation Period)

Transformation frequencies proportional to its parameters like bacterial concentration, inoculation time, co cultivation etc (Zia et al.2010). A range of bacterial density is used to conduct studies to find out optimal bacterial density for transformation. In this study better result found in OD_{600} 1.0 in the range of OD_{600} (0.6-1.3) for both explants. Lori A.*et al*., (1994) and Rohini and Rao (2000) also used bacterial density more than 1.0 in their transformation experiment for better result. Similar result also found in Thomas *et al.,* (1993) for lentil , S.prakash *et al*., (1998) for peanut, Zhang *et al.,* (1997) for common bean.

Infection period is very crucial for transformation frequency. Time length beyond and beneath the optimal limit shows decrease in efficiency (Sarker *et al*., 2000;Oliveria *et al*., 2003). Sarker and Islam (1999) found 60 min infection duration optimal for leaflet explant. Here 90 min infection time found better for decapitated half embryo despite 60 min of infection time. But in case of decapitated whole embryo infection time rise up to 15hrs, Which resembles to Chi(1987),Valvekens *et al.,* (1988),and Rohini and Rao (2000) .

Co-cultivation period 48 hrs showed better result here, which is similar to Montagu *et al.,* (1988), Egnin *et al.,*(1998), Venkatachalam *et al.*(,2000). Warketin *et al.,* (1992) and Alan M.Lloyd *et al* (1993) got better result in same co cultivation duration. But Sarker(2000), Anuradha *et al*.,(2006) showed longer length, 3day of co cultivation. John M. Sherman(2003) also showed 3 day of co cultivation duration in their experiment infection time was lower than present study. It may state that shorter infection period with longer co-cultivation period for better transformation frequency.

4.2 Application of antibiotics

For the eliminations of excess bacteria and non transformed plants from the study two type of antibiotic usually used bacteriostatic and bacteriocidal antibiotic. In present study 200 mg\l cefotaxim was used in selection media for all peanut varieties. It worked as a bacteristatic antibiotic, prevented bacterial over growth without compromising regeneration potential. Mathaias(1986), Borrelli and Di Fonzo(1992), Rafael *et al.,(*2005) and Amugune and Anyango(2011) used cefotaxim as bacteriosatic agent in their experiment.

pK7WG2_AtNHX1_1.6 and *pK7WG2_OsNHX1_1.6* ,both vector contains kanamycin resistance gene. Kanamycine is the mostly reported bacteriocidal antibiotic in transformation event. In this study 100 mg/l kanamycine used as optimal concentration for selection of transformed plant. Susan Eapen *et al.,* (1994) and P.Venkatachalam *et al.,* (2000) used 75 mg/l kanamycine as optimal concentration for cotylidonary node transformation. D.Valvekens *et al.,(*1988), S. Prakash *et al.,* (1998), Rohini and Rao (1999) and K. Sharma *et al*.,(2000) used 125mg/l kanamycin on their experiment for cotylidonari node,leaflet explants.According to Mathews,(1988) and Dekeyser and coles (1989) optimal concentration of kanamycine varies with plant species, explants age and type.

4.3 Shoot regeneration

Shoot regeneration numbers slightly differ in two explants. Highest mean shoot number got in BINA Chinabadam 6 (2.7±0.81). After transformation which shoots continued to grow under selection pressure of kanamycin for one month state putatively transformed plant.

4.4 Rhizogenesis and Transplantation

All three Auxin (IAA,NAA, and IBA) reported to give positive response and gave healthy roots. Sarker and Islam (2000) and Akhter (2006) found best result with IAA. Susan (1993), Kiran(2000)Venkatachalam(2003) got best response with NAA. Auxin preference depends upon genotype of peanut. Hormone IBA was reported to give better response for local BARI and BINA varities (honey et al., 2003) .Kavipriya and Venkatachalam also got better bresult with IBA hormone in different concentrations.

4.5 Determination of baseline salinity tolerance test

Salt stress affects crop germination rate, plant growth, development and yield (Ifediora,2014). Before transformation natural germline salt stress tolerance were tested. High concentration of salt in media alters osmotic potential, causing more energy drain, how salinity affects germination (Shahid et al., 2011). Osmotic potential cease water uptake and Na^+ and Cl^- ions toxic effects influences on seed germination Turhan and Ayaz (2004). Germionation rate is more susceptible than germination percentage to saline stress Khodadad et al,.(2012).Thet found germination rate lowers with the increase of salt concentration in Sun flower. Germination rate of okra al;so decreases with higher concentration of salt (parve3z et al,. 2011) ,which resembles to this study.

4.6 Marker gene selection

In this study both vector contains nptII marker gene which stands for neomycin phosphotransferase gene. It gives transgenic plant resistance against kanamycine. nptII gene is the sequence that encodes the enzyme having the ability to inactive kanamycin (Suratman and Ughude, 2013). So when plants of experiment exceeds optimal level of kanamycin susceptibility; then the plant called transgenic plant. Present study that thresh hold level was 100mg/l kanamycine. Those plant that grown on medium containing 100 mg/l kanamycin were containing nptII gene, in other words they are transformed plant. Use of nptII gene showed in lots of reports some are- K. Sharma(2000),
P.Venkatachalam(2000), S. Prakash *et al*(1998),D. Valvekens *et al* (1988). In other crops use of nptII gene also reported like in safflower (Rohini and Rao,2000),marula(Margaret *et al.,*2004),chili (Ismail *et al*.,2005), chick pea (Reddy *et al.,* 2007), cotton (Rohini Rao,2008), pigeon pea (Rohini and Rao, 2008), soybean (Zia *et al.,*2010) and common bean (Amugune *et al.*,2011) etc.

4.7 Salt tolerance analysis through leaf disk senescence assay

For testing salt tolerance potential leaf disk senescence assay done in both transformed and non transformed plant according to Fan *et al* (1996). Bleaching as weel as clorosis rate of leaf disk observed for 14 days in presence of different concentration of NaCl ranging from 5mM- 100mM. Leaf disk of control plant showed extensive and quick bleaching whereas the transgenic line took longer time and less demage occure. Seraj *et al.,* (2015) found no bleaching up to 12 days at the concentration of 200mM in Bangladeshi rice varity-Binnatoa and BR-49. Leidi *etal.,(*2010) found salt tolerance up to 100mM in tomato. Use of leaf disk assay for other transgene also reported.Nguyent *et al.,(*2011) used it of detecting antifungal gene. Manvella *et al.,* (2009) used it for detecting *HAHB4* gene.

4.8 Salt tolerance analysis on shoot development and rhozogenesis

Shoot and root growth under salt stress also examined in this study in putative transgenic plant and control plant. Shoot death and bleaching rate increased with the increase of NaCl concentration in control shoots. Sopory *et al,*. (2003) found normal growth of root and shoot in transgenic tobacco plants up to 150mM of NaCl. Zhang *et al.,* (1997) found normal growth up to 120mM NaCl stress.

4.9 Transformation frequency

In present study highest transformation frequency 66% was obtained by BINA Chinabadam 3 with vector pK7WG2_OsNHX1_1.6 based on regeneration percentage of explants on selection media. Similar result found by M. Lloyd *et al*., (2010) 60% for leaf explants. Higher percentage of transformation efficiency also found in K.Sharma *et al*.,(200) 55% for cotyledon explants, P.Venkatachalam *et al*(2000) 47% for cotyledon explants. Rohini and Rao (2000) found only 33% GUS positive plant by non tissue culture based transformation procedure. Lori A.(1994) found 25% transformation frequency with leaf explants. S.prakash(1998) found 25% transformation frequency for cotyledon explants.

Significant progress has been made towards developing salinity tolerant plant. Abiotic stress is a multigenic trait, but reports suggested that salt tolerance plant could be produced by transfer of single gene through transgenic approaches(Zhang *et al.,* 2011;Kasuga *et al.,* 1999; Saijo *et al.,* 2002). The NHX gene works against salinity by maintain ion and osmotic homeostasis (Khan, 2011). The expression of NHX gene was upregulated by

salinity in cotton (Wu *et al.,* 2000), rice (Fukuda *et al.,* 1999;Seraj *et al.,* 2012), Wheat(Saqib *et al.,* 2005). Present work also found that insertion and expression of Na^{+}/H^{+} antiporter gene in peanut improved tolerance to salt stress, the effect of salt treatment on leaf disk was also observed by monitoring phenotypic changes. Wild type plants display progressive chlorosis, reduced leaf area and general growth inhibition when treated with high salt concentrations in compare to transformed plants. Moreover these explants allowed the transformation to be targeted to regeneration competent tissue. This transformation protocol is cost effective and can be routinely used in studies of gene expression. In future development of transgenic salt tolerant peanut will bring a big impact in crop productivity in this semi arid tropics specially in Bangladesh. In which this could be first step.

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