# STUDY OF *IN VITRO* REGENERATION AND TRANSFORMATION PARAMETERS FOR THE DEVELOPMENT OF TRANSGENIC TOMATO (*Solanum lycopersicum* L.)


A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN

PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE IN BIOTECHNOLOGY

Submitted by

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# DECLARATION

I hereby solemnly declare that the research work embodying the results reported in this thesis entitled **"Study of *in vitro* regeneration and transformation parameters for the development of transgenic tomato (*Solanum lycopersicum* L.)"** submitted by Fahima Akter Mukta 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.

**Certified by:**

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Fahima Akter Mukta

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# ABBREVIATIONS

| | |
|---|---|
| *A.* | *Agrobacterium* |
| BAP | 6-Benzylaminopurine |
| CaMV35S | Cauliflower mosaic virus 35 S |
| Conc. | Concentration |
| DAT | Day after transplantation |
| DNA | Deoxyribo nuclic acid |
| EDTA | Etylene diamine tetra acetic acid |
| gm | Gram |
| GUS | β glucuronidase |
| h. | Hour |
| Ha. | Hectare |
| hpt | Hygromycin phosphotransferage |
| Fig | Figure |
| 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 |
| NAA | Napthalene acetic acid |

| | |
|---|---|
| NaOH | Sodium Hydroxide |
| $Na_2$-EDTA | Sodium salt of Ferric Ethylene Diamine Tetra Acetate |
| Ng | Nano gram |
| Nos | Nopaline synthetase |
| *npt* II | 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-β-D-glucuronide |
| YMB | Yeast Manitol Broth |
| µl | Micro litre |
| µM | Micro Mole |

# ABSTRACT

Investigation was carried out to develop an *in vitro* regeneration protocol using three locally grown farmer popular tomato varieties namely BARI Tomato 2, BARI Tomato 9 and Bahar. Later *Agrobacterium* mediated transformation study was performed to find out the best transformation parameters using these three varieties along with BARI tomato 7 variety. Tomato seeds were sterilized with 24 hours continuous shaking as it took less time for germination and cause less damage to embryo. During *in vitro* culture BARI tomato 2 and BARI tomato 9 variety showed better response in presence of 1 mg/l BAP (germination percentage 96%, 13 days for germination initiation and 24.8 shoots/explant and germination percentage 93%, 12.6 days for germination initiation and 19 shoots/explants respectively). Bahar variety respond well in 1 mg/l BAP + 0.1 mg/l IAA containing MS media combination (germination percentage 100%, 10 days for germination initiation and 39.6 shoots/explant). All three varieties showed cent percent rooting response in ½ MS + 0.2 mg/l IAA. However, there was no significant difference in rooting response in relation to shooting media. Seeds collected from well ripened fruits of *in vitro* regenerated plants showed 95-100% viability in germination test. Thus, indicates the reproducibility of the protocol. Transformation efficiency was found to be increased with the increase of optical density of *Agrobacterium* suspension. All the varieties gave 80-100% GUS positive expression in GUS assay at 0.62 $OD_{600}$ and minimum 47% was found in BARI tomato 9 variety with 0.49 $OD_{600}$. In the present study 20 minutes of incubation period with higher $OD_{600}$ (0.62) gave better transformation in BARI tomato 2, BARI tomato 7 and BARI tomato 9 varieties. Interestingly, Bahar variety produced highest number of GUS positive explants (98%) in 30 minutes incubation period at similar OD. Among the co- cultivation periods 48 hours was found best for all four varieties in transient GUS assay. The highest regeneration percentage in presence of antibiotic (kanamycin, 150 mg/l) following transformation with pBI121 found to be 56% in Bahar variety. Again, when transformation was done by pH7WG2_OsNHX1_1.6 highest regeneration was achieved (23%) in Bahar variety when cultured in hygromycin, more than 4 mg/l.

# CONTENT

# LIST OF TABLES

# LIST OF FIGURES

# Chapter 1
# Introduction

## 1.1 General overview of tomato:

Botanically tomato belongs to family Solanaceae and named scientifically as *Solanum lycopersicum* L. It is recognized as a highly valuable and nutritious food. It is the second most popular vegetable crop next to potato in the world (Bhatia *et al*., 2004; Foolad, 2004). This family also includes chili, peppers, potato, eggplant etc. Like all known species of the genus Solanum, tomato is a diploid, it has 2n=24 chromosomes, and a genome size of 950 Mbp, which is composed of 77% heterochromatin and 23% euchromatin (Peterson *et al*., 1996). Tomato plants are vines typically growing six feet or more above ground if supported. Most tomato plants have compound leaves, the leaves are 10-25 centimeter (4-10 inc) long odd pinnate, with 5-9 leaflets on petioles, each leaflet up to 8 centimeters (3 inc) long, with a serrated margin, both the stem and leaves are densely glandular–hairy. Their flowers appear on the apical meristem and self-fertilizing. The flowers are 1-2 centimeters (0.4-0.8 Inc) across, yellow, with five petioled lobes on the corolla; they are borne in cymes of 3 to 12 together. Tomato fruit is classified as berry. The fruit is edible, bright colored, soft and succulent. Fruit size is generally 1-2 inches diameters in wild plants and commonly much larger in cultivated form (Islam, 2007).

## 1.2. Origin and distribution:

The tomato is native to South America. Genetic evidence shows that the progenitors of tomatoes were herbaceous green plants with small fruit and in the high land of Peru is the centre of diversity (Cox, 2000; Smith and Andrew, 1994). Tomato is growing in tropical, sub-tropical and temperate areas (Atherton and Rudich, 1986). The wild cherry tomato species was transported to Mexico, where it was grown and consumed (Sink and Reynolds, 1986). Spanish explorers introduced tomato to Europe in the 1500s. European took the tomato to China, and South and South East Asia in the $17^{th}$ century (Siemonsama and Piluek, 1993). At present tomatoes grow widely in China, USA, Turkey, Russia, Egypt, India, Spain, Mexico and many other countries of the world.

### 1.3. Uses and nutrient content:

Tomato is achieving tremendous popularity as vegetables. It can be used in various ways. Green and ripe tomato used as vegetables, ripe tomato also used as salad, sauce, jam, ketchup, pickle etc. Tomato is very nutritious and beneficial for our health. They are also very low in any fat contents and zero cholesterol levels. None the less they are excellent source of antioxidants, dietary fibers and minerals. Because of their all round qualities, dieticians and nutritionists often recommended them to be included in cholesterol controlling and weight reduction programs. The antioxidants present in the tomatoes are scientifically found to be protective against cancers including colon, prostate, breast, endometrial, lung, and pancreatic tumors etc. (Rudrappa, 2009). It is an excellent source of vitamins and other minerals (Bose and Som, 1990). Well ripen tomato contains 94.2 gm water, 23 cal energy, 1.00 gm calcium, 7.00 gm magnesium, 1000 IU vitamin A, 22.00 mg ascorbic acid, 0.09 mg thiamine, 0.03 mg riboflavin and 0.80 mg niacin per 100 gm fresh weight (MacGillivery, 1961).Vitamin A deficiency in humans represents a global health problem affecting approximately one third of the countries of the world (Mayer *et al.*, 2008). Every year, 30 thousand children are affected by night blindness due to the deficiency of vitamin A in Bangladesh (FAO, 2002). They can get 7% of total vitamin A from tomato as cheap source of vitamin (Begum and Mia, 1993).

### 1.4. Status of tomato production in the world:

In 2011, about 150 million tons of tomatoes were produced in the world in 47,51,530 Ha of land with an average yield 3,35,359 Kg/ha (FAOSTAT, 2011). Tomatoes are the world's 2$^{nd}$ important crop in terms of production. At present, it is growing in more than 120 countries of the world. China is the largest producer followed by United States and India.

### 1.5. Status of tomato production in Bangladesh:

In Bangladesh, tomato is grown usually in winter. At present, some tomato varieties are grown extensively in summer while some are grown in both the seasons. The demand of tomato is increasing day-by-day in the agro-and food industries of Bangladesh. In

Bangladesh 17,813.8 ha of land was under tomato cultivation that produces 1, 20,000 metric tons having an average yield 6.7 metric tons per hectare (BBS, 2010).The average yield of our country is quite low as compared to other leading tomato producing countries.

**1.6. Constrains of tomato production:**

The tomato cultivation area is increasing day-by-day. This has lead to increased farmers income. However, complexes of pest and diseases and environmental stress as well as post harvest loss threaten the stability of the production. There are more than 200 pathogens, like, fungi, bacteria, virus, nematode etc. that cause tomato disease (Watterson, 1986). Fungal diseases (especially, early blight, late blight and *Fusarium* wilt), bacterial diseases (bacterial wilt, bacterial spot) and viral diseases (tobacco mosaic virus, leaf curl, spotted wilt, etc.) are a serious problem in several countries. Tomato is sensitive to a number of environmental stresses, especially extreme temperature, salinity, drought, excessive moisture and environmental pollution. High temperature can causes significant losses in tomato production due to reduced fruit setting, and smaller and lower quality fruits (Stevens and Rudich, 1978). High temperatures causing fruit set failure in tomato; this includes bud drop, abnormal flower development, poor pollen production, ovule abortion and poor viability, reduced carbohydrate availability, and other reproductive abnormalities (Hazra *et al.,* 2007). In addition, significant inhibition of photosynthesis occurs at temperatures above optimum, resulting in considerable loss of potential productivity. Salt stress is reflected in loss of turgour, growth reduction, wilting, leaf curling and epinasty, leaf abscission, decreased photosynthesis, respiratory changes, loss of cellular integrity, tissue necrosis, and potentially death of tomato plant (Jones, 1986; Cheeseman, 1988). Flooded tomato plants accumulate endogenous ethylene that causes damage to the plants and flooding with rising temperatures cause rapid wilting and death of tomato plants (Drew, 1979; Kuo *et al.,* 1982). There is a need to develop varieties that can withstand such environmental stress. Apart from environmental stresses, tomato yield is also affected breeding techniques, varieties, growth habit, etc.

**1.7. Alternative way of regeneration:**

Tomato has high degree of self pollination and non availability of suitable germplasm. Moreover, it is a per-dominantly inbreeding species and its genetic variation tends to decrease. So, these problems hamper to improve tomato characters through conventional breeding program. Besides, this method takes long time, extending over seven to eight years involving crossing and selection of desirable traits. *In vitro* regeneration technique helps to provide unique possibilities for overcoming the barriers of incompatibility between remote species and it facilitates rapid introduction of new varieties (Parveen, 2011). For raising transgenic crops with useful traits efficient *in vitro* plant regeneration protocol is necessary. As far as tomato is concerned, a good deal of tissue culture work has been done to regenerate plants through *in vitro* culture systems. However, standard regeneration protocol with farmer popular tomato varieties of Bangladesh has not been explored extensively. In few labs of Bangladesh including Dhaka University and BRAC University are working with establishment of reproducible regeneration protocol of locally grown tomatoes. (Chowdhury, 2008; Das, 2011; Ferdous, 2012 and Sarker, 2013) Tomato is quite amenable and responsive to *in vitro* regeneration (Fari *et al.*, 1992). *In vitro* technique help to overcome the barrier of self incompatibility facilitates rapid introduction of new traits (Taji *et al.*, 2002) and development of disease free plant (Moghaleb *et al.*, 1999). For *in vitro* regeneration researchers have used various types of explants sources viz, cotyledon (Schutze and Wieczorrek, 1987), hypocotyls (Plastira and Perdikaris, 1997; Gunay and Rao 1980), pedicel/peduncle (Compton and Veilleux, 1991), leaf (Duzyaman *et al*., 1994), stem sections and inflorescence (Applewhite *et al.,* 1994). In tomato, adventitious shoot regeneration can be achieved either directly (Dwivedi *et al.*, 1990) or indirectly through an intermediate callus phase (Behki and Lesley, 1980; Geetha *et al.*, 1998). However, both callus and shoots may be produced together (Bhatia, 2004). Fari and her colleges reported a simple and efficient organogenetic mechanism of shoot regeneration via seedling decapitation method for tomato (Fari *et al.*, 1991).

In order to develop efficient and reliable procedure for regeneration, from different tomato varieties, various types of explants were cultured in MS media supplemented with various hormones in different concentrations and combinations. Response towards

regeneration ability was found to vary in various genotypes. In Bangladesh while working with tomato varieties Begum and Miah use leaf explants of two (E 6 and S 1) strains on MS medium supplemented with 2mg/l IAA and 4mg/l kinetin for regeneration of calli (Begum and Miah, 1993). Colyledonary leaf explants were used by Chowdhury and Islam while working with popular Bangladeshi varieties (BINA tomato 3, BINA tomato 5, Bahar) and Indian variety Pusa Ruby, they found MS medium supplemented with 2mg/l BAP for best shoot regeneration of all four varieties (Chowdhury and Islam, 2012). Das mentioned MS media containing 1.5mg/I BAP and 0.2mg/I IAA showed best result with lowest callus formation and increased number of shoot in BINA Tomato 3, BARI tomato 3, Bahar and Pussa Rubby varieties (Das, 2011). Ferdous, also mentioned that MS media supplemented with 2 mg/l BAP found to be best for shoot regeneration for BARI Tomato 2, BARI Tomato 3, BARI Tomato 14, BARI Tomato 15 and BINA Tomato 3 variety (Ferdous, 2012). It was found by (Sarker, 2013) that MS medium containing 2 mg/l BAP showed the best result with highest number of shoots in BARI Tomato-3 and BINA Tomato-3 and 1 mg/l BAP containing MS medium showed the best result with highest number of shoots in BARI Tomato-7 variety.

**1.8. Tomato transformation:**

The most widely used method for transferring genes into tomato plants is *Agrobacterium*-mediated transformation and chloroplast transformation using particle bombardment (Ruf *et al.*, 2001). Since 1986, a number of reports have been published describing the use of *Agrobacterium tumefaciens*-mediated transformation and regeneration of different tomato cultivars (McCormick *et al*., 1986; Fillati *et al*., 1987; Chyi *et al*., 1987). Transformation procedure is used for the production of stress tolerant plants, insect and disease resistant plants, herbicide tolerant plants and finally for the production of improve quality fruits. In most cases, neomycin phosphotransferase (*NPTII*) has been used as plant selection marker and ß-glucuronidase (*GUS*) as a reporter gene. Transformation of tomato shows widely variable rates of success, depending on different factors, like, plant variety (genotype) (Ultzen *et al*., 1995; Ellul *et al.*, 2003), explant type (McCormick *et al*., 1986, Bird *et al*., 1988), explant size (Davis *et al*., 1991; Frary and Earle, 1996), explant orientation (Frary and Earle, 1996), the age of explant (Patil *et al*., 2002, Hamza and

Chupeau, 1993), plant growth regulators (McCormick *et al*., 1986), carbon source (Madhulatha *et al*., 2006), bacterial concentration (Shanin *et al*., 1986) and *Agrobacterium vir* gene inducers (Bolton *et al*., 1986). For successful transformation using *Agrobacterium*, effective elimination of bacteria from the culture is necessary as soon as their presence is no longer required. Carbenicillin, cefotaxime and augmentin are extensively used antibiotics for this purpose. An ideal antibiotic for inhibiting *Agrobacterium* species should be highly effective, inexpensive, without a negative effect on plant growth and regeneration (Cheng *et al*., 1998). A liquid culture system for *Agrobacterium*-mediated transformation of tomato has been developed and found to be better for transformation, because of the better distribution of the selective agent in the liquid cultures (Velcheva *et al*., 2005). The most critical factors affecting the regeneration of transformed explants are genotype/ variety, explants type, plant growth regulators, selection system, use of feeder cells or acetosyringone, *Agrobacterium* density, duration of infection, co-cultivation period and antibiotics that effects the regenerated explants (Khoudi et al., 2009). However, there is no universal protocol for transformation of wide range of tomato varieties, which became the subject of fascination for the researchers.

**1.9. Improvement of biotic and abiotic stress tolerance:**

A wide range of insect pests and pathogens are known to attack tomato. Hence several studies have been undertaken to confer significant resistance against such biotic stresses to remedy significant yield losses in commercial tomato via *Agrobacterium*-mediated transformation. Tomato is severely damaged by lepidopteran insect pest *Helicoverpa armigera*, which also called tomato fruit borer (Atwal, 1986). The introduction of the synthetic *cry*1*Ac* gene and *cry1Ab* gene into tomato conferred high levels of protection against *H.armigera* infestations (Mandaokar *et al.,* 1999). Combined expression of defense genes such as potato protease inhibitors (*PI-II*) and carboxypeptidase inhibitors (*PCI*) were reported in tomato against multiple insect resistances (Abdeen *et al*., 2005). Likewise, the introduction of viral nucleoprotein gene into tomato conferred resistance against tomato spotted wilt virus (Nervo et al. 2003) and coat protein (*CP*) gene of tomato leaf curl virus (TLCV) against TLCV (Raj *et al.,* 2005). There are very few reports of transgenic tomato for fungal resistance. For instance, stilbene synthase (*Vst 1*

and *2*) gene (Thomzik *et al.,* 1997) and *Thi* 2.1 gene (Chan *et al.,* 2005) have been used to create resistance against *Phytophthora infestans* and phytopathogens, respectively. Glyphosate (herbicide) tolerance using *aro A* gene has also been attempted in tomato (Fillatti *et al.,* 1987).

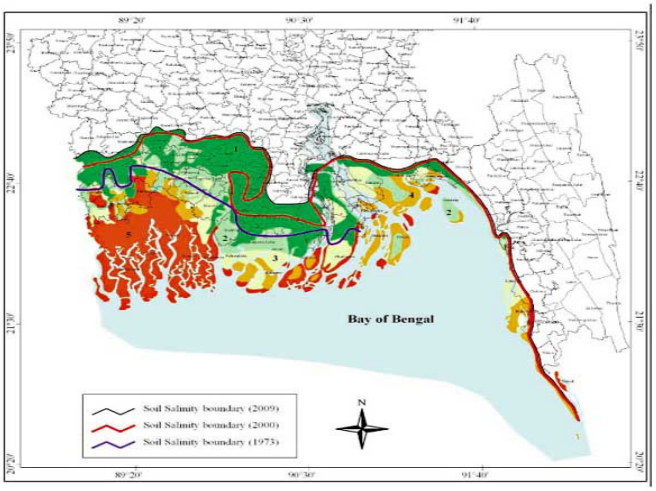
Tomato plants are not only susceptible to biotic stresses but also affected by abiotic stresses. Tomato transgenic lines have been developed to combat abiotic stress by engineering the trehalose biosynthetic pathway. The introduction of the yeast trehalose-6-phosphate synthase gene (*TPS1*) resulted in pleiotropic changes such as thick shoots, rigid dark-green leaves, erected branches and an aberrant root development in transgenic plants. These plants showed improved tolerance under drought, salt and oxidative stress conditions as compared to wild type plants (Cortina and Carolina, 2005). Most of the tomato cultivars are moderately sensitive to salts (Cuartero and Munoz, 1999). There are very few reports published on the engineering of salt tolerance in tomatoes (Foolad, 1999). Tomato has no glycinebetaine synthesis pathway (Weretilnyk *et al*., 1989) to synthesize glycinebetaine (a osmoprotectant), betaine aldehyde dehydogenase (*BADH*) gene from *Atriplex hortensis* (Weretilnyk *et al*., 1989, Xiao *et al*., 1995) has been introduced into tomato that allowed the biosynthesis of glycinebetaine to maintain an osmotic balance with the environment and also to withstand the salinity stress (Zhand and Blumwald, 2002; Robinson and Jones 1986). Other examples for salt-tolerance in tomato include introduction of *HAL1* (Gisbert *et al.,* 2000) and *HAL2* (Arillaga *et al*., 1998) to maintain high internal K+ concentration and decreased intracellular Na+ concentration during salt-stress. Waterlogging is another stress for which *ACC* deaminase gene has been introduced to confer tolerance to flood (Grichko and Glick 2001).

**1.10. Salinity and its effect on crop production:**

A significant change in global climate has occurred. This is impacting agriculture and thus affecting the world's food supply. Climate change is not always harmful in every case; the problems arise from extreme events that are difficult to predict (FAO, 2001). More erratic rainfall patterns and high temperature spells may consequently reduce crop productivity. Latitudinal and altitudinal shifts in ecological and agro-economic zones, land degradation, extreme geophysical events, reduced water availability, and rise in sea

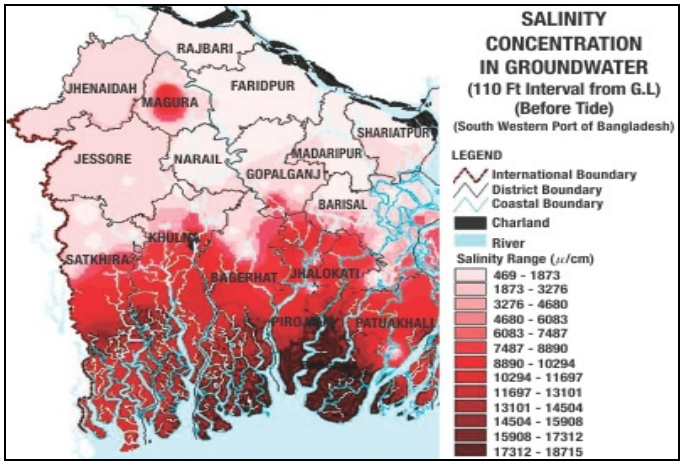
level and salinization are postulated (FAO, 2004). Unless measures are undertaken to mitigate the effects of climate change, food security in developing countries in the tropics will be under threat.

Soil salinity is a worldwide problem. Bangladesh is no exception to it. In Bangladesh, crop production is greatly hampered by salinization problem. Bangladesh is a deltaic country and its coastal area constitutes 20% of the country of which about 53% are affected by varying degrees of salinity. In these salinized areas agricultural land use is very poor. Salinity decline land productivity and nutrient balance of soil which became the main concerns with food security problem in the country (Haque, 2006). Among 64 thanas of 13 districts covering 8 agro-ecological zones (AEZ) of the country are under the coastal saline soils. Shatkhira, Khulna, Bagerhat, Barguna, Patuakhali, Pirojpur and Bhola are high saline porn area in the west. In recent years, the districts of Narail, Jessore and Magura are also extended actually making a total of 16 districts (SRDI, 2001). The smaller portion of the saline area lies in the districts of Chittagong, Cox's bazar, Noakhali, Lakshimpur, Feni and Chandpur (Panaullah, 1993). Neogi reported the overall salization situations of Bangladesh over the year 1973, 2000 and 2009 (Neogi, 2012).




Source: The Daily-Sun, September19, 2012

According to a research by Bangladesh Agricultural Development Corporation (BADC) in 2010 the groundwater of Dhaka was 170 feet below the sea level. The researchers estimated sea water intrusion by analyzing salt density in groundwater extracted from 200 feet below the sea level from coastal areas up to Magura, adding that salt density at several places in Bagerhat and Khulna had increased in the last two years (Asif, 2013). Salinity problem received very little attention in the past. Nevertheless, symptoms of such land degradation with salinization are becoming too pronounced in recent years to be ignored. Increased pressure of growing population demand more food. It has become imperative to explore the possibilities of increasing potential of these (saline) lands for increased production of food crops. Thus combating land salinization problem is vital for food security in the country through adoption of long-term land management.




Source: The Daily Star, August 25, 2013

### 1.11. Objective of the study:

According to the present situation of environment due to climate change it become great concern of salinity problem in Bangladesh and this problem greatly hampers tomato production, so in this study the main objective is to develop salinity tolerant tomato plant and as a prerequisite of transformation establishment of a reproducible *in vitro* regeneration protocol is unavoidable.

The present study was undertaken to achieve the following objectives,

- To develop a protocol for *in vitro* regeneration and to determine suitable concentrations and combinations of plant growth regulators in locally grown three tomato varieties for high frequency plantlet regeneration.
- To analyze factors affecting transformation efficiency for the development of an *Agrobacterium* mediated transformation protocol.
- To regenerate putative transgenic tomato plants using pBI121 and pH7WG2_OsNHX1_1.6. by *Agrobacterium* mediated genetic transformation.

# Chapter 2
# Materials and Methods

## 2.1. Materials:

In the present study following plant materials and *Agrobacterium* strains were used.

### 2.1.1. Plant Material:

Seeds of four varieties of tomato (*Solanum lycopersicum* L.) were used in tissue culture and transformation study. These are BARI tomato 2, BARI tomato 7, BARI tomato 9 and Bahar. Among these four varieties three varieties were used for tissue culture experiment and all four varieties were used in transformation experiment. BARI tomato 2, BARI tomato 7 and BARI tomato 9 were collected from BARI (Bangladesh Agricultural Research Institute, Gazipur, Dhaka) and Bahar was collected from BINA (Bangladesh Institute of Nuclear Agriculture, Mymensingh). Important Characteristics of all the verities are given in Table 2.1.

### 2.1.2. *Agrobacterium* strain and plasmid vectors:

*Agrobacterium tumefaciense* strain LBA4404 with plasmids constructs, pBI121 and pH7WG2_OsNHX1_1.6 were used for infection in the transformation experiment.

#### 2.1.2.1. 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.1 A, B).

#### 2.1.2.2. Plasmid pH7WG2_OsNHX1_1.6:

The $Na^+/H^+$ antiporter gene (*OsNHX1_1.6*) cloned from rice was immobilized to Gateway vector, pH7WG2_OsNHX1_1.6. This final construct pH7WG2_OsNHX1_1.6 was transformed into *A. tumefaciense* LBA4404 to be used in tomato transformation. It contains hygromycin

resistance for selection in plants and spectinomycin and streptomycin resistance for selection in bacteria. (Fig.2 A, B)

**Table 2.1. List of tomato varieties used (Ferdous, 2012; Chowdhuri, 2009; BARI website; BINA website)**

| **Description** | **BARI Tomato 2 (Roton)** | **BARI Tomato 7 (Apurba)** | **BARI Tomato 9 (Lalima)** | **Bahar** |
|---|---|---|---|---|
| Year of release | 1986 | 1988 | 1998 | 1992 |
| Developed by | Olericulture Division, HRC, Gazipur. | Olericulture Division, HRC, Gazipur. | Olericulture Division, HRC, Gazipur. | Bangladesh Institute of Nuclear Agriculture, Mymensingh |
| Yield (ton/ha) | 80-85 | 95-100 | 85-90 | 65-75 |
| Identifying Characteristics | The average plant height is 75-85 cm and average fruit weight is 80-90gms. Leaf color is light green, fruits are high yielding, longer shelf life, tolerant to bacterial wilt and can cultivate all over Bangladesh | Average fruit weight is 145-155gm and Yield is about 3-3.5 kg/plant. Leaf color is light green, high yielding, longer shelf life, tolerant to bacterial wilt and can cultivate all over Bangladesh. | Average fruit weight is 75gm. Suitable area for cultivation is all over Bangladesh. It has prolific bearer (80-85 fruits/plant) with longer shelf life of fruits (2-3 weeks), Suitable for longer transportation for its compactness, thick and hard skin. This variety has the resistant against bacterial wilt. | Average fruit weight is 110 g. Plants are determinate in habit. Fruits are large, fleshy, tastier and contain less number of seeds. Vitamin C content is 21.2 mg/100 g. Suitable area for cultivation is all over Bangladesh |
| Sowing time | September-October | September-October for winter and May –June for summer | September-October for winter and May –June for summer | September-October |
| Fruit size and colour | Oval, red | Semi-globe, attractive orange | Oval, attractive red | Round, red |
| Crop duration | 120-130 days (DAT) | 120-130 days (DAT) | 110-120 days (DAT) | 90-100 days (DAT) |

DAT: Day after Transplantation.

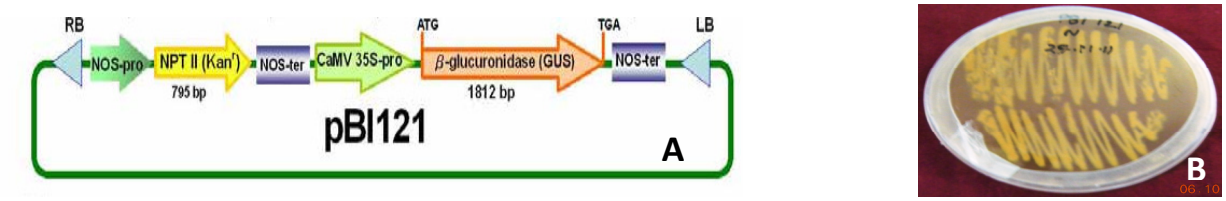



**Fig.1. A**. Schematic diagram of the T-DNA region of binary vector pBI121, **B**. Pure culture plate of *Agrobacterium tumefaciense* LBA4404 with pBI121 on YMB medium.

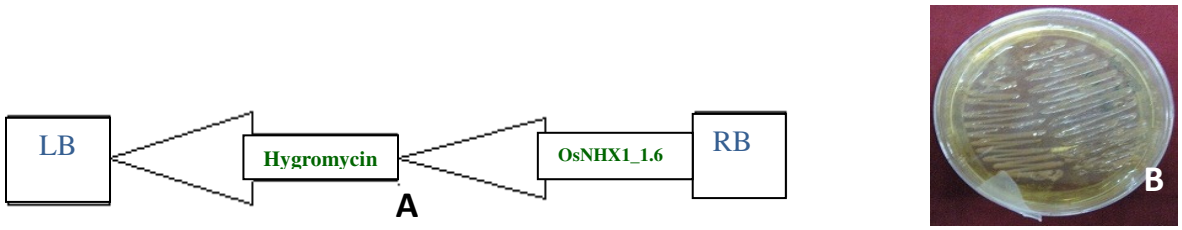



**Fig. 2. A**. Schematic diagram of constructed vector of pH7WG2_OsNHX1_1.6. **B.** Culture plate of *A. tumefaciense* LBA4404 with pH7WG2_OsNHX1_1.6. on YMB medium.

### 2.1.3. Different culture media used:

#### 2.1.3.1. 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.

##### 2.1.3.1.1. Seed germination and seedling development medium:

For seed germination MS basal medium solidified with agar or phytogel were used.

##### 2.1.3.1.2. Regeneration initiation and shoot differentiation media:

For regeneration initiation cotyledonary leaf explants were cultured on MS media supplemented with different concentrations and combinations of various growth regulators, such as, BAP, IAA. After shoot initiation same or reduced concentration of hormone containing MS media were used for shoot elongation.

##### 2.1.3.1.3. Root induction media:

For induction of root from the *in vitro* grown multiple shoots, half strength of MS basal medium supplemented with different concentrations of IAA were used.

#### 2.1.3.2. Transformation media:

##### 2.1.3.2.1. *Agrobacterium* culture media:

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

**2.1.3.2.2. Co-culture media:**

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

**2.1.3.2.3. Selection media:**

For *Agrobacterium* culture, three antibiotics (kanamycin for *Agrobacterium* containing pBI121, and hygromycin for pH7WG2_OsNHX1_1.6 plasmid containing *Agrobacterium* and streptomycin and spectinomycin for *Agrobacterium* strain LBA4404) were used. Cefotaxime (Duchefa Bioc) was used after co-cultivation as bacteriostatic against *Agrobacteria*. Appropriate concentrations of two antibiotics, namely, hygromycin and kanamycin (Duchefa Bioc) were used as selectable agaents with the best regeneration media.

**2.2. Methods:**

**2.2.1. Stock solution preparation:**

Different components were required for the preparation of stock solution in MS (Murashige and Skoog, 1962) media **(Appendix-1).**

**2.2.1.1. 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 (500 ml) 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.

**2.2.1.2. Micro nutrients stock solution preparation:**

The solution was made 100 times of their full strength. 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.

**2.2.1.3. 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 as well for making this solution. This solution was made and preserved at 4ºC in amber bottle as it is light sensitive.

**2.2.1.4. 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 next. Then it was stored at 4ºC.

**2.2.2. Stock solutions of growth hormones:**

**2.2.2.1. BAP stock solution preparation:**

The BAP (Sigma) stock solution was prepared by dissolving 10 mg of BAP in 1 ml or 2 ml of 1 N 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.

**2.2.2.2. IAA stock solution preparation (1 mg/ml):**

First, 200 mg of IAA (Sigma) was dissolved with 1 drop of absolute ethanol. 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.

**2.2.3. Preparation of one liter of MS medium:**

Murashige and Skoog (MS) medium (1962) was used as basal tissue culture medium for tomato regeneration.

**Table 2.2. Different components needed for preparation of 1 litre of full strength MS media**

| Components (stock conc.) | Amount (for 1000 ml) |
|---|---|
| Macronutrients (10x) | 100 ml |
| Micronutrients (100x) | 10 ml |
| Vitamin/Organic (100x) | 10 ml |
| Fe- EDTA (100x) | 1 0ml |
| Myo-inositol | 0.1 g |
| Sucrose | 30 g |

All components (**Table 2.2**) were added to a conical flask and volume up to one liter with dd$H_2O$. The pH was adjusted to 5.8 with 1N NaOH or HCl as needed. For solid medium agar (Sigma) was added in 0.6% (w/v) ratio. To dissolve solidifying agents quickly the whole mixture was heated in a microwave oven (LG, China, MH-65SR). Finally the media was aliquate in appropriate vessel plugged and marked the media.

**2.2.4. Media sterilization**:

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

**2.2.5. Preparation of seed germination and seedling development medium:**

Solid MS medium without hormone supplementation was used for seed germination and to support seedling development.

**2.2.6. Shoot regeneration media preparation:**

Different concentrations and combinations of growth hormones were added in plant regeneration media. To determine the effect of phytohormones on shoot regeneration MS media was supplemented with BAP (1, 2, 5 and 7 mg/l) with/without IAA (0.1mg/l)**.**

### 2.2.7. Subculture media preparation:

Regenerated explants need to subculture every 3 to 4 weeks. Media with same and reduced hormonal supplementation and in some cases only MS basal medium was used during subculture.

### 2.2.8. Rooting media preparation:

Half strength of MS medium was used as basal medium for rooting. The medium was supplemented by various concentration of IAA. For solidification, 0.3% (w/v) phytagel (Sigma) was used instead of agar in rooting. Three different concentrations (0.1, 0.2, 0.5 mg/l) of IAA were tested for rooting response.

### 2.2.9. 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 on for 15 minutes to one hour. Then the laminar hood was cleaned with 70% ethanol spray and hands were washed with antimicrobial hand wash (Hexisol®, ACI Ltd.). The instruments (forcep, 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.

### 2.2.10. Axenic culture:

#### 2.2.10.1. Seed sterilization:

Surface sterilization of seeds was the first step of axenic culture. Seeds were immersed in 70% ethanol for 3 minutes followed by 1.575% sodium hypochloride (30% Clorox) treatment with 2 drops of Tween-20 and shaken continuously for 15-20 minutes till its slimy layer is removed completely. The seeds were then washed with autoclaved distilled water for three times up-to 30 minutes to washout the detergent and visualize the embryo. Seeds were then either kept in a rotary shaker (Wise Cube-Wis 20R, Korea) for 24-36 hr in 180 rpm at 28ºC or in the normal

laboratory condition in 25ºC without shaking for 24-36 hrs, while immersed in sterile distilled water to observe the effect of germination on shaking.

**2.2.10.2. Explant culture and shoot regeneration:**

Explants were collected from seedlings for *in-vitro* shoot regeneration. Each cotyledonary leaf was transversely cut into small pieces giving three pieces and used as explants for multiple shoot regeneration. The explants were placed in different regeneration media in dorsal position of cotylidonary leaf.

**2.2.10.3. Subculture:**

Regenerated explants were subcultured into fresh media containing the same hormonal supplement or reduced concentration of hormonal supplementation for further proliferation and development. Subculture was performed regularly at an interval of three or four weeks for maintenance. Cultures were routinely examined for different morphogenic development and data were recorded after 12-15 days and 45-50 days of inoculation.

**2.2.10.4. Rooting:**

Well developed shoots around 3-4 cm long, were placed individually in the rooting medium to obtain sufficient root formation. Data were recorded after 15-20 days of placement in the rooting media.

**2.2.10.5. Plant hardening procedure:**

Hardening 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 a forcep when the roots were 3 to 6 cm long. The agar attached to their root part was gently washed with running water to make sure that the entire agar was removed completely to avoid any contamination.

b. Then the plants were transferred in a pot containing autoclaved soil. Perforated plastic bags were taken to cover the potted plantlets. Inside the bags water was sprayed to

maintain the humidity inside the bag and to protect from moisture shock. Plantlets were kept inside the culture room for 15 days. During these 15 days the moisture inside the bags were maintained constantly.

c. After 15 days the bags were removed and the plantlets were kept for next 15 days inside culture room. Four weeks after transplantation, plants were then kept in a shade place outside the culture room each day for 2 hours for 1 week.
d. On the eighth week, the plants were exposed to direct sunlight for 2 hours a day. This treatment was continued for 2 more weeks.
e. Lastly the plants were placed in natural environment. At this stage leaves were dark green than it was before and stem had secondary thickness. Finally, the plants were transferred to pots containing soil and peat (3:1) in net house.

**2.2.10.6. Analysis of reproductive response of the regenerated plantlets:**

Following acclimatization of regenerated plantlets survivability, flowering and fruiting response in natural environment were assessed by observing flowering and fruiting response. Fruit weights, number of fruits per plant etc. data were collected.

**2.2.10.7. Seed viability test:**

Twenty four hours later of surface sterilization seeds were taken out from shaker and from normal condition then separately placed on sterilized filter paper and transferred to solidified germination media in 25±2ºC with 16 h photoperiod. Time required for germination initiation and regeneration percentage was recorded.

**2.2.11. Antibiotics stock solution preparation (25 mg/ml):**

Kanamycin sulfate, hygromycin, 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.

### 2.2.12. *Agrobacterium tumefaciense* culture media preparation:

YMB medium was prepared to culture *Agrobacterium tumefaciense* strain LBA4404.

**Table 2.3. Components for YMB medium preparation:**

| Components | Amount (g/l) |
|---|---|
| Mannitol | 10.0 |
| $K_2HPO_4.3H_2O$ | 0.5 |
| Yeast extract | 0.4 |
| $MgSO_4.7H_2O$ | 0.2 |
| NaCl | 0.1 |

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 the *Agrobacterium tumefaciense* containing pBI121 binary vector, kanamycin was added at 200 mg/l to the medium. For the *Agrobacterium tumefaciense* containing pH7WG2_OsNHX1_1.6, 100 mg/l streptomycin and 200 mg/l spectinomycin were added to each 100 ml medium.

### 2.2.13. 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 tomato varieties was added to this medium. No antibiotics were added here.

### 2.2.14. Media for kanamycin or hygromycin sensitivity test:

Regeneration media with different concentrations of kanamycin (0 mg/l, 50mg/l, 100mg/l, 150mg/l and 200mg/l) or hygromycin (0mg/l, 1mg/l, 2mg/l, 4mg/l, 6mg/l, 8mg/l, and 10mg/l) were used for plant sensitivity tests.

### 2.2.15. Selection media preparation:

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

### 2.2.16. Tomato transformation procedure:

**Day 1:** YMB solid media was prepared with required antibiotics (kanamycin for *Agrobacterium* strain with pBI121 and both streptomycin and spectinomycin for *Agrobacterium* strain with pH7WG2_OsNHX1_1.6) for *Agrobacterium* stock maintenance.

**Day 2:** A single colony of *Agrobacterium tumefaciense* (containing the desired construct) was streaked on an antibiotic containing YMB media plate with a sterilized loop. The Petri-dish was sealed with Para-film and kept upside down at 37°C for 48 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 YMB medium was prepared for liquid culture of bacteria. MS media was prepared for transferring explants after infection.

**Day 4:** Explants were cut and placed on regeneration media for pre-culture. Single colony was picked from *Agrobacterium* culture to inoculate with an inoculation loop in 100 ml of antibiotic containing liquid YMB 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 YMB 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 10, 20 and 30 minutes for infection and then placed on co-cultivation medium and kept there for next 1 to 2 days (co-cultivation period).

**Day 6**: The Petri-plates were checked for bacterial overgrowth.

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

After 2 weeks, explants were placed on kanamycin or hygromycin 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.

### 2.2.17. Preparation of reagents for performing histochemical GUS assay:

#### 2.2.17.1. Preparation of MES buffer:

2.44 g MES were weighed into a clean dry beaker. 20 ml dd$H_2O$ was added and mixed well to dissolve MES completely. The pH was adjusted to 5.6 with 5 M KOH. Final volume was made up to 25 ml and stored at room temperature.

#### 2.2.17.2. Preparation of fixation solution:

**Table 2.4. Different components for preparation of fixation solution**

| Component | Stock concentration | Final concentration |
|---|---|---|
| Formaldehyde (40%) | 0.75% (v/v) | 0.3% |
| 0.5 M MES (pH 5.6) | 0.002% (v/v) | 10 mM |
| Mannitol | 5.46% (w/v) | 0.3 M |

750 µl of Formaldehyde, 2 µl of 0.5 M MES (pH 5.6) and 5.46 g of Mannitol were weighed into a beaker. Then dd$H_2O$ was added to make final volume up to 100 ml. Stored at room temperature for next three months or until precipitate appears.

**2.2.17.3. Preparation of phosphate buffer:**

**Solution A:** 156.01 g of $NaH_2PO_4.2H_2O$ (acidic) was required for 1 M 1 liter solution.

**Solution B:** 141.96g $Na_2HPO_4$ (basic) was required for 1 M 1 liter of solution.

39 ml solution A and 59 ml solution B were mixed well to prepare 50 mM phosphate buffer. pH was adjusted to 7.0 by adding low pH solution A or high pH solution B as necessary. Filter sterilization was needed and then stored it at 4°C.

**2.2.17.4. 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, Duchefa) 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.

**2.2.18. Histochemical GUS assay:**

To observe transformation explants treated with pBI121 were subjected to histochemical assay for *GUS* gene. 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 which is the characteristic expression of GUS (β-glucuronidase) gene in the plant tissue. X-Gluc solution was discarded and 70% ethanol was added and again incubated at 37°C for 48 hours for degreening. Transformed explants were observed under microscope for *GUS* gene expression.

# Chapter 3

## Results

In the present study, *in vitro* regeneration response of three varieties of tomato (*Solanum lycopersicum* L.), namely BARI Tomato 2, BARI Tomato 9 and Bahar were studied and transformation study was performed by using these three varieties and BARI tomato 7 variety to developed *in vitro* regeneration and transformation protocol.

### 3.1. *In vitro* regeneration:

The cotyledonary leaf explants were used for direct regeneration. They were collected from aseptically grown seedlings following germination. Successfully developed shoots were cultured for root formation. Regenerated plantlets were acclimatized in soil and were allowed to grow under field conditions to obtain flowers and fruits. Finally, viability of seeds collected from matured fruits of these regenerated plants was studied.

#### 3.1.1. Effect of different types of agitation on germination:

These experiments were performed to find out an easy and proper procedure for germination of seeds to obtain maximum germination under *in vitro* conditions.

When seeds were kept in normal laboratory condition at 25 ºC for 24, 30 and 48 hours without shaking, BARI Tomato 2 varieties showed the highest germination response (88%, 92%, and 95%) (Table 3.1) and it took 3-4 days to germinate. When seeds were shaken continuously in rotator shaker for 24, 30 and 48 hours after seed sterilization, it was observed that in case of 24 and 30 hours of shaking, BARI Tomato 2 again showed highest germination response (95%, and 97%, respectively) and in case of 48 hours of shaking this variety showed highest germination response (97%) and required less day requirement for germination initiation (2-3 days). Bahar variety showed less germination response and more time requirement in all these condition (Table 3.1).Therefore, it is understandable that germination percentage has no significant difference. However, the time requirement is considerably decreased after 48 hours of shaking, but 48 hours treatment leads to embryo damage. For this reason 24 hours continuous shaking was

adopted for further studies. Germinated seedlings of different tomato varieties are shown in Fig.3 A-D.

**Table 3.1. Effect of agitation on time requirement for germination in all three varieties of tomatoes**

| Tomato Varieties | Time duration (hr.) in shaking condition after sterilization | | % of seed germination | Days required for germination initiation |
|---|---|---|---|---|
| | Without shaking | Continuous shaking on a shaker | | |
| BARI Tomato 2 | 24 | - | 88 | 3-4 |
| | 30 | - | 92 | 3-4 |
| | 48 | - | **95** | **3-4** |
| | - | 24 | 95 | 3-4 |
| | - | 30 | 97 | 3-4 |
| | - | 48 | **97** | **2-3** |
| BARI Tomato 9 | 24 | - | 79 | 3-4 |
| | 30 | - | 86 | 3-4 |
| | 48 | - | **89** | **3-4** |
| | - | 24 | 88 | 3-4 |
| | - | 30 | 89 | 3-4 |
| | - | 48 | **91** | **2-3** |
| Bahar | 24 | - | 74 | 5-6 |
| | 30 | - | 75 | 4-5 |
| | 48 | - | **77** | **4-5** |
| | - | 24 | 78 | 5-6 |
| | - | 30 | 78 | 5-6 |
| | - | 48 | **80** | **4-5** |

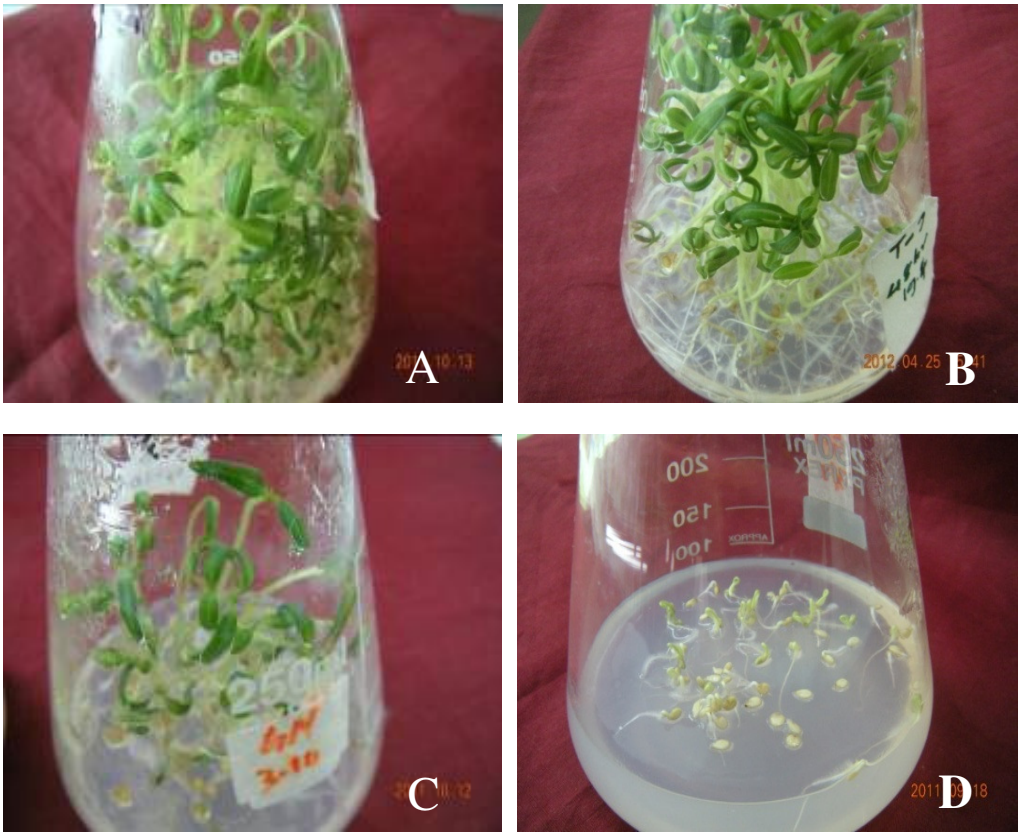



**Fig. 3**. Germinated seedlings of different tomato varieties. **A**. 7 days old seedlings of BARI tomato 2 variety, **B**. 7 days old seedlings of BARI tomato 9 variety, **C**. 10 days old seedlings of Bahar variety, **D**. Germination initiation in BARI tomato 2 variety.

### 3.2. Determination of suitable medium for *in vitro* regeneration of shoots:

These experiments were performed to determine suitable MS medium supplemented with various concentrations of auxins and cytokinins for *in vitro* shoot regeneration. In the present study, effect of different concentration of hormonal combinations on regeneration initiation and number of shoot formation per explants were observed.

### 3.2.1. Response of cotyledonary leaf explants towards multiple shoot regeneration using different concentrations of BAP and IAA in MS medium for all three varieties:

MS medium supplemented with different concentrations and combinationsof BAP and IAA were used for induction of multiple shoots using cotyledonary leaf explants. Regeneration initiation took place from all varieties within 12 to 18 days. Results of this experiment are presented in (Table 3.2). By doing two way ANOVA it was found that development of multiple shoots was influenced by various concentrations and combinations of BAP and IAA.

#### 3.2.1.1. BARI Tomato 2:

In BARI Tomato 2 variety it was found that when MS medium was supplemented with 1mg/l BAP, highest numbers of shoots per explants were obtained and it was statistically similar with combination of 2 mg/l BAP + 0.1 mg/l IAA and 2 mg/l BAP. Lowest number of shoots was obtained when the explants were placed in 0.5 mg/l BAP containing media. This variety also showed an interesting response called albinism, which was observed in case of 2 mg/l BAP supplementation. In 5 mg/l BAP, BARI tomato 2 forms callus but in the MS media with 7 mg/l BAP it forms normal shoots (Table 3.2)

Among those different concentrations and combinations of BAP and IAA containing MS media this variety showed early regeneration when the media was supplemented with combination of 2 mg/l BAP + 0.1mg/l IAA. This is statistically similar with the combination of 1mg/l BAP + 0.1 mg/l IAA, 7mg/l BAP and 1mg/l BAP containing MS medium. It was observed that media containing combination 0.5 mg/l BAP + 0.1 mg/l IAA showed highest time requirement to regenerate which is statistically similar with combination of 5 mg/l BAP and 2 mg/l BAP containing MS media.(Fig. 4 A-G).

Among these concentrations and combinations of hormone finally, 1 mg/l BAP concentration was found to be the best in case of regeneration initiation and number of shoot production in BARI Tomato 2 variety.

### 3.2.1.2. BARI Tomato 9:

BARI Tomato 9 variety produced highest shoots in 1 mg/l BAP containing MS medium which is statistically similar with 2 mg/l BAP containing MS media. Lowest shoots response found in 7 mg/l BAP containing MS medium which is statistically similar with combination of 0.5 mg/l BAP + 0.1 mg/l IAA (Table 3.2).

The lowest day requirement was observed when the MS media was supplemented with combination of 1 mg/l BAP + 0.1 mg/l IAA. This is statically similar with other concentrations and combinations of hormone. It was also observed that the media containing 7 mg/l BAP showed highest time requirement for regeneration initiation (Fig. 5 A-G)

Finally, BARI Tomato 9 produced highest number of shoot and required lowest days for regeneration initiation in 1mg/l BAP containing MS medium.

### 3.2.1.3. Bahar:

Bahar variety produced highest number of shoots in combination of 1mg/l BAP + 0.1 mg/l IAA containing MS media when compare to other supplementation. Lowest number of shoots was found at 7 mg/l BAP containing MS media which is statistically similar with 5 mg/l BAP containing MS media (Table 3.2).

When the MS media was supplemented with combination of 1 mg/l BAP + 0.1 mg/l IAA, lowest day requirement was observed. This is statically similar with combination of 0.5 mg/l BAP + 0.1 mg/l IAA, 2 mg/l BAP + 0.1 mg/l IAA and 2 mg/l BAP and highest day requirement observed when the MS media was supplemented with 7 mg/l BAP. (Fig. 6 A-G).

From the statistical analysis we can see that Bahar variety produced highest number of shoot and require lowest day requirement in combination of 1 mg/l BAP + 0.1 mg/l IAA containing MS medium. So this hormonal combination may consider as best for this variety.

**Table 3.2. Response of explants of BARI Tomato 2, BARI Tomato 9 and Bahar towards multiple shoot regeneration using different concentration of BAP in MS medium**

| Tomato varieties | Concentration of hormones (mg/l) | | Total no. of explants inoculated | No. of responsive explants | % of explants respond | Average time Requiredfor initiation of regeneration (days) | Average no. of shoots taken after 40 days |
|---|---|---|---|---|---|---|---|
| | BAP | IAA | | | | | |
| BARI Tomato 2 | **1.0*** | - | **31** | **30** | **96** | **13.0** | **24.8** |
| | 2.0* | - | 31 | 29 | 93 | 13.8 | 18.4 |
| | 5.0 | - | 31 | 21 | 67 | 16.4 | 03.8 |
| | 7.0 | - | 30 | 18 | 60 | 11.6 | 13.0 |
| | 0.5 | 0.1 | 30 | 20 | 66 | 13.6 | 12.4 |
| | 1.0 | 0.1 | 30 | 23 | 76 | 11.2 | 10.0 |
| | **2.0** | **0.1*** | **30** | **29** | **96** | **10.2** | **23.0** |
| BARI Tomato 9 | **1.0*** | - | **58** | **54** | **93** | **12.6** | **19.4** |
| | 2.0* | - | 22 | 20 | 91 | 14.4 | 18.6 |
| | 5.0 | - | 28 | 21 | 75 | 20.2 | 07.0 |
| | 7.0 | - | 31 | 06 | 19 | 32.2 | 02.2 |
| | 0.5 | 0.1 | 31 | 22 | 71 | 13.2 | 04.6 |
| | **1.0** | **0.1*** | **31** | **24** | **77** | **12.2** | **10.2** |
| | **2.0** | **0.1*** | **31** | **23** | **74** | **13.8** | **15.4** |
| Bahar | **1.0*** | - | **30** | **28** | **93** | **13.8** | **21.8** |
| | 2.0* | - | 30 | 29 | 97 | 11.8 | 20.2 |
| | 5.0 | - | 30 | 21 | 70 | 15.2 | 07.6 |
| | 7.0 | - | 30 | 25 | 83 | 23.2 | 03.2 |
| | 0.5 | 0.1* | 30 | 28 | 93 | 12.4 | 11.8 |
| | **1.0** | **0.1*** | **30** | **30** | **100** | **10.0** | **39.6** |
| | 2.0 | 0.1* | 30 | 28 | 93 | 11.4 | 23.2 |
| LSD(0.05) | - | | - | - | - | 3.00 | 4.67 |
| CV | - | | - | - | - | 16.35% | 25.15% |

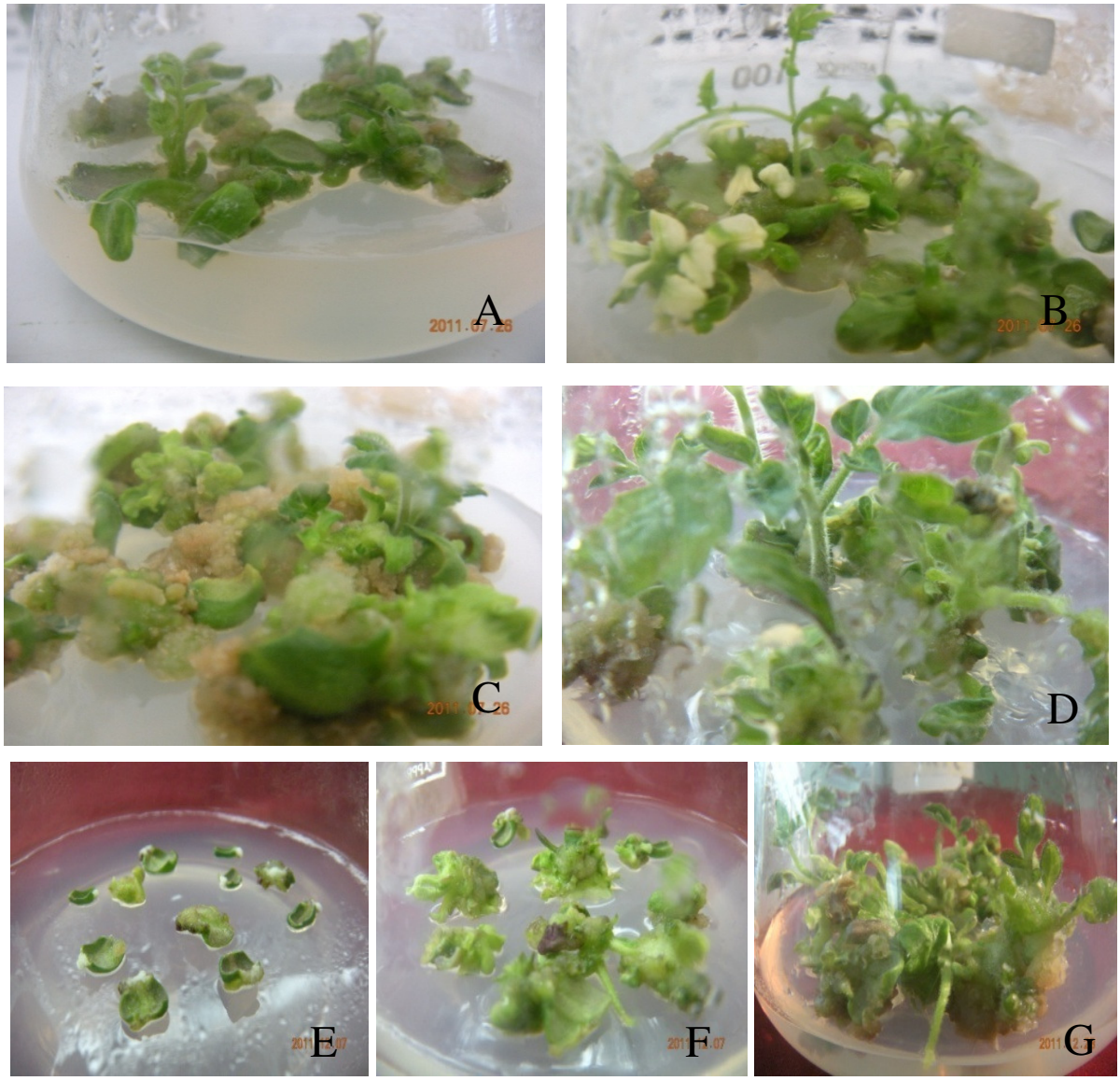



**Fig. 4**. Regeneration response of BARI tomato 2 variety. **A**. Regenerated explants in 1mg/l BAP containing media after 40 days of their inoculation, **B**. Albino shoots in 2 mg/l BAP supplemented media, **C**. Formation of callus in 5mg/l BAP (Photographs A, B and C were taken 40 days after inoculation), **D**. Regenerated explants in 7 mg/l BAP containing media (Photograph taken after 45 days of inoculation). **E.** in 0.5mg/l BAP $_+$ 0.1 mg/l IAA, **F**. in 1mg/l BAP $_+$ 0.1 mg/l IAA and **G.** in 2 mg/l BAP $_+$ 0.1 mg/l IAA containing MS media. (photographs were taken 40 daysafter inoculation).

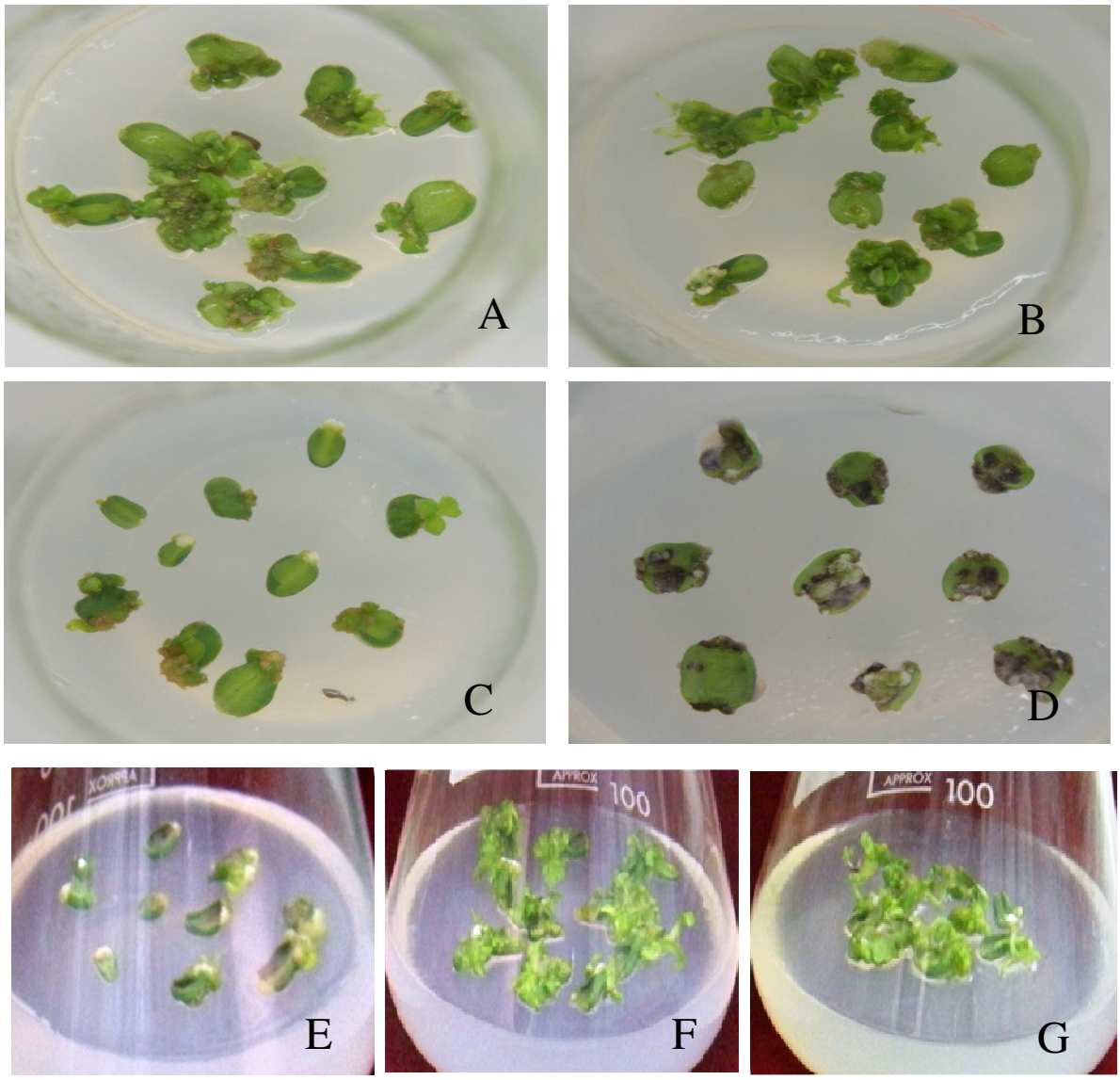



**Fig.5**. Regeneration response of BARI tomato 9 variety. **A.** in 1 mg/l BAP, **B.** in 2 mg/l BAP, **C.** in 5 mg/l BAP, **D.** in 7 mg/l BAP containing MS media. (Photographs were taken after 30 days of inoculation). **E.** in 0.5mg/l BAP + 0.1 mg/l IAA, **F**. in 01mg/l BAP + 0.1 mg/l IAA and **G.** in 2 mg/l BAP + 0.1 mg/l IAA containing MS media. (photographs were taken 40 daysafter inoculation).

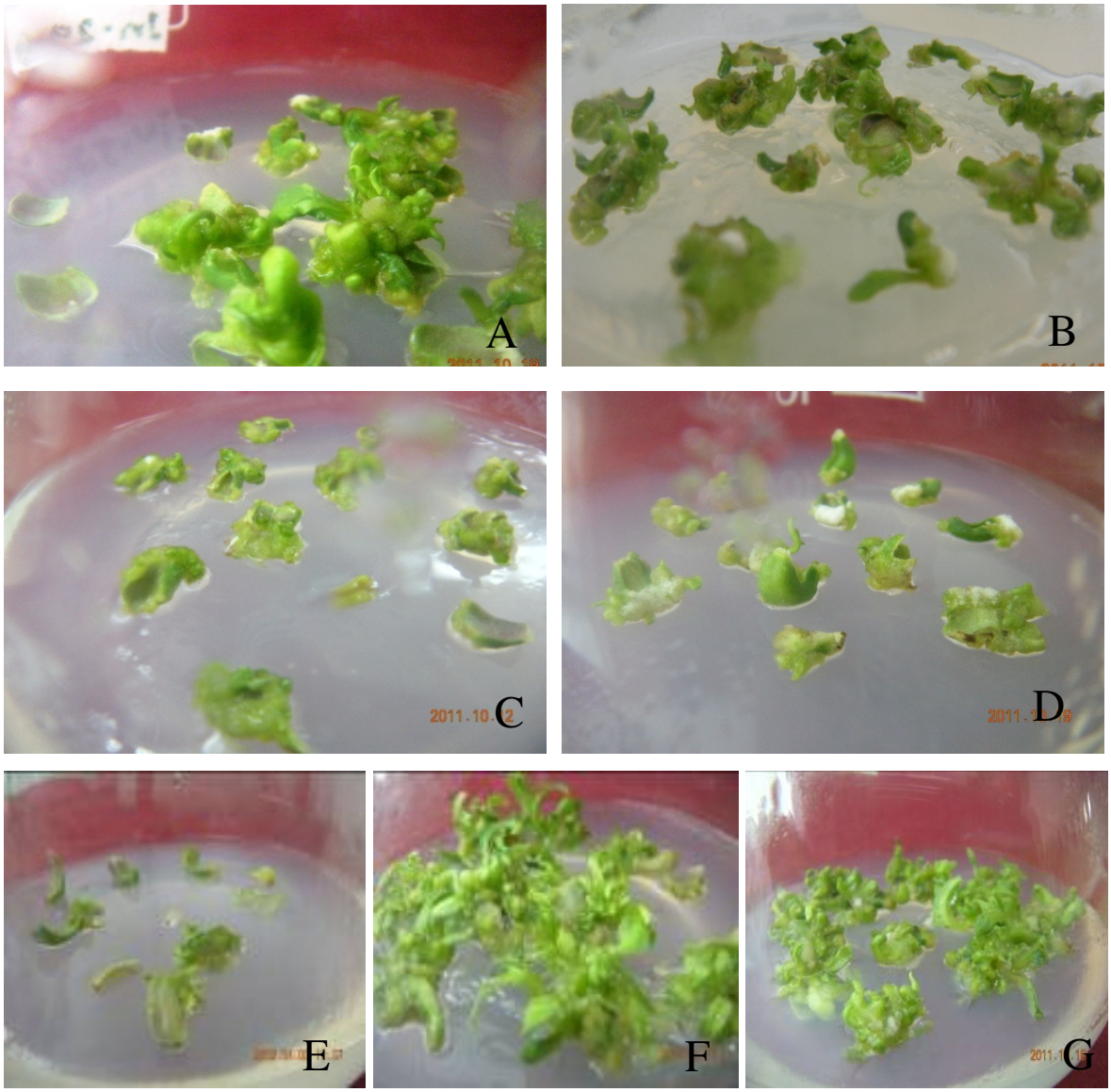



**Fig. 6**. Regeneration response of Bahar variety. **A.** in 1 mg/l BAP, **B.** in 2 mg/l BAP, **C.** in 5 mg/l BAP, **D.** in 7 mg/l BAP containing MS media. (Photographs were taken 40 days after inoculation). **E**. in combinetion of 0.5 mg/l BAP $_+$ 0.1 mg/l IAA containing MS medium. **F**. in 01 mg/l BAP $_+$ 0.1 mg/l IAA and **G**. in 2 mg/l BAP $_+$ 0.1 mg/l IAA containing MS media. (photographs were taken 40 daysafter inoculation).

### 3.3. Subculture of regenerated shoots:

Shoot regeneration was not similar in all the varieties and in all the media. In the BAP and BAP with IAA supplemented media, shoots were initiated in non-synchronized manner. In all the varieties some shoots were found to attain 3-4 cm length within one month of culture. While, many shoot remain small. For better shoot growth and development, explants were sub-cultured in the fresh media containing the same hormonal concentration. In this study it was found that BARI tomato9 produce multiple shoot after several subcultures. It was observed to give up to 20 shoots/explant after three to four sub-cultures but the appearance of the shoot were not healthy. To improve the morphology of shoots, subculture was done in lower 2X $KNO_3$ containing MS media without hormone and in only MS media without hormone. Shoot, subcultured in MS media produced root and in 2X $KNO_3$ produced both healthy shoot and root (Fig.7).

### 3.4. Effect of IAA on root formation in different tomato varieties:

Root formation is an essential step to achieve plantlets.All the*in vitro* regenerated shoots did not produce root spontaneously. So, the elongated shoots were excised and cultured to ½ MS media supplemented with different concentrations of IAA (0.1, 0.2 and 0.5 mg/l) to produce roots (Table 3.4). In all the three varieties, it was found that when the rooting media was supplemented with 0.2 mg/l IAA minimum day requirement for root initiation was observed and percentage of rooting was 100%. Average number of roots was found to be 13-16 and average root length was 6.1-6.5 cm after 14 days after inoculation. In reduced concentration of IAA (0.1 mg/l IAA) containing media all three varieties required more days (7-8) for root initiation and after 14 days the average number of roots were lowest (9-11) and average root length was also less, only 3.4-4.4 cm. When the concentration of IAA was increased, then highest number of root was formed (15-30) but it required highest day for root initiation and all the roots were short (average length 1.5-3) and fibrous. Rooting response of Bahar variety in IAA (0.1, 0.2 and 0.5mg/l) supplemented rooting media shown in (Fig. 8).

**Table 3.4. Effect of IAA on root regeneration in different tomato varieties**

| Tomato Varieties | Concentration of IAA mg/l | No. of shoot inoculated | Percentage of shoot producing root | Days required for root initiation | Types of root | Average no. of roots(after 14 days) | Average root length (cm) |
|---|---|---|---|---|---|---|---|
| BARI tomato 2 | 0.1 | 08 | 75 | 7.13 | Tap root | 9 | 4.3 |
| | **0.2** | **25** | **100** | **3.60** | **Tap root** | **13** | **6.1** |
| | 0.5 | 09 | 88 | 9.86 | Fibrous | 15 | 2.4 |
| BARI tomato 9 | 0.1 | 07 | 72 | 8.20 | Tap root | 8 | 4.4 |
| | **0.2** | **33** | **100** | **4.52** | **Tap root** | **15** | **6.2** |
| | 0.5 | 10 | 80 | 9.40 | Fibrous | 20 | 3 |
| Bahar | 0.1 | 10 | 90 | 8.50 | Tap root | 11 | 3.4 |
| | **0.2** | **40** | **100** | **4.82** | **Tap root** | **16** | **6.5** |
| | 0.5 | 10 | 100 | 11.1 | Short & fibrous | 30 | 1.5 |

In the present experiment, it was found that half strength of MS medium containing 0.2 mg/l IAA was suitable for *in vitro* root formation for all 3 varieties of tomatoes. Healthy shoots collected from all these 3 varieties were placed in rooting media and response was observed (Table 3.5). Well-developed roots were found to be initiated from the cut end at the base of the shoots (Fig.9 A). Minimum day requirement for root formation was observed in shoots collected from 1 mg/l BAP containing MS media in BARI tomato 2 variety and BARI tomato 9 variety and both of the varieties produced spontaneous roots (Fig.9 B-C) within 40-45 days. However, shoot regeneration media containing both BAP and IAA fail to initiate same response. BARI tomato 2 and Bahar varieties produced highest number of roots (15-18) and long root (Fig.9 D-E). In all three varieties it was found that there were no significant differences in response of root formation of shoots collected from different shooting media from which shoots were collected.


**Fig. 7. A.** Multiple shoots of BARI tomato 2 variety after two subculture and observed to be ready for rooting. **B**. Multiple shoots of BARI tomato 9 variety, **C**. Shoot of BARI tomato 9 subcultured in MS media containing 2X $KNO_3$ produce healthy shoot and root. **D**. Shoot of BARI tomato 9 subcultured in MS media.


**Fig. 8.** Rooting response of Bahar variety in IAA (**A.** 0.1 mg/l IAA**, B.** 0.2mg/l IAA and **C.** 0.5mg/l IAA) supplemented rooting media. Photographs were taken 15 days after inoculation.

**Table 3.5. Rooting response of shoots regenerated in various hormonal supplementation in all three varieties in 0.2 mg/l IAA containing ½ strength MS media**

| Tomato varieties | Hormone supplementation for shoot formation(mg/l) | | Spontaneous roots in shooting medium | No. of shoot inoculated in rooting medium | Days required for root initiation | Percentage of shoots producing root | Average length of roots after 14 days | Roots per shoot |
|---|---|---|---|---|---|---|---|---|
| | BAP | IAA | | | | | | |
| | 1.0 | - | + | 20 | 0-3 | 100 | 6.5 | 15.27 |
| BARI | 2.0 | - | - | 15 | 3-4 | **100** | **6.2** | **16.23** |
| tomato2 | 5.0 | - | - | 14 | 3-4 | 93 | 5.5 | 11.40 |
| | 0.5 | 0.1 | - | 2 | 3-4 | 100 | 5.7 | 12.99 |
| | 1.0 | 0.1 | - | 7 | 3-4 | 100 | 5.5 | 10.84 |
| | 2.0 | 0.1 | - | 13 | 4-5 | 100 | 5.5 | 11.13 |
| | 1.0 | - | + | 23 | 0-5 | **100** | 6.2 | **15.50** |
| BARI | 2.0 | - | - | 13 | 4-5 | 100 | **6.5** | 10.34 |
| tomato9 | 0.5 | 0.1 | - | 2 | 2-3 | 50 | 5.8 | 14.22 |
| | 1.0 | 0.1 | - | 6 | 4-5 | 84 | 5.7 | 13.62 |
| | 2.0 | 0.1 | - | 4 | 5-6 | 75 | 5.8 | 10.12 |
| | 1.0 | - | - | 10 | 3-4 | **100** | 6.3 | **15.81** |
| | 2.0 | - | - | 4 | 3-4 | 75 | **6.5** | 12.54 |
| Bahar | 5.0 | - | - | 2 | 5-6 | 100 | 6.0 | 14.00 |
| | 0.5 | 0.1 | - | 5 | 3-4 | 100 | 7.0 | 14.78 |
| | 1.0 | 0.1 | - | 17 | 3-4 | 100 | 7.3 | 13.81 |
| | 2.0 | 0.1 | - | 11 | 4-5 | 100 | 6.9 | 12.21 |

+, positive response; -, negative response

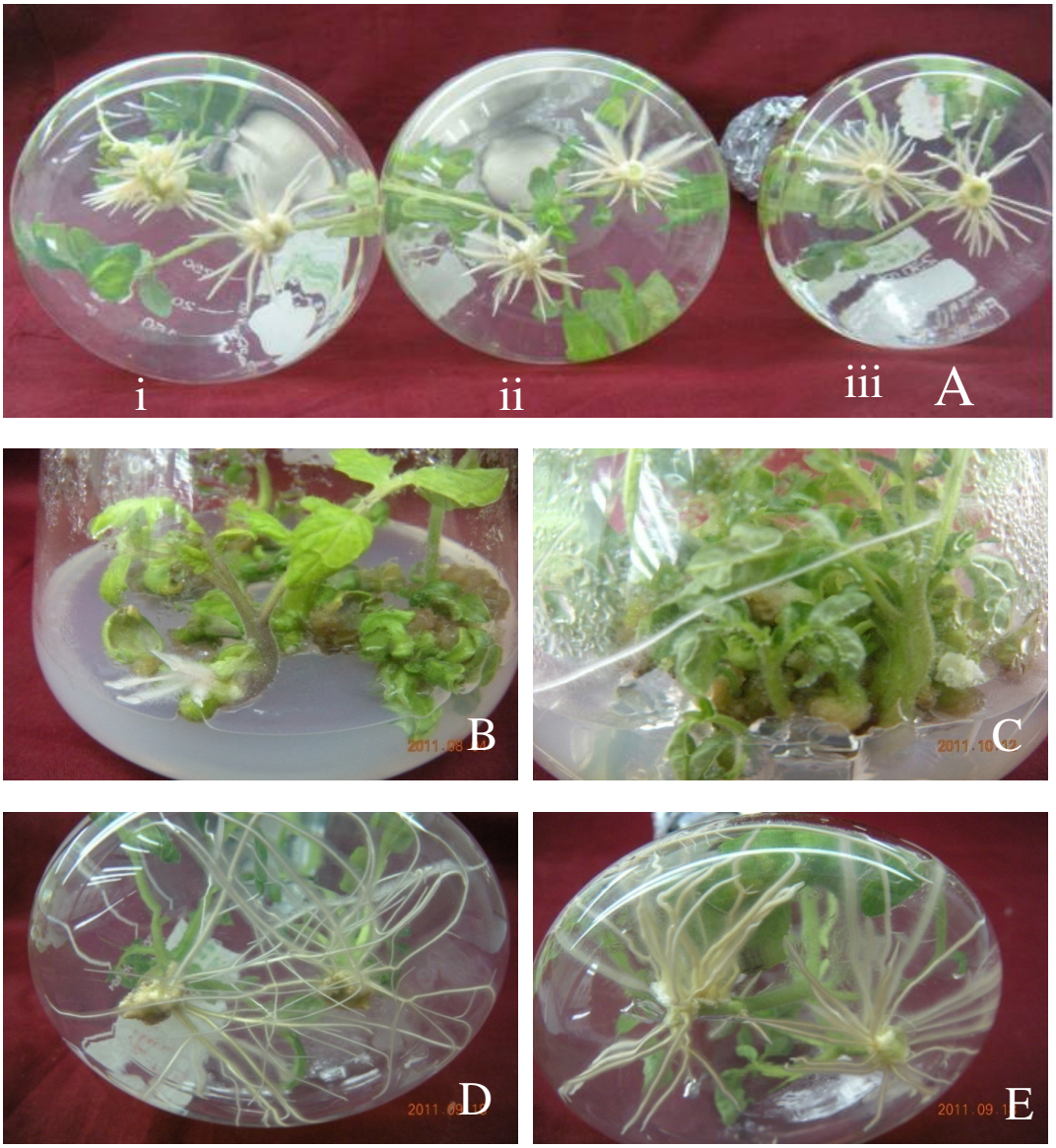



**Fig. 9. A**. Well developed roots of BARI tomato 2 variety (collected from i. 0.5, ii, 1 and iii.2 mg/l BAP containing MS medium) in rooting media which contain ½ MS + 0.2 mg/l IAA. Spontenious root, **B**. In BARI tomato 2, **C**. In BARI tomato 9, (photographs were taken after 45 days of inoculation). **D**. Healthy and long roots in BARI tomato 2. **E**. Healthy and long rootin Bahar variety (D and E photographs were taken after 14 days of rooting).

### 3.5. Establishment and performance of regenerated plantlets in the natural environment:

Healthy well developed rooted plantlets of all three tomato varieties were successfully transplanted into plastic pot containing soil (Fig.10 A). Rooted plantlet survival rate was found to be highest in Bahar variety. Following proper acclimatization the plantlets were transferred to larger pots for their further growth (Fig.10 B-C). The survival rate of plantlets in the larger pots where found to be cent percent. The plantlets flowered within 7-9 weeks after transplantation to the larger pots (Fig.10 D-F).Time requirement for plantlet development, flowering, and numbers of flower per plant were shown in the (Table 3.6). In the natural environment average time requirement for fruit setting was 14-16 days after flowering. In case of BARI tomato 2 and BARI tomato 9, time requirement is 15-20 days but for Bahar variety it was required more (30-35 days) for fruit setting after flowering. It took 3-7 weeks to obtain mature fruits (Figs.10 G-I) in case of BARI tomato 2, BARI tomato 9, Bahar variety required 6-7 weeks for fruit maturation. Fruits per plant were 10, 14 and 6 in BARI tomato 2, BARI tomato 9 and Bahar variety respectively. The average weight of fruits was found highest (41 gm) in Bahar variety followed by 38 gm in BARI tomato 2 and in case of BARI tomato 9 it was 32 gm. BARI tomato 9 contained highest average number of seeds per fruit (38 seeds). BARI tomato 2 and Bahar contained 30 and 20 seeds per fruit, respectively (Table 3.6).

**Table 3.6. Time required for plantlet development and reproductive response in all three tomato varieties**

| Tomato varieties | Days required for seedling development | Days required for regeneration ignition | Matured developed shoot (weeks) | Initiation of roots (days) | Fully developed roots (days) | Flowering after transplantation. (weeks) | Average no of flower/plant after 12 weeks | Average time required for fruit setting after flowering (days) | Average time required for fruit maturation (weeks) | Fruits /Plant | Average weight of fruits (gm) | Average seed number/ Fruit |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BARI tomato 2 | 9-10 | 10-12 | 5-7 | 3-4 | 16-18 | 7-9 | 21 | 14-16 | 3-7 | 10 | 38 | 30 |
| BARI tomato 9 | 8-9 | 11-13 | 5-7 | 4-5 | 16-18 | 8-10 | 21 | 15-20 | 3-7 | 14 | 32 | 38 |
| Bahar | 15-20 | 10-12 | 4-5 | 2-3 | 12-14 | 4-7 | 14 | 30-35 | 6-7 | 6 | 41 | 20 |

### 3.1.12. Viability response of seed collected from ripened fruits of tomato varieties:

The seeds of all three varieties collected from fruits of regenerated plantlets were checked and found viable in the viability test (Table 3.6). BARI tomato 2 and BARI tomato 9 varieties showed 100 percent viability, and Bahar variety showed 96 percent viability in germination test.

**Table 3.7. Viability response of seeds**

| Tomato varieties | Total number of seed taken for germination | Number of seeds germinated | % of germination |
|---|---|---|---|
| BARI Tomato 2 | 50 | 50 | 100 |
| BARI Tomato 9 | 50 | 50 | 100 |
| Bahar | 50 | 48 | 96 |


**Fig. 10.** Acclimatization and reproductive response of regenerated plantlets. Regenerated plantlet of **A.** BARI tomato 2 variety transplanted into soil in small pot covered with plastic bag. **B**. BARI tomato 9 variety transplanted into soil in small pot. **C**. BARI tomato 9 variety transplanted into soil in larger pot. **D.** Flower bloosom of BARI tomato 2 plant. **E**. BARI tomato 9. and **F**. Bahar variety. **G**. Mature fruits on in vitro regenerated plantlets of BARI tomato2. **H**. BARI tomato 9 and **I.** Bahar variety.

### 3.2. *Agrobacterium* mediated genetic transformation of different tomato varieties:

Four tomato varieties, namely, BARI tomato 2, BARI tomato 7, BARI tomato 9 and Bahar and two genetically engineered *Agrobacterium* strainsware used in these study:

1. *Agrobacterium* strain LBA4404 containing pBI121.

2. *Agrobacterium* strain containing pH7WG2_OsNHX1_1.6.

#### 3.2.1. Determination of factors affecting transformation efficiency of four tomato varieties:

In this investigation, *Agrobacterium* strain LBA4404 harboring pBI121 was used to test its compatibility with four different tomato varieties. GUS histochemical assay was done to observe the transfer of marker gene *uidA* (β-glucoronidase). All four varieties were tested with different parameters for the achievement of optimum condition of transformation. Using these suitable parameters genetically engineered *Agrobacterium* strains pH7WG2_OsNHX1_1.6 was used to transform tomato varieties to make them salinity tolerant. Putative transgenic tomato varieties were achieved by the end of this study.

##### 3.2.1.1. Effect of bacterial culture density on transformation:

The effect of optical density (OD) of *Agrobacterium* suspension on transformation efficiency of cotyledonary leaf explants was investigated in this experiment. Bacterial suspension with optical density 0.62, 0.54 and 0.49 were used in this experiment to show its effect on transformation of four tomato varieties. Transformation efficiency was found to be increased with the increase of optical density of *Agrobacterium* suspension. Maximum percentage of GUS positive explants were found at $OD_{600}$ 0.62 and minimum percentage of GUS positive was at $OD_{600}$ of 0.49 in all most all varieties. In BARI tomato7, Bahar, and BARI tomato2, BARI tomato 9 gave 80-100% GUS positive expression in GUS assay at 0.62 $OD_{600.}$ In contrast to this at $OD_{600}$ 0.537, highest number of GUS positive explants was obtained in BARI tomato-7 (87%) (Table 3.8). GUS positive regions were detected mostly at the cut ends (Fig.11 A-C) of the cotyledonary leaf surface, in the mid rib (Fig.11 D-E), within the internal vines (Fig. 11 F) and even in the internal tissues away from the cut surface (Fig.11 G-H) of all four varieties which indicates successful transformation of LBA4404 within the varieties. Here control did not give the blue color (Fig.11 I)

### 3.2.1.2. Effect of incubation period on transformation:

To determine the effect of incubation period on transformation efficiency three different incubation periods, like, 10, 20 and 30 minutes against $OD_{600}$ nm of 0.5-0.7 were tested and it was observed that longer incubation period (20 min) with higher $OD_{600}$ gave better transformation in most of the cases. Interestingly Bahar variety produced highest number of GUS positive explants (98%) in 30 minutes incubation period and 0.62 $OD_{600}$ and in case of BARI tomato-9 it produce highest percentage (85%) of GUS positive transformation was observed in 20 minutes of incubation period and $OD_{600}$ 0.54(Table 3.8).

### 3.2.1.3. Effect of co-cultivation period on transformation efficiency of cotyledonary explant of four tomato varieties:

Three co-cultivation periods were tested in this study, like, 24, 48 and 72 hrs to find out the effect of it on transformation and subsequent regeneration capacity. Among this co- cultivation period of 48 hours were found to be the best for all most all varieties. In transient GUS assay it was found that BARI tomato 7 and Bahar shows highest response, 90 percent and 89 percent, respectively (Table3.9). With the decrease of co-cultivation period the percentage of GUS expression also decreases. Explants showed over growth of bacteria when co-cultivation period increased by 3 or more days and it was also found that the explants fail to regenerate afterwards due to necrosis (Fig.12). From this experiment it was found that 48 hours of co-cultivation period was the best for transformation.

**Table 3.8. Effect of optical density ($OD_{600}$) and incubation period of *Agrobacterium* suspension on transformation efficiency of four tomato varieties**

| Tomato varieties | $OD_{600}$ | Incubation period (minutes) | Percentage of GUS positive explant |
|---|---|---|---|
| BARI tomato 2 | 0.49 | 10 min | 60 |
| | | 20 min | 80 |
| | | 30 min | 70 |
| | 0.54 | 10 min | 50 |
| | | 20 min | 80 |
| | | 30 min | 80 |
| | 0.62 | 10 min | 60 |
| | | **20 min** | **92** |
| | | 30 min | 90 |
| BARI tomato 7 | 0.49 | 10 min | 65 |
| | | 20 min | 70 |
| | | 30 min | 68 |
| | 0.54 | 10 min | 60 |
| | | 20 min | 75 |
| | | 30 min | 87 |
| | 0.62 | 10 min | 75 |
| | | **20 min** | **100** |
| | | 30 min | 98 |
| BARI tomato 9 | 0.49 | 10 min | 45 |
| | | 20 min | 50 |
| | | 30 min | 50 |
| | 0.54 | 10 min | 56 |
| | | **20 min** | **85** |
| | | 30 min | 70 |
| | 0.62 | 10 min | 70 |
| | | **20 min** | **80** |
| | | 30 min | 75 |
| Bahar | 0.49 | 10 min | 50 |
| | | 20 min | 60 |
| | | 30 min | 60 |
| | 0.54 | 10 min | 65 |
| | | 20 min | 70 |
| | | 30 min | 70 |
| | 0.62 | 10 min | 75 |
| | | 20 min | 85 |
| | | **30 min** | **96** |

Total 20 explants were used and out if that 9-12 were tested for GUS activity.

**Table 3.9**. **Influence of co-cultivation periods on transformation efficiency of different tomato varieties**

| Tomato varieties | Co-cultivation period (hours) | No. of explants assayed in GUS assay | % of GUS +ve explants |
|---|---|---|---|
| BARI tomato 2 | 24 | 6 | 66 |
| | 48 | 8 | **88** |
| BARI tomato 7 | 24 | 7 | 71 |
| | 48 | 10 | **90** |
| BARI tomato 9 | 24 | 5 | 60 |
| | 48 | 8 | **88** |
| Bahar | 24 | 6 | 83 |
| | 48 | 9 | **89** |

Total no of 20 explants used in each case. Infection time 20 minutes with 0.62 $OD_{600.}$

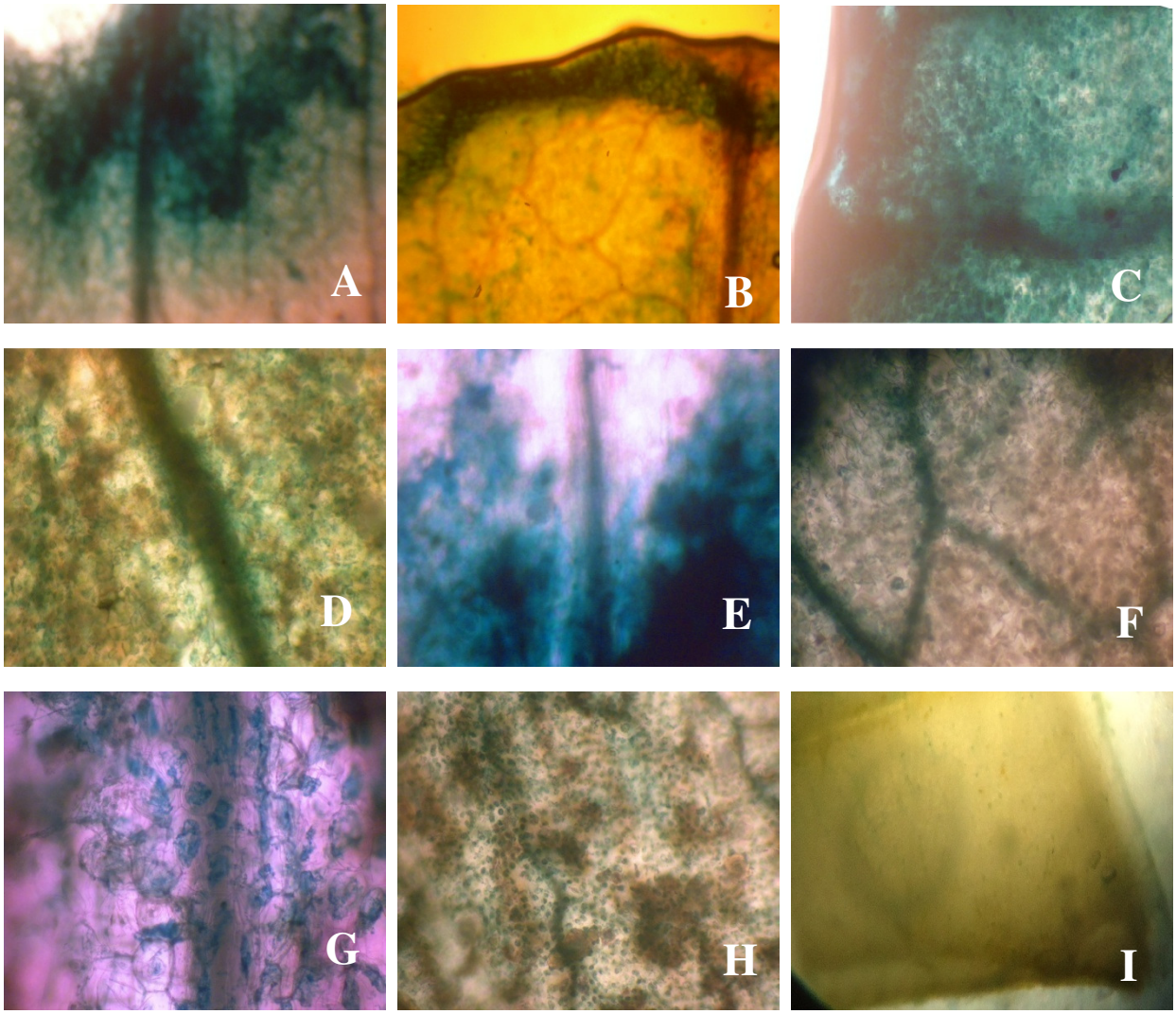



**Fig. 11.** Color representation in GUS histochemical assay. **A-C**. Stereomicroscopic views of GUS assay at the cut surface in BARI tomato 7, BARI tomato 9 and Bahar variety, respectively. **D-E**. GUS expression at the midrib region BARI tomato 2 and BARI tomato 7, **F**. Blue color region within the inner veins of BARI tomato 2, **G-H**. GUS activity within the internal tissues of BARI tomato 7 and Bahar variety**. I**. Control.

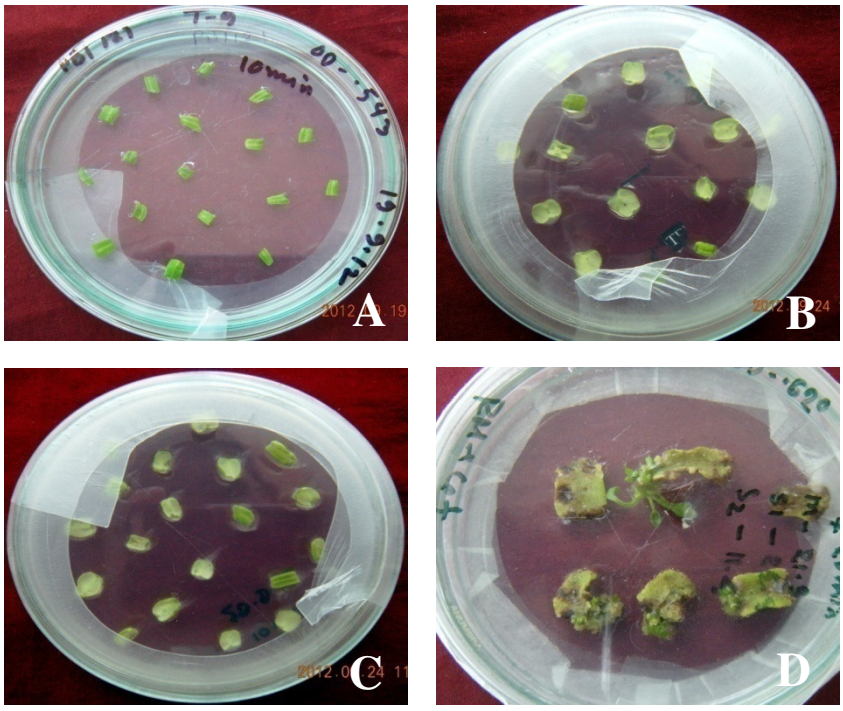



**Fig. 12**. Factors affecting transformation **A**. Infected explants of BARI tomato 9 variety, **B**. Bacterial growth in BARI tomato 7 variety, **C**. Bacterial over growth after three days incubation, **D**. Necrosis due to bacterial over growth of bacteria.

### 3.2.2. Determination of antibiotic concentration for selection medium:

Cotyledonary leaf explants of BARI tomato 2 variety were tested for antibiotic sensitivity. Various concentrations of kanamycin and hygromycin antibiotics were used in this experiment. Control treatment (without antibiotic) was also used to compare the effect.

#### 3.2.2.1. Kanamycin sensitivity test:

Different concentrations of kanamycin (0, 50, 100, 150 and 200mg/l) were tested in this study. It was found that the survival percentage decrease with the increase of kanamycin concentration in the regeneration medium. The explants become albino at 100mg/l kanamycin and become brown and dark brown and finally died at 150 mg/l and 200 mg/l kanamycin concentrations respectively (Fig 13. A-F).

So, the optimum kanamycin concentration was found to be 150 mg/l for the selection of transformed shoots. Shoot surviving in this selection pressure for more than 15 days, will be considered as putative transformed.

**3.2.2.2. Hygromycin sensitivity test:**

Different concentrations of hygromycin (1, 2, 4, 6, 8 and 10mg/l and control) were tested in this study. It was done to identify the minimum tolerance level of cotyledonery leaf explants (Table 3.10). In the selection medium it was found that 40% explants survived when it was subjected to 1 mg/l hygromycin containing medium. The survival rate was reduced to 20% when it was in 2mg/l hygromycin containing media and the survival rate fallen to 0% when the hygromycin concentration was more than 4mg/l, which means no explants can survive at 4mg/l and more than that hygromycin concentration (Fig. 14 A-F).

**Table 3.10**. **Effect of various concentrations of hygromycin as a selection medium**

| Hygromycin concentration (mg/l) | Percentage of shoot formation | Percentage of survival | Visual appearance |
|---|---|---|---|
| 0 | 60 | 100 | Green |
| 1 | 25 | 40 | Green |
| 2 | 10 | 20 | Greenish brown |
| 4 | 0 | 0 | Albino |
| 6 | 0 | 0 | Albino and brown |
| 8 | 0 | 0 | Albino and brown |
| 10 | 0 | 0 | Brown |

Total no of 20 explants used in each case. Data was collected after 45 days of inoculation.

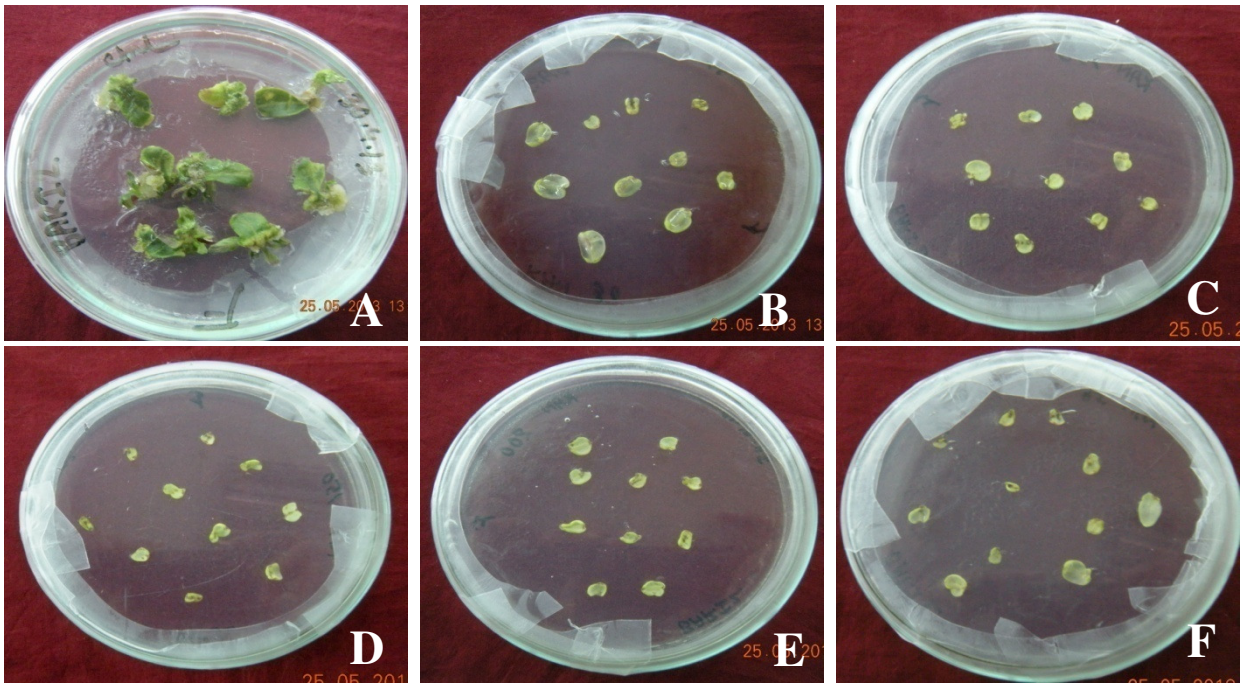



**Fig. 13.** Effect of various concentrations of kanamycin on cotyledonary leaf explants, **A**. Control (without kanamycin), **B**. 50 mg/l kanamycin in BARI tomato 2 variety, **C**. 100 mg/l kanamycin in BARI tomato 2 variety, **D**. 150 mg/l kanamycin effect in BARI tomato 2 variety, **E**. 200mg/l kanamycin in BARI tomato 2 variety, **F**. 200 mg/l kanamycin effect in case of BARI tomato 9 variety. (Photographs were taken 40 days after inoculation).

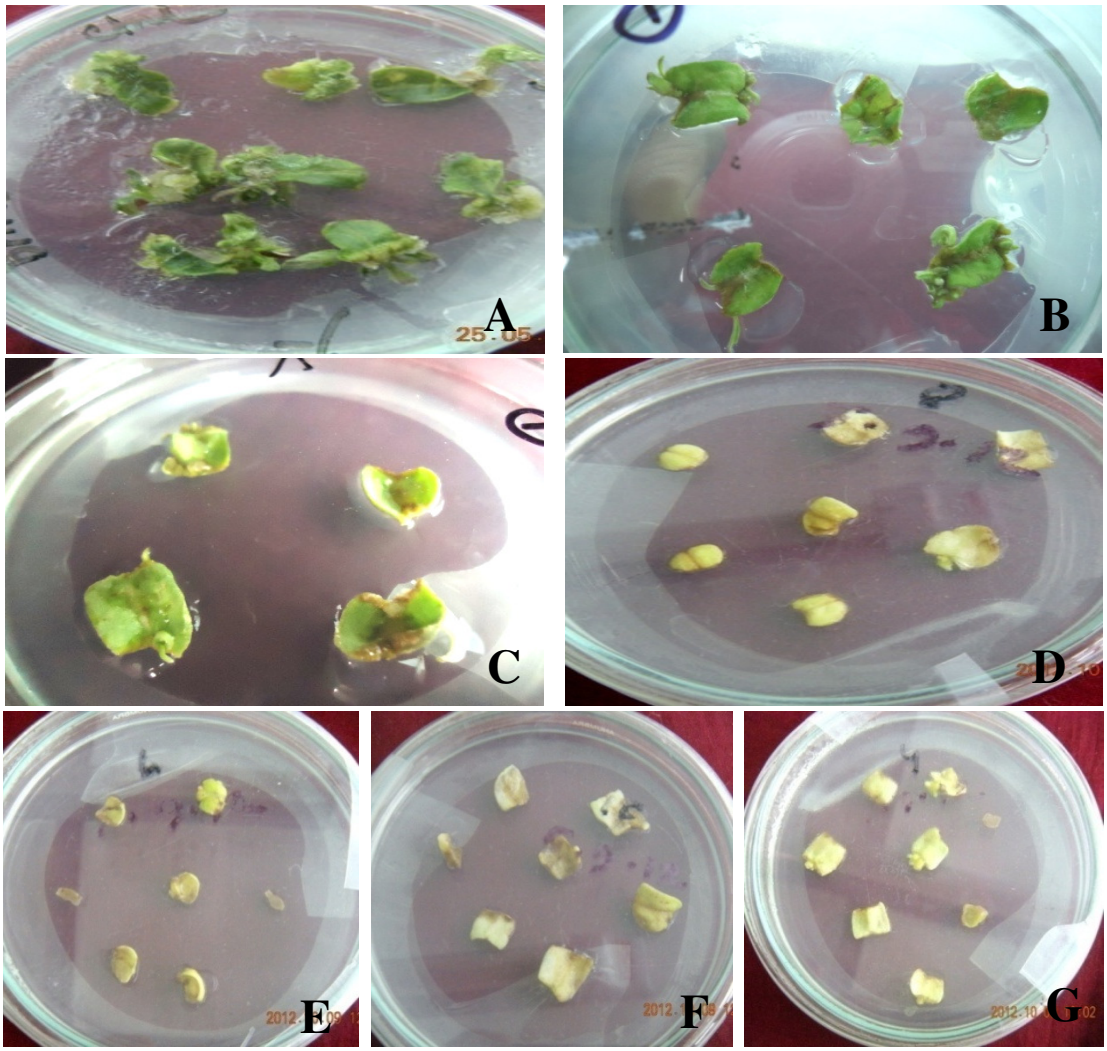



**Fig. 14**. Effect of various concentrations of hygromycin on cotyledonary leaf explants in Bahar variety, **A**. Control (without hygromycin), **B**. 1 mg/l hygromycin, **C**. 2 mg/l hygromycin, **D**. 4 mg/l hygromycin, **E**. 6 mg/l hygromycin, **F**. 8 mg/l hygromycinand **G**. 10 mg/l hygromycin effect. (Photographs were taken 40 days after inoculation).

### 3.2.3. Percentage of regeneration following infection of four tomato varieties (transformed with pBI121) on selection medium:

After transformation, explants were placed on the regeneration medium (MS + 1 mg/l BAP) containing 200 mg/l cefotaxime to control Agrobacterial overgrowth. After one week explants were placed in the selection media containing cefotaxime and 50mg/l kanamycin to obtained regeneration from transformed explants. Green explants which survived in the selection medium were then placed in the media containing increased amount of kanamycin 100mg/l and again after two weeks in 150mg/l kanamycin containing selection medium. However, control experiment without infection also carried out (Fig.15 A). In the selection medium some shoots become albino and some shoots died after browning. (Fig.15 B-D). Thus, shoot survived in the selection pressure for more than 15 days were considered as putative transformed plants (Fig.15 E-I)

The highest average regeneration rate was found 56% in Bahar variety and second highest was found 46% in BARI tomato 2 and both varieties were transformed well at $OD_{600}$ 0.56, BARI tomato 7 and BARI tomato 9 also transformed well (43%, 30%) in kanamycin supplemented media. All four varieties showed better result in 30 min incubation period than that of 20 min. Results are shown in (Table 3.11).

**Table 3.11. Percentage of regeneration following infection on media containing kanamycin**

| Tomato varieties | Incubation period (min) | No. of explants inoculated | No. of explants retain green colour | Percentage of transformation |
|---|---|---|---|---|
| BARI tomato 2 | 20 min | 30 | 12 | 40 |
| | 30 min | 30 | 14 | 46 |
| BARI tomato 7 | 20 min | 30 | 11 | 36 |
| | 30 min | 30 | 13 | 43 |
| BARI tomato 9 | 20 min | 30 | 8 | 26 |
| | 30 min | 30 | 9 | 30 |
| Bahar | 20 min | 30 | 10 | 33 |
| | 30 min | 30 | 17 | 56 |

### 3.2.4. Transformation frequency of four tomato varieties (transformed with pH7WG2_OsNHX1_1.6) based regeneration on selection media:

All four tomato varieties were infected with *Agrobacterium* harboring pH7WG2_OsNHX1_1.6 with higher $OD_{600}$ value, and 20 minutes and 30 minutes incubation period. Regeneration media (MS + 1mg/l BAP) containing hygromycin (4 mg/l) was used as selection media. (Fig. 16 A-F). The highest rate of transformation was found in Bahar variety (23%) with 20 min incubation period and $OD_{600}$ 0.76, while BARI tomato 7 shows lowest regeneration response (6%) with 30 minutes incubation period and $OD_{600}$ 0.78 (Table3.12).

**Table 3.12. Percentage of regeneration following infection on media containing hygromycin**

| Tomato varieties | $OD_{600}$ | Incubation period (min) | No. of explants inoculated | No. of explants retain green color | Percentage of transformation |
|---|---|---|---|---|---|
| BARI tomato 2 | 0.62 | 30 min | 30 | 4 | 13 |
| BARI tomato 7 | 0.52 | 20 min | 30 | 3 | 10 |
| | 0.78 | 30 min | 30 | 2 | 6 |
| BARI tomato 9 | 0.52 | 20 min | 30 | 5 | 16 |
| Bahar | 0.76 | 20 min | 30 | 7 | 23 |

### 3.2.5. Effect of selection media on root induction from transformed shoots:

Antibiotics (kanamycin 100 mg/l or hygromycin 4 mg/l) selection pressure found to have negative effect on rooting. After proper shoot development, they were transferred to rooting media with kanamycin selection, however no root formation was observed. To achieve rooting, hormonal concentration was increased/ changed to 0.4 mg/l IBA. But that condition was also found not to be suitable for rooting. Interestingly, it was found that prolong culture of shoots at selection pressure in shooting media resulted root formation (Fig. 16 G-L).

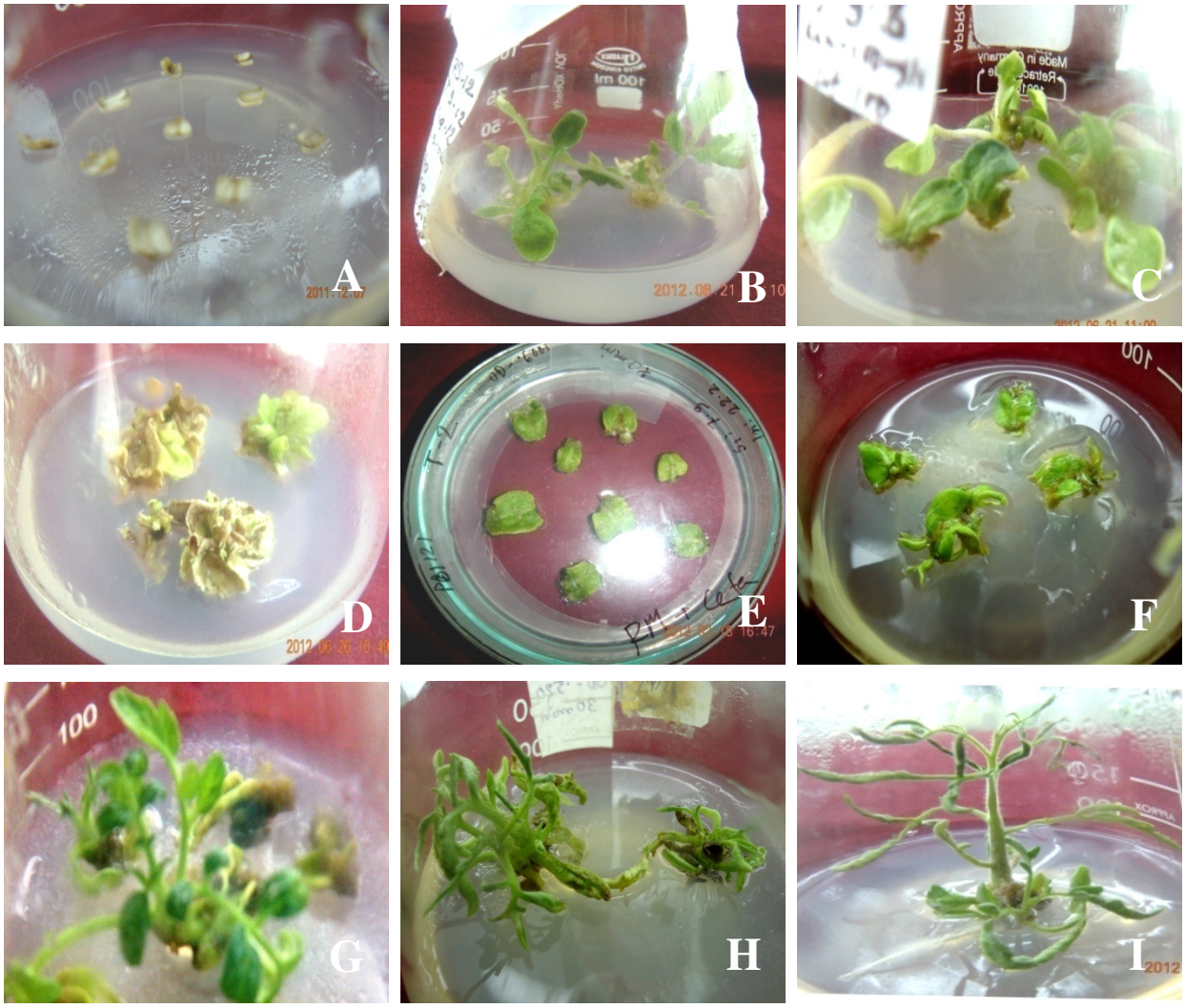



**Fig. 15.** Regeneration response on selection media. **A.** no regeneration in control (uninfected explants in 150 mg/l kanamycin). **B-C**. Albinism in the untransformed region in 100 mg/l kanamycin **B**. BARI tomato 9, C. BARI tomato 7, **D**. Browning of explants in Bahar variety, **E**. BARI tomato 2 with 50 mg/l selection pressure, **F**. Bahar variety with 100 mg/l kanamycin selection, (Photographs were taken after 30-45 days of incubation), **G-I**. Shoot formation in selection media containing 150 mg/l kanamycin **G**. BARI tomato 2, **H**. BARI tomato 7 and **I**. BARI tomato 9 variety (Photographs were taken after 60 days of incubation).


**Fig. 16**. Hygromycin sensitivity in different varieties. **A**. Bahar variety, **B**. BARI tomato 7, **C.** BARI tomat 2, **D**. BARI tomato 9 in 6 mg/l hygromycin containing media. (Photographs taken 30-45 days after infection). **E-F**. Putative transformed shoot in 10 mg/l hygromycin containing media, **E.** BARI tomato 7, **F**. Bahar variety (Photographs taken 60 days after incubation). **G**. Rooting response of putative transformed shoots of Bahar variety in ½ strength MS media with 0.2 mg/l IAA was not produced any root after 7 days of rooting. **H**. BARI tomato 7 produced only 2 long roots in 0.4 mg/l IBA containing rooting media. **I-J**. Spontaneous root in Bahar variety. **K**. Long spontaneous root in Bahar variety. **L**. Potted putatively transformed plant.

# Chapter 4

## Discussion

The purpose of the present investigation was to study *in vitro* regeneration response of three varieties of tomato (*Lycopersicon esculentum* Mill.), namely BARI Tomato 2, BARI Tomato 9 and Bahar and establishment of an *Agrobacterium*-mediated transformation protocol using these three varieties along with BARI tomato 7 variety. It was done in two phases. In the first phase of this study, *in vitro* regeneration protocol was studied and in the second phases using this protocol transformation study was carried out. In case of transformation pBI121 (containing *nptII* marker gene and *uidA* gene) and pH7WG2_OsNHX1_1.6 construct (Containing OsNHX1, Na+/H+ antiporter gene, cloned from rice) was used to determined the optimum conditions for transformation and to obtain transgenic salt tolerant tomato varieties.

Tissue culture protocol is a prerequisite for genetic transformation and the tissue culture protocol starts with selection of appropriate hormonal supplementations for *in vitro* regeneration.

The seeds were grown aseptically in MS medium. In the present study, after seed sterilization different types of agitation were performed to evaluate the effect on seed germination response. When seeds were kept in laboratory conditions (after sterilization) at 25ºC with or with continuous shaking in an orbital shaker, it was found that there was no significant difference among different types of agitations. 24 hours continuous shaking was adopted for further studies as it gave good regeneration percentages and took less time for germination and damage less embryo in all three varieties. Chowdhury (2009) and Das (2011) found the similar germination response in BINA Tomato 3 when seeds were shaken continuously on a shaker for few hours after surface sterilization before placing on germination media. Ferdous (2012) found 24 hours of continuous shaking best in case of BARI Tomato 2, BARI Tomato 3, BARI Tomato 14, BARI Tomato 15 and BINA Tomato 3 varieties which support my findings. While Sarker (2013) found highest germination response in both without shaking and continuous shaking condition in BARI Tomato 3, BARI Tomato 7 and BINA Tomato 3 varieties when seeds were kept for 48 hours.

*In vitro* regeneration of tomato varieties using various explants, viz. cotyledons, hypocotyls, epicotyls, meristem, leaf, stems, roots, internodes, petiole, anthers and inflorescences has been

reported (Padmanabhan *et al*., 1974; Behki *et.al*., 1980; Kartha *et al*., 1976; Ohki *et al*., 1978; Fray and Earl, 1996; Gubis *et al*., 2003; Raj *et al*., 2005; Islam, 2007; Chowdhury, 2009; Das, 2011; Ferdous, 2012; Sarker, 2013). Among these explants cotyledonary leaf segments have reported to be the most responsive explants for tomato regeneration in various tomato varieties including BARI Tomato 2, BARI Tomato 3, BARI Tomato 5, BARI Tomato 7, BARI Tomato 14, BARI Tomato 15, BINA Tomato 3, Bahar, Pussa Rubby and Maple (Islam, 2007; Chowdhury, 2009; Das, 2011; Ferdous, 2012; Sarker, 2013). For this reason in the present study, cotyledonary leaf explants were collected from aseptically grown seedlings. MS medium was used as basal media for *in vitro* regeneration, as it is reported to be the most effective media for tomato regeneration (Mirgish *et al*., 1995; Costa, 2000; Gubis *et al*., 2003; Islam, 2007; Chowdhury, 2009; Das, 2011; Ferdous, 2012; Sarker, 2013).

Cotyledonary leaf explants of several verities reported to give best *in vitro* shoot regeneration response when Zeatin was added in addition to IAA in MS media (Costa *et al*., 2000; Ahasan *et al*., 2007) but in the present study, cotyledonary leaf explants were excised into several pieces and placed in various concentrations and combinations of BAP and IAA containing regeneration media for development of shoots.

Shoot initiation was found in all three varieties. Among all the combinations, 1 mg/l BAP containing MS medium showed the best result with highest number of shoots in BARI Tomato 2 and BARI Tomato 9 and combination of 1 mg/l BAP and 0.1 mg/l IAA containing MS medium showed the best result with highest number of shoots in Bahar variety. In a report, Chowdhury (2009) reported that 2.0 mg/l BAP containing MS media was found to be the best for shoot formation for BINA tomato 3 variety. She also reported that 2.0 mg/l BAP and 0.1mg/l IAA containing MS media were also found to be the best for shoot formation for varieties BINA 5, Bahar and Pussa Rubby. However, a different report came from Ferdous, (2012) where 7mg/l BAP was found to be the best for shoot formation in BARI Tomato 2 variety. But such high BAP supplementation was found to give abnormal morphology in the present varieties.

Subculture of explants was essential for achieving elongated and maximum shoot. Shoot regeneration found to be suppressed by callus formation at the base of the regenerated shoot if subculture was not done. Similar result was reported earlier in tomato regeneration (Rashid and Bal, 2010). In this study it was found that BARI tomato 9 produced multiple shoot after several

subcultures in the same media. It was observed to give up to 20 shoots/explant after three to four sub-cultures but the appearance of the shoot were not healthy. To improve the morphology of shoots, subculture was done in 2X $KNO_3$ containing MS media without hormone and in only MS media. Shoot sub-cultured in MS media produced root and in 2X $KNO_3$ produce both healthy shoot and root. Abnormal morphology, such as, abnormal leaf formation, brunching and vitrificatin was found by Chowdhury and Islam (2012) when subculture was done in same media and rooting of explants was found when only MS media was used for regeneration (Chowdhury, 2009).

Root formation is an essential step for producing plantlets. Rooting occur on IAA, IBA and NAA supplemented media but IAA has been reported to be more preferred rooting hormone by others (Jawahar *et al.*, 1997; Oktem *et al.*, 1999; Costa., 2000; Sheeja *et al.*, 2004; Islam., 2007; Chowdhury, 2009; Das, 2011; Ferdous, 2012). Oktem *et al.* (1999) and Costa *et al.* (2000) used IAA in full strength of MS media for rooting whereas Sheeja *et al.* (2004) used IAA with half strength MS media which is similar to the present findings. On the other hand Zagorska *et al.* (2004) found rooting in half strength MS media with 0.2 mg/l IBA and 0.5 mg/l $GA_3$. In the present study, *in vitro* rooting experiment was carried out in half strength of MS medium with different concentration of auxin and it was observed that ½ strength MS media containing 0.2mg/l IAA showed best response for all three varieties, such as, BARI tomato 2, BARI tomato 9 and Bahar varieties which is similar to the findings of Chowdhury (2009) and Das (2011). In the present experiment, rooting media (half strength of MS medium containing 0.2 mg/l IAA) further analysis was done this suitable to check the response of root formation of shoots regenerated in different combinations and concentrations of BAP and IAA containing MS media. All these three varieties respond almost equally to this medium. There were no significant differences in response of root formation of shoots collected from different combination and concentration of BAP and IAA containing MS medium which supported by Sarker (2013).

After completion of rooting stage, plantlets were acclimatized in natural condition where they flowered and set fruits. The seeds from all varieties collected from fruits regenerated plantlets were found viable in the viability test. Seeds of BARI Tomato 2 and BARI Tomato 9 variety showed 100 percent, while Bahar showed 96 percent viability in germination test. Chowdhury (2009) also found 90% to 100% viability in germination test of seeds collected from Bahar,

BINA Tomato 3, BINA tomato 5 and Pussa Rubby varieties. Das, (2011) found the highest percentage of germination (82.5%) in BINA Tomato 3 and the lowest percentage of germination in Maple variety. Ferdous, (2012) also found 82% viability in BINA Tomato 3 variety, the highest percentage of germination in BARI Tomato 14 (92%) and the lowest percentage of germination in BARI Tomato 3 variety. Sarker (2013) found 100% viability in BARI Tomato 7 and 95% viability in BARI 3 and BINA Tomato 3 in germination test. So in general our viability test is in agreement of previous result and also similar to parent material.

Among all the varieties, BARI Tomato 2 and BARI Tomato 9 showed the highest number of flowers (21) per plant and Bahar showed 14 flowers per plant. Das (2011) reported that BARI Tomato 3 and BINA Tomato 3 showed the highest number of flower (9).

BARI tomato 9 showed the highest number of fruits per plant and the highest number of seeds per fruit. However, Islam (2007) and Das (2011) reported BINA tomato 3 showed the highest number of seeds per fruit. Ferdous (2012) also mentioned in her MS thesis that BARI Tomato 14 showed highest number of fruit per plant and highest number seeds per fruit, while Sarker (2013) found highest number of fruit and highest number of seeds per fruit in BARI Tomato 7 when they working with BARI tomato 2, BARI tomato 7, BARI tomato 14, BARI tomato 15 and BINA tomato 3 varieties.

Finally, for all the three varieties, 1 mg/l BAP containing MS medium showed the best result with highest number of shoots in BARI Tomato 2 and BINA Tomato 9 and combination of 1 mg/l BAP and 0.1 mg/l IAA containing MS medium showed the best result with highest number of shoots in Bahar variety. There were no significant differences in response of root formation of shoots collected from different combination and concentration of BAP and IAA containing MS medium.

From the above experiment, it is evident that, this study has established *in vitro* regeneration methodology and has proved the effectiveness of various plant growth regulators on all the three varieties. This study will help to carry on further research on these tomato varieties for improvement by using gene transfer technology.

In the second phase of this study *Agrobacterium*-mediated genetic transformation of tomato was done to determine a suitable transformation protocol and finally the main goal was to obtain transgenic salt tolerant tomato varieties.

Four tomato varieties, namely, BARI tomato 2, BARI tomato 7, BARI tomato 9 and Bahar were tested with *Agrobacterium* strain LBA4404 containing pBI121 (containing *nptII* marker gene and *uidA* gene) for the determination of factors affecting transformation. Bacterial concentration, inoculation period, co-cultivation period and selection antibiotic concentrations were optimized in this study which is supported by number of report available (Islam, 1998; Sharma *et al*., 2009; Gou *et al.,* 2012*).*

Transformation efficiency was found to be increased with the increase of optical density of *Agrobacterium* suspension; all the varieties gave 80-100% GUS positive expression in GUS assay at 0.62 $OD_{600}$ and minimum 47% was found in BARI tomato 9 variety with 0.49 $OD_{600.}$ Similar result was reported by Islam *et al.* (2010) and Ferdous (2012) in Bahar, BINA tomato 3, BINA tomato 5, BARI tomato 3, BARI tomato 14, BARI tomato 15 and Pusa Ruby varieties.

Incubation period influence the efficiency of *Agrobacterium*-mediated transformation system (Men *et al*., 2003) and it differ among the plant species (Mendel and Mansch, 1995). 20 minutes incubation period with higher $OD_{600}$ 0.62 gave better transformation (80%-100%) in BARI tomato 2, BARI tomato 7 and BARI tomato 9 variety, interestingly Bahar variety produced highest number of GUS positive explants 98% in 30 minutes incubation period and 0.62 $OD_{600.}$ 30 minutes was reported to be optimum for tomato varieties BARI tomato 3, BARI tomato 14, BINA tomato 3, Pusa Ruby, Akra Viska and Sioux when transformed with *Agrobacterium* strain by Ferdous (2012) and Sharma *et al.* (2009).

Among three co-cultivation periods 48 hours was found best (88%-90%) for all four varieties in transient GUS assay. 48 hours co-cultivation period was also supported by the reports of Ferdous (2012); Paramesh *et al*. (2010); Mythili *et al*. (2011) and Patil *et al*. (2002) for BARI tomato 3, BARI tomato 14, BARI tomato 15, BINA tomato 3, Megha (L 15), Pusa Ruby and Arka vikas. In this study it was also found that longer co-cultivation period delay growth of transformed explants due to over growth of bacteria which supported by Christoph *et al*. (1997).

Antibiotics are required to use in the regeneration medium for the elimination of *Agrobacterrium* after co-cultivation. A commonly used antibiotic for the removal of A. *tumifaciens* from plant tissue is cefotexime (Ling *et al*., 1998). In the present study 200 mg/l cefotaxim was used in selection medium which prevented bacterial over growth completely in the tomato varieties. Similar approach was also reported in tomato varieties BARI tomato 3, BARI tomato 14, BARI tomato 15, BINA tomato 3 (Ferdous, 2012; Patil *et al*., 2002).

In the present study, kanamycin sensitivity was tested using different concentrations of this antibiotic in the regeneration media which was compared with control experiment (without kanamycin) and finally 150 mg/l were used for selection. Ferdous (2012) used the same kanamycin concentration for selection of transformed tomato varieties. A higher concentration (200 mg/l) of kanamycin was used by Chowdhury ( 2009) in Bahar, BINA tomato 3, BINA tomato 5 and Pusa Ruby varieties. Lower concentrations 100 mg/l and 50 mg/l were used in different studies (Patil *et al*., 2002; Mythili *et al*., 2011).

Different concentrations of hygromycin were tested in this study and finally more than 4mg/l hygromycin were used for selection because the survival rate fallen to 0%. Ferdous (2012) found more than 5mg/l hygromycin for selection of transformed tomato varieties. However Choudhry and Rashid (2010) reported 25 mg/l hygromycin as a lethal dose for selection of cultivars, Riogrande, Roma and Summer Set. Increased concentration of hygromycin (40 mg/l) was also reported during selection of drought tolerant cv. Pusa Ruby (Roy *et al.,* 2006).

In the present study, while calculating regeneration percentage of transformed shoot, it was found that, transformation of four tomato varieties with *Agrobacterium* strain containing pBI121, gave higher transformation frequencies and the highest regeneration percentage was found 56% in Bahar variety. Again, while transformation was done by pH7WG2_OsNHX1_1.6 then highest transformation percentage (23%) was also found in Bahar variety.

Previous studies on the genetic transformation of tomato have reported transformation efficiencies ranging from 6 to 37% (Hamza and Chupeau, 1993; Van Roekel *et al*., 1993; Frary and Earle, 1996; Ling *et al*., 1998; Vidya *et al*., 2000; Hu and Phillips, 2001; Park *et al*., 2003). The transformation efficiency exceeded 40% of the explants in Micro-Tom, while working with cotyledon explants of tomato inoculated with *Agrobacterium tumefaciens C58C1 Rif R* harboring

the binary vector pIG121Hm (Sun *et al*.,2006). Transformation frequency of 41.4% was confirmed for tomato varieties Pusa Ruby, Arka Vika sand Sioux (Sharma *et al.,* 2009). *Agrobacterium*-mediated transformation protocol of tomato *cv.* 'Arka Vikas' using dreb 1A gene under Rd 29A promoter in pCAMBIA2301 binary vector was optimized transformation efficiency (34%) (Manamohan *et al*., 2011). 49.5% transformation efficiency was in tomato cultivar Pusa Ruby when transformation was performed with TLCV-CP construct (Raj *et al.*, 2005).

# Chapter 5
# Reference


Abdeen A, Virgos A, Olivella E, Villanueva X, Aviles R, Gabarra, Prat S (2005) Multipleinsect resistance in transgenic tomato plants over-expressing two families of plant proteinase inhibitors. Plant MolBiol 57: 189-202.

Ahsan N, Lee SH, Lee DG, Anisuzzaman M, Alam MF, Yoon HS, Choi MS, Yang JK, Lee BH (2007) The effects of wounding type, preculture, and infection method and co-cultivation temperature on the *Agrobacterium*-mediated gene transfer in tomatoes. Annual Applied Biology 151: 363-372.

Applewhite PB, Sawhney RK, Galston AW (1994) Isatin as an auxin source favoring floral and vegetative shoot regeneration from calli produced by thin layer explants of tomato pedicel. Plant Growth Regul 15: 17–21.

Arillaga I, Mascarell GR, Gisbert C, Sales E, Montesinos C, Serrano R, Moreno V (1998) Expression of the yeast *HAL2* gene in tomato increases the *in vitro* salt tolerance of transgenic progenies. Plant Sci 136: 219-226.

Asif S (2013) Salinity reaches up to Magura archive.thedailystar.net/beta2/news/salinity-eaches-up-to-magura/ Accessed 25 August, 2013.

Atherton JG, Rudich J (eds) (1986). In: Tomato crop; Scientific Basis for Improvement. Chapman and Hall, London, New York. pp.661.

Atwal SA (1986) Agricultural pests of India and South East Asia. Kalyani Publishers, New Delhi.

BBS, (2010). Statistical Year Book of Bangladesh. Bangladesh Bureau of Statistics, Ministry of Planning, Government of People Republic of Bangladesh, Dhaka Bangladesh.

Begum S, Miah AJ (1993) Study on callus induction and plant regeneration in tomato. Plant Tissue Culture 3(2): 81-84.

Behki RM, Lesley SM (1980) Shoot regeneration from leaf callus of *Lycopersicon esculentum*. Z. Pflanzenphysiol 98: 83–87.

Bhatia P, Ashwath N, Senaratna T, Midmore D (2004) Tissue culture studies of tomato (*Lycopersicon esculentum*). Plant Cell Tissue Organ Culture 78: 1-21.

Bird CR, Smith CJS, Ray JA, Moureau P , Bevan MJ, Birds AS, Hughes S, Morris PC, Grierson D, Schuch W (1988) The tomato polygalacturonase gene and ripening specific expression in transgenic plants. Plant Mol Biol 11: 651–662.

Bolton GW, Nester EW, Gordon PM (1986) Plant phenolic compounds induce the expression of the *Agrobacterium tumefaciens* loci needed for virulence. Science 232: 983- 985.

Bose TK, Som MG (1990) Vegetable Crops in India. Naya Prokash, 206 BidhanSarani, Calcutta, India, pp: 249.

Chan YL, Prasad V, Sanjaya, Chen KH, Liu PC, Chan MT, Cheng CP (2005) Transgenic tomato plants expressing an *Arabidopsis thionin* (Thi2.1) driven by fruitinactive promoter battle against phytopathogenic attack. Planta 19: 1432-2048.

Chaudhury Z, Rashid H (2010) An improved Agrobacterium mediated transformation in tomato using hygromycin as a selective agent. African journal of biotechnology. 9 (13): 1882-1891.

Cheeseman JM (1988) Mechanisms of salinity tolerance in plants. Plant Physiol 87:57-550.

Cheng ZM, Schnurr JA, Kapaun JA (1998) Timentin as an alternative antibiotic for suppression of *Agrobacterium tumefaciens*in genetic transformation. Plant Cell Rep 17:646–649.

Chowdhury J. (2009) Establishment of *in vitro* regeneration and transformation protocol in tomato (*Lycopersicon esculentum* Mill). MS Thesis, BRAC University, Dhaka.

Christoph F, Marc K, Lutz N (1997). Stable transformation of an *Arabidopsis* cell suspention culture with firefly luciferase providing a cellular system for analysis of chaperone activity *in vivo*. The plant cell. 9: 2171-2181.

Chowdhury J, Islam A (2012) A comparativestudy on in vitro regeneration frequency of four locally grown popular tomato ((*Lycopersicon esculentum* Mill) varieties of Bangladesh. 2nd Annual Biotechnology Conference on Advances in Biotechnology (Bio Tech 2012), doi 10.5176/2251-2489_BioTech42.

Chyi YS, Phillips GC (1987) High efficiency *Agrobacterium*-mediated transformation of *Lycopersicon* based on conditions favourable for regeneration. Plant Cell Rep. 6: 105–108.

Compton ME,Veilleux RE (1991) Shoot, root and flower morphogenesis on tomato inflorescence explants. Plant Cell Tiss Org Cult 24: 223–231.

Cortina C, Culianez-Macia FA (2005) Tomato abiotic stress enhanced tolerance by trehalose biosynthesis. Plant Sci 169: 75-82.

Costa GMC, Nogueira FTS, Otoni WC, Brommonschenkel SH (2000) *In vitro* regeneration of processing tomato (*Lycopersicon esculentum* Mill.). “IPA-5” and “IPA-6”. Ciencia-e-Agrotechnologia 24(3): 671-678.

Cox S, (2000) I Say Tomayto, You Say Tomahto. http://lamar.colostate.edu/~samcox/Tomato.html. Accessed 26 June 2013.

Cuartero J, Munoz RF (1999) Tomato and salinity. SciHorti 78: 83-125.

Das A (2011) Effect of growth regulators on shoot induction and plant regeneration in tomato (*Lycopersicon esculentum* Mill.). MS thesis, BRAC University, 2011.

Davis MR, Miller LD (1991) Temporal competence for transformation of *Lycopersicon esculentum* Mill. cotyledons by *Agrobacterium tumefaciens*: relation to wound healing and soluble plant factors. J Exp Bot 42: 359–364.

Drew MC (1979) Plant responses to anaerobic conditions in soil and solution culture. CurrAdv Plant Sci 36:1-14.

Duzyaman E, Tanrisever AGunver G (1994) Comparative studies on regeneration of different tissues of tomato *in vitro*. Acta Hort. 366: 235–242.

Dwivedi K, Srivastava P, Verma HN, Chaturvedi HC (1990) Direct regeneration of shoots from leaf segments of tomato (*Lycopersiconesculentum*) cultured *in vitro* and production of plants. Indian J Exp Biol. 28: 32–35.

Ellul P, Garcia-Sogo B, Pineda B, Rios G, Riog LA, Moreno V (2003) The ploidy level of transgenic plants in *Agrobacterium*-mediated transformation of tomato cotyledons (*Lycopersicon esculentum* Mill.) is genotype and procedure dependent. TheorAppl Genet 106 (2): 231-238.

FAO (2001) Climate variability and change: A challenge for sustainable agricultural production. Committee on Agriculture, Sixteenth Session Report, 26-30 March, 2001. Rome, Italy.

FAO (2004) Impact of climate change on agriculture in Asia and the Pacific. Twenty-seventh FAO Regional Conference for Asia and the Pacific. Beijing, China, 17-21 May 2004.

FAO (2002) Quarterly Bulletin of Statistics. Food and Agriculture Organization. Rome. Vol. 20 (1/4.).

FAOSTAT (2011) Food and Agricultural Organization of the United Nations, Statistics. http://faostat.fao.org/site/408/default.aspx.

Fari M, Peredi BA, Csanyi TM (1991) Highly efficient *in vitro* shoot regeneration system in tomato and eggplant via seedling decapitation method (SDM). ISHS Acta Hort. International symposium on plant biotechnology and its Contribution to Plant Development, Multiplication and Improvement pp. 238.

Fari M, Szasz A, Mityko J, Nagy I, Csanyi M, Andrasfalvy A (1992) Induced organogenesis via the seedling decapitation method (SDM) in three solanaceous vegetable species. *Capsicum* Newsl pp. 243–248.

Ferdous MEM (2012) Establishment of *in vitro* regeneration and transformation protocol to develop salinity stress tolerant tomato (*Lycopersicon esculentum* Miller). MS thesis, BRAC University, 2012.

Fillati JJ, Kiser J, Ronald R, Comai L (1987) Efficient transfer of glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. Biotechnology 5: 726–730.

Foolad MR (1999) Genetics of salt and cold tolerance in tomato: quantitative analysis and QTL mapping. Plant Biotechnol. 16: 55-64.

Foolad MR (2004) Recent advances in genetics of salt tolerance in tomato. Plant Cell, Tissue Organ Cult. 76: 101-119.

Frary A, Earle ED (1996) An examination of factors affecting the efficiency of *Agrobacterium*-mediated transformation of tomato. Plant Cell Rep 16: 235–240.

Geetha N, Venkatachalam P, Reddy PS, Rajaseger G (1998) *In vitro* plant regeneration from leaf callus cultures of tomato (*Lycopersicon esculentum* Mill.). Adv Plant Sci 11: 253-257.

Gisbert C, Rus AM, Bolarin C, Lopez-Coronado JM, Arrillaga I, Montesinos C, Caro M, Serrano R, Moreno V (2000) The yeast *HAL1* gene improves salt tolerance of transgenic tomato. 123: 393-402.

Gou M, Zhang YL, Meng ZJ, Jiang J (2012) Optimization of factors Affecting *Agrobacterium* mediated transformation in Micro-Tom tomatoes. Genetics and molecular research. 11(1): 661-671.

Grichko VP, GlickBR (2001) Flooding tolerance of transgenic tomato plants expressing the bacterial enzyme ACC deaminase controlled by the 35S, rolD or PRB-1b promoter. Plant Physiol. Biochem 39: 19-25.

Gubis J, LajchovaZ, Farago J, Jurekova Z (2003) Effect of genotype and explant type on shoot regeneration in tomato (*Lycopersicon esculentum* Mill.) *in vitro*. Czech Journal of Genetic Plant Breeding 39(1): 9-14.

Gunay AL, Rao PS (1980) *In vitro* propagation of hybrid tomato plants (*Lycopersicon esculentum* L.) using hypocotyl and cotyledon explants. Ann Bot 45: 205–207.

Hamza S, Chupeau Y(1993). Re-evaluation of conditions for plant regeneration and *Agrobacterium*-mediated transformation from tomato (*Lycopersicon esculentum)*. J Exp Bot 269: 1837–1845.

Haque SA (2006) Salinity problem and Crop Production xin Coastal Regions of Bangladesh. Department of Soil Science, Bangladesh Agricultural University, Mymensingh, Bangladesh Pak. J. Bot., 38(5): 1359-1365.

Hazra P, Samsul HA, Sikder D, Peter KV (2007) Breeding tomato (*Lycopersicon esculentum* Mill) resistant to high temperature stress. Int J Plant Breed 1(1).

http://www.bari.gov.bd/home/commmodity_detail_pop/688.

http://www.bina.gov.bd/index.php?option=com_content&view=article&id=77&Itemid=91.

Hu W, Phillips G C (2001) A combination of overgrowth-control antibiotics improves *Agrobacterium tumefaciens*-mediated transformation efficiency for cultivated tomato (*L. esculentum*); *In Vitro* Cell Dev. Biol. Plant 37 12–18.

Islam A, Chowdhuri J, Seraj ZI (2010) Establishment of optimal conditions for an Agrobacterium mediated transformation in four tomato (*Lycopersicon esculentum* Mill) varieties grown in Bangladesh. Journal of Bangladesh Achademy of Sciences. 34(2): 171-179.

Islam A (1998) *In vitro* regeneration and genetic transformation of peanut (*Arachis hypogaea* L.). MS Thesis, Department of Botany, University of Dhaka, Bangladesh.

Islam K (2007) *In vitro* regeneration and *Agrobacterium*- mediated genetic transformation of tomato (*Lycopersicon esculentum* Mill). MS thesis, Department of Botany, University of Dhaka, Bangladesh.

Jawahar M, Mohammed SV, Jayabalan N (1997) A simple protocol for efficient plantlet regeneration from tomato (*Lycopersicon esculentum* Mill.) hypocotyl derived callus. Plant Tissue culture 7(1): 35-39.

Jones RA (1986) The development of salt-tolerant tomatoes: breeding strategies. Acta Horticulturae 190: Symposium on Tomato Production on Arid Land.

Kartha KK, Gamborg OL, Shyluk JP, Constable F (1976) Morphogenic investigations *on in vitro* leaf culture of tomato (*Lycopersicon esculentum* Mill cv. Starfire) and high frequency plant regeneration. Z Pflanzenphysiol 77: 292-301.

Khoudi H, Khemakhem AN, Gouiaa S, Masmoudi K (2009) Optimization of regeneration and transformation parameters in tomato and improvement of its salinity and drought tolerance African Journal of Biotechnology Vol. 8 (22), pp. 6068-6076.

Kuo DG, Tsay JS, Chen BW, Lin PY (1982) Screening for flooding tolerance in the genus *Lycopersicon*. Hort Science 17(1):76-78.

Ling HQ, Kriseleit D, Ganal MW (1998) Effect of ticarcillin/potassium clavulanate on callus growth and shoot regeneration in *Agrobacterium*-mediated transformation of tomato (*Lycopersicon esculentum* Mill.). Plant Cell Rep. 17: 843–847.

MacGillivary J H (1961) Vegetable production. McGraw Hill Book Company, New York, USA.

Madhulatha P, Pandey R, Hazarika P, and Rajam MV (2006). High transformation frequency in *Agrobacterium*-mediated genetic transformation of tomato by using polyamines and maltose in shoot regeneration medium. Plant Sci (communicated).

Manamohan M, Prakash MN, Chandra SG, Asokan R, Nagesha SN (2011) An improved protocol for rapid and efficient Agrobacterium-mediated transformation of tomato (*Solanum lycopersicum* L.). J. Appl. Hortic. 13: 3–7.

Mandaokar AD, Kumar PA, Sharma RP, Malik VS (1999) Bt-transgenic crop plants-Progress and Prospectus. In: V.L. Chopra, V.S. Malik, and S.R. Bhat (Eds.), Applied Plant Biotechnology. Oxford & IBH publishing Co, New Delhi, pp. 285-300.

Mayer JE, Pfeiffer WH, Beyer P (2008) Biofortified crops to alleviate micronutrient malnutrition. Current Opinion in Plant Biology 11:166–170.

McCormick S, Niedermeyer J, Fry B, Barnason A, Horch R, Farley R (1986) Leaf disk transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Rep 5: 81-84.

Men S, Ming X, Liu R, Wei C, Li Y (2003) *Agrobacterium*-mediated genetic transformation of a *Dendrobium* orchid. Plant cell tissue Org. Cult. 75: 63-71.

Mendel RR, Mansch RM (1995) Gene transfer to higher plants. In Molicular Method in plant pathology, RP Singh and US Singh, editors. CRC press, Florida, USA. Pp 189-200.

Mirghis E, Mirghis R, Lacatus V (1995) Analysis of tomato cultivars and hybrids for *in vitro* callus formation and regeneration. Acta Horticulturae 412: 111-116.

Moghaleb REA, Saneoka H, Fjita K (1999) Plant regeneration from hypocotyls and cotyledon explants of tomato (*Lycopersicon esculentum*). Soil Sci. plant Nutr. 45:639-646.

Murashige T, Skoog F(1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15: 473-479.

Mythili JB, Sariprasad GVS, Naveena C, Rajeev PR, Upreti KK (2011) Differential response of tomato and tobacco to Agrobacterium mediated transformation with cytokinin independent -1 (CKI-1) gene as influenced by cytokinin levels Indian J Exp Biol. 49(12):909-18.

Neogi (2012) Salt-tolerant rice in changing climate. http://www.daily-sun.com/details_yes__Salt-tolerant-rice-in-changing-climate_266_2_17_1_0.htmlSep 19, 2012.

Nervo G, Cirillo C, Accotto GP, Vaira AM (2003) Characterisation of two tomato lines highly resistant to tomato spotted wilt virus following transformation with the viral nucleoprotein gene. J Plant Pathol 85: 139-144.

Ohki S, Bigot C, Mousseau J (1978) Analysis of shoot-forming capacity *in vitro* in two lines of tomato (*Lycopersicon esculentum* Mill) and their hybrids. Plant Cell Physiol 19: 27–42.

OktemHA, Bulbul Y, Oktem E, Yucel M (1999) Regeneration and *Agrobacterium*-mediated transformation studies in tomato (*Lycopersicon esculentum* Mill.). Turkish Journal of Botany 23: 345-348.

Padmanabhan V, Paddock EF, Sharp WR (1974) Plantlet formation from *Lycopersicon esculentum* leaf callus. Canadian Journal of Botany 52: 1429-1432.

Panaullah GM (1993) 'Soil salinity and associated problems in connection with crop production in the coastal regions of Bangladesh', Proceedings of the workshop on, 'Coastal salinity and crop production in Bangladesh', February 17, 1993, Bangladesh Rice Research Institute publication, pp 1-30.

Paramesh H, Fakrudin B, Kuruvinashetti MS (2010) Genetic transformation of local variety of tomato using GUS gene: An efficient genetic transformation protocol for tomato. Journal of Agricultural technology. 6(1): 87-97.

Park S H, Morris J L, Park J E, Hirschi K D, Smith R H (2003) Efficient and genotype-independent *Agrobacterium*-mediated tomato transformation; J. Plant Physiol. 160 1253–1257.

Parveen F (2011) *In vitro* regeneration of three local potato varieties of Bangladesh. MS thesis, BRAC University, 2011.

Patil RS, Davey MR, Power JB, Cocking EC (2002) Effective Protocol for *Agrobacterium*-mediated Transformation in Tomato (*Lycopersicon esculentum* Mill.). Indian J Biot 1: 339-343.

Peterson DG, Price HJ, Johnston JS and Stack SM (1996) DNA content of heterochromatin and euchromatin in tomato *(Lycopersicon esculentum)* pachytene chromosomes. *Genome* 39: 77-82.

Plastira VA, Perdikaris AK (1997) Effect of genotype and explant type in regeneration frequency of tomato *in vitro*. ActaHort 231–234.

Raj SK, Rachana S, Pandey SK Singh BP (2005) *Agrobacterium*-mediated tomato transformation and regeneration of transgenic plants expressing tomato leaf curl virus coat protein gene for resistance against TLCV infection. CurrSci 33: 1674-1679.

Rashid R, Bal SS (2010) Effect of hormone on direct shoot regeneration in hypocotyl explants of tomato. Notulae Scientia Biologicae 2: 70-73.

Robinson SP, Jones GP (1986) Accumulation of glycinebetaine in chloroplasts provides osmotic adjustment during salt stress. Aust J Plant Physiol 13: 659–668.

Roy R, Purty R, Agrawal V, Gupa S (2006) Transformation of tomato cultivar 'Pusa Ruby' with *bspA* gene from *Populastermula* for drought tolerance. Plant Cell. Tiss. Org. Cult. 84: 55-67.

Rudrappa U (2009) Tomato nutrition facts. www.nutrition-and-you.com

Ruf S, Hermann M, Berger IJ, Carrer H, Bock R (2001) Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. Nature Biotechnol 19: 870-875.

SRDI (2001) Soil Salinity in Bangladesh-2000. p-113.

Sarker U (2013) Effect of growth regulators on plant regeneration and shoot induction in tomato (*Solanum lycopersicum* L.). MS thesis, BRAC University, 2013.

Schutze R,Wieczorrek G (1987) Investigations into tomato tissue cultures. I. Shoot regeneration in primary explants of tomato. Arch Zuchtungsforschung 17: 3–15.

Shanin EA, Sukhapinda K, Simpson RB (1986) Transformation of cultivated tomato by a binary vector in *Agrobacterium rhizogenes*: transgenic plants with normal phenotypes harbour binary vector T-DNA, but no Ri -plasmid T-DNA. TheorAppl Genet 72: 770–777.

Sharma MK, Solanke AU, Jani D, Singh Y, Sharma AK (2009) A simple and efficient *Agrobacterium*- mediated procedure for transformation of tomato. J Biosci. 34: 42-433.

Sheeja TE, Mondal AB, Rathore RKS (2004) Efficient potential regeneration in tomato (*Lycopersicon esculentum* Miller). Plant Tissue Culture 14(1): 45-53.

Siemonsma, JS Piluek K (1993) Plant resources of southeast Asia, No.8: Vegetables. The Netherlands: Pudoc Scientific Publishers pp. 1199-1205.

Sink KC, Handley LW, Niedz RP, Moore PP (1986) Protoplast culture and use of regeneration attributes to select tomato plants. Genet Manip 405–413.

Sink KC, Reynolds JF (1986) Tomato (*Lycopersicon esculentum* Mill.). Biotechnology in agriculture and forestry. Vol. 2: Crops I: 319-344 (ed. By Y. P. S. Bajaj). Springer Verlag Berlin Heidelberg.

Smith, Andrew F (1994) The tomato in America: early history, culture, and cookery. Columbia, S.C, USA: University of South Carolina Press.

Stevens MA, Rudich J (1978) Genetic potential for overcoming physiological limitations on adaptability, yield, and quality in tomato. Hort Science 13:673-678.

Sun HJ, Uchii S, Watanabe S, Ezura H (2006) A Highly Efficient Transformation Protocol for Micro-Tom, a Model Cultivar for Tomato Functional Genomics, Plant Cell Physiol. 47(3): 426–431.

Taji A, Kumar PP, Lakshmanan P (2002) In Vitro Plant Breeding. Food Products Press, New York. 167 pp.

Thomzik JE, Stenzel K, Stocker R, Schreier PH, Hain R, Stahl DJ (1997) Synthesis of a grapevine phytoalexin in transgenic tomatoes (*Lycopersicon esculentum* Mill.) conditions resistance against *Phytophthora infestans*. PhysiolMol Plant Pathol 51:265-278.

Ultzen T, Gielen J, Venema F, Westerbroek A, Haan P De, Tan M, Schram A, Van Grinsven M, Golbach R (1995) Resistance to tomato spotted wilt virus in transgenic tomato hybrids. Euphytica 85: 159–168.

VanRoeke JSC, Damm B, Melchers LS, Hoekema A (1993). Factors influencing transformation frequency of tomato. Plant Cell Rep. 12: 644-647.

Velcheva M, Faltin Z, Flaishman M, Eshdat Y, Perl A (2005) A liquid culture system for *Agrobacterium*-mediated transformation of tomato (*Lycopersicon esculentum*nMill.). Plant Sci 168: 121-130.

Vidya CSS, ManoharanM, Kumar CTR, Savithri HS, Sita GL (2000) *Agrobacterium*-mediated transformation of tomato plants (*Lycopersicon esculentum* var. Pusa Ruby) with cote-protein gene of *Physalis* mottle tymovirus. J Plant Physicol. 156:16-110.

Watterson JC (1998) Diseases of tomato crops. Atherton and Rudich (eds), Champan and Hill Ltd, New York, pp 461-462.

Weretilnyk EA, Bednrek S, McCUe KF, Rhodes D (1989) Comparative biochemical and immunological studies of the glycinebetaine synthesis pathway in diverse families of dicotyledons. Planta 178:342-352.

Xiao G, Zhang GY, Liu FH, Wang J, Chen SY, Li C, Geng HZ (1995) Study on BADH gene from *Atriplex hortensis* L. Chin Sci Bull 40: 741–745.

Zagorska NA, Shtereva LA, Kruleva MM, Stoirova VG, Baralieva DL, Dimitrov BD (2004), Induced androgenesis in tomato (*Lycopersicon esculentum* Mill.). Characterization of the regenerants. Plant Cell Rep 22: 449-456.

Zhand H, Blumwald E (2002) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Nature Biotechnol 19: 765-768.

# Appendix

**Appendix-1. Different components for preparation of stock solutions in MS media**

| **Component** | **Amount** |
|---|---|
| **Macro nutrients (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 micro element (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 |
| Thaimin HCl | 0.1 |
| Glycin | 2.0 |