ESTABLISHMENT OF *IN VITRO* REGENERATION AND TRANSFORMATION PROTOCOL TO DEVELOP SALINITY STRESS TOLERANT TOMATO (*LYCOPERSICON ESCULENTUM* MILLER)

M.S. THESIS

A DISSERTATION SUBMISSION TO THE BRAC UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOTECHNOLOGY

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DECLARATION

I solemnly declare that the research work entitled **'Establishment of *in vitro* regeneration and transformation protocol to develop salinity stress tolerant tomato (*Lycopersicon esculentum* Miller)'** presented in this thesis has been done by myself under joint supervision of Professor Zeba Islam Seraj, Plant Bitechnology Laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka and Dr. Aparna Islam, Biotechnology Program, Department of Mathematics and Natural Sciences (MNS), BRAC University, Dhaka. It is further declared that this research work presented here is original and any part of this thesis has not been submitted to any other institution for a degree or diploma.

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LIST OF ABBREVIATIONS

A.	*Agrobacterium*
CaMV35S	Cauliflower mosaic virus 35 S
Cm	Centimeter
DAT	Day after transplantation
DNA	Deoxyribose nucleic acid
EDTA	Ethylene diamine tetra acetic acid
g	Gram
GUS	β glucuronidase
h	Hour
hpt	Hygromycinphosphotransferase
Hyg	Hygromycin
IAA	Indole-3-acetic acid
Kb	Kilo bases
Kg	Kilogram
mg/l	Milli gram/Litre
mM	Milli mole
mL	MilliLitre
MS	Murashige and Skoog, 1962
NaOH	Sodium Hydroxide
NAA	α- Naphthalene acetic acid
ng	Nano gram
Nos	Nopalinesynthetase
npt II	Neomycin phosphotransferase

OD	Optical Density
PCR	Polymerase chain reaction
pH	-log H^+
RM	Regeneration medium
T-DNA	Transfer DNA
Ti-Plasmid	Tumor inducing plasmid
X-Gluc	5-Bromo-4-Chloro-3-indoyl-β-D-glucuronide
YMB	Yeast Extract Mannitol Broth
µl	Micro litre
µM	Micro mole
%	Percentage

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Abstract

Tomato (*Lycopersiconesculentum* Miller), a nutritious plant, can be substituted to overcome malnutrition and offers much higher income for the farmers in Bangladesh because of its high demand throughout the year. However, there is a considerable loss in their production due to environmental stress. Such as, salinity affected one tenth of the total cultivable lands of Bangladesh due to the climate change and sea level rise. It afflicted every aspect of plant physiology and biochemistry thus economic yield. Therefore, a simple and efficient transformation protocol is necessary to develop salt tolerant tomatoes. In the present study, a reproducible and efficient regeneration protocol was obtained as a prerequisite for tomato transformation. Among five varieties tested in this study, BARI Tomato 15 and BARI Tomato 2 showed equally best regeneration response (96%). Highest shoot number was obtained in MS+2 mg/l BAP. BARI Tomato 14, BARI Tomato 15 and BINA tomato 3 showed highest rooting response (100%). Maximum number of shoot was observed while explants placed in the regeneration media at 1.5 cm distances apart for tested varieties namely, BARI Tomato 3 and BARI Tomato 15. Seeds were collected from ripen fruits of regenerated plants and their viability was tested. These tissue cultured plants were found as same as naturally grown plants. In *Agrobacterium*-mediated transformation, these five tomato varieties were transformed with pBI121 (containing *nptII* selectable marker gene and *uidA* gene) for optimization of various factors. Co-cultivation of explants with a bacterial concentration of OD_{600} 0.68 for two days resulted in the highest transformation frequency of 47% in BARI Tomato 3. Then transformation was performed using these proper conditions with *Agrobacterium* strain containing pH7WG2_OsNHX1_1.6 (*OsNHX1*antiporter gene, cloned from rice) to make salinity

tolerant tomato plants. In this case, highest transformation frequency of 20.5% was obtained by BARI Tomato 3. Transformation of putative transformed shoots was confirmed by *β-glucuronidase* (GUS) assay and PCR. The optimized transformation procedure is simple, efficient and does not require any feeder layer or acetosyringone.

Chapter 1

INTRODUCTION

Vegetable crops can be the best substitute to overcome malnutrition and provides much higher income for the farmers (1). Tomato (*Lycopersicon esculentum* Mill), a member of the Solanaceae family is a major vegetable crop and consumed all over the world. It can play a vital role in mitigating malnutrition problem (2). The current annual tomato production worldwide is about 145 million tons with a cultivable land of 4 million hectares (3). It ranks first among all fruits and vegetables in nutritional contribution (4) and ranks second in economic importance after potato worldwide (5). It is highly nutritive and consumed both directly and processed. Tomato is rich in vitamin A, vitamin C and a good source of important dietary components like β-carotene, amino acids, natural sugars and minerals (6). It also provides natural antioxidant lycopene which helps to prevent cancer (7) and gives protection against sunburn and skin ageing according to a research led by Newcastle University (8).

1.1. Tomato production in Bangladesh

In Bangladesh, the area of cultivation is about 13,066 hectares with the production of about 74,000 metric tons (9). The average yield of tomato in this country is very low (7.42 t/ha) in comparison with that of other countries (10). Bangladesh government imported 9395.14 metric tons tomato to fulfill local demand in 2000-2001 (11). Farmers usually grow 50 Indian and Bangladeshi varieties available in the market (12).

1.2. Tomato production constraints

Tomato plants are sensitive to a number of biotic and abiotic stresses. Over 200 diseases were found in tomato which caused by pathogenic fungi, bacteria, virus and nematodes etc. (13). Plant growth and development adversely affected by abiotic stress conditions, especially, extreme temperature, drought, salinity and inadequate

moisture stresses (14) (15). These stresses reduce agricultural production world-wide by more than 50% of average yield in most major crops (16) (17).

In Bangladesh, the year round tomato production is constrained by two factors, seasonality and multiple diseases (18). A study took place in Jessore area, Bangladesh, reported that the 'insect and disease attack' as one of the main constraints to higher tomato production according to 40 percent farmers (19). Among environmental stresses, excessive rainfall thus flooding affects the production by accumulating an increased production of a 4-ethylene precursor; 1-aminocyclopropane-1-carboxylic acid (ACC) in the roots that damages plants (20). A short period of severe flood with increased high temperature and soil salinity causes leaf curling, rapid wilting, tissue necrosis and finally potential death of tomato plants (21) (22) (23).

1.3. Tomato regeneration

Tomato regeneration protocol is an important tool for the development of virus free plant and introduction of new traits (24) (25). Although, tomato regeneration protocol has been reported by different authors for several decades to optimize regeneration system but no standard protocol has been established so far for farmer popular tomato varieties of Bangladesh. It may be due to the fact that *in vitro* regeneration depends on many factors including genotype, explant, composition of basic medium, growth regulators, gelling agent, light intensity and quality, photoperiod, temperature etc. (26).

Researchers reported tomato shoot formation from hypocotyls, apical meristem, cotyledons, stem petioles, leaves, anthers and inflorescences explants (27). Response for various explants has been compared by Durzan (28) and found leaves to be most responsive followed by

cotyledons, hypocotyls, while the order is hypocotyls, cotyledon and leaves (29) (30). Cotyledonary leaves are the most commonly used explants (31). However, Gubiš (32) reported up to 100% regeneration with hypocotyls and epicotyls.

Different cytokinin and auxin were used in various concentrations in tomato regeneration protocol. Medium containing zeatin as the cytokinin was found to be the best medium and the most used concentration is 1 mg/l. IAA is the most popular auxin to be used in tomato regeneration medium and the preferred concentration is 0.05 mg/l (33). An increase in the concentrations of the cytokinin level results in the decrease of percentage of shoot formation since higher cytokinin level produces excessive callus and failed to increase the efficiency of shoot multiplication (34). Good and faster response has also been reported with the use of equimolar concentrations of auxin and cytokinins (35). The auxin/cytokinin one fourth in proportion was also found to result in higher frequency of callus induction when NAA (0.5 mg/l) and BAP (2 mg/l) were used (36). Maximum callogenesis was also obtained for MS medium containing IAA (2 mg/l), NAA (2 mg/l), BAP (2 mg/l) and Kinetin (4 mg/l) (37). High callus induction has been reported when BAP (3 mg/l) was used alone in MS medium while multiple adventitious shoots formed at 0.1 mg/l IAA and 2.5-5.0 mg/l BAP containing medium (38).

Media containing different concentrations of BAP has also been evaluated and the optimum concentration was found to be 2 mg/l. BAP was found to be superior to kinetin for maximum shoot bud differentiation as well as multiple shoot induction (39). Medium containing kinetin (0.5 mg/l) and BAP (0.5 mg/l) can also lead to high percentage of shoot regeneration (40).

Some studies reported that thidiazuron (TDZ), a substituted phenyl urea is more potent than BA (Benzyl adenine) for *in vitro* shoot regeneration of dicotyledonous species. Therefore, MS medium supplemented with TDZ was studied for tomato regeneration and 3.4 μM of TZD produced the highest frequency of shoot formation (41). Similar results were found when MS medium was supplemented with 3.0 mg/l TDZ. However, addition of IAA (1 mg/l) and IBA (2 mg/l) were found essential to induce highest number of roots and longer roots (42).

Effect of the use of gibberellins (GA_3), a natural plant hormone has also been studied for tomato regeneration. GA_3 is known to be associated with cell enlargement and division which leads to internodes elongation in stem. IAA (0.5 mg/l) and BAP (0.5-2.5 mg/l) were used alone or in combination GA_3 (2 mg/l) on MS medium and medium containing GA_3 reduced the days for regeneration (20-25 days) as compared to medium without GA_3 (40-45 days) (43).

Diverse media, namely, Murashige and Skoog (MS), Chu's N6, Linsmaier & Skoog (LS) and Gamborg's B5 were also studied for tomato regeneration in which N6 was found to show maximum plantlet regeneration, shoot length and whole plantlets, number and length of shoot. MS basal media was found to be most appropriate for development of maximum healthy tall whole plantlets and LS basal media found good for faster regeneration (44).

MS medium containing IAA at the concentrations of 0.1, 0.2 and 0.3 mg/l showed the adventitious rooting while 0.1 mg/l was found to be the best concentration which showed formation of milky white healthy roots (45). Unfortunately, medium containing NAA at the concentrations of 0.1, 0.2 and 0.3 mg/l did not improve the root formation rather slowed root formation and resulted in grey, short and weak roots. While MS medium was found to be the best for shoot formation, ½ MS medium was found best for root induction (46) (47). Good rooting was also observed for ½ MS

medium supplemented with IBA (0.1 mg/l) and BAP (0.0025 mg/l) (48). Basal nutrient support for ½ MS medium has been reported by other authors as good rooting medium but addition of IAA (1 mg/l) was found essential to induce longer roots (49).

Coconut water has also been tested as a supplement in MS medium. Significant decrease of time for callus formation leading to shoot regeneration has been observed when 12% coconut water was added to the medium. Higher survival rate for the plantlets were obtained with the addition of coconut water (50). Other adjuvants, for example, biotin, folic acid were used in MS medium and found to enhance plantlet regeneration (51).

In conclusion, numerous paper has been published on the regeneration of tomato, low regeneration frequency, instability and high proportion of deformity bud are still remain the challenging factors (52).

1.4. Tomato Transformation

It has a relatively small genome (0.7-1.0 pg), well developed classical (53) and molecular genetics maps (54) and a complete genomic library in yeast artificial chromosomes. Due to its relatively small DNA content, tomato plant has served as model plant for cloning agronomically important genes in dicotyledonous crop plants (55). Since the first report of *Agrobacterium*-mediated tomato transformation by Horsch *et al.* (56) and McCormick *et al.* (57), there have been many reports of tomato being engineered for a variety of purposes (58). Tomato is playing an important role in map-based cloning of the first disease resistance (59) and identification of quantitative trait loci (60) for agronomical, morphological and developmental traits (61) (62) (63). One of the first crops was tomato which had a molecular genetics map

(64) it is recently subjected *to* functional genomics, proteomics, and metabolomics (65).

Some of the varieties of *Lycopersicon esculentum* or its hybrid that has been transformed include ES58, LA1563, UC82, UC 134-1-2, UC82B, UC204A, UC204B, VF36, VF145B-78-79, WC156, 981 X TY-6, Ailsa Craig, Arka Vikas, BARI Tomato-3, Beefmaster, Betterboy VFN, Bina Tomato-3, Bina Tomato-5, Bahar, Burpee's Bigboy, CastleRock, Floramerica VF, Green grape, Koma, Micro-MsK, MicroTom, Rutgers, Swifty Belle, Starfire, Shalimar, Rio Grande, Seokwang, Pusa Ruby, Summer set, Margobe, Mocross Surprise, Perfect Peel, Roma Italian Canner and Sioux. Successful transformation has also been carried out with other species, like, *Lycopersicon peruvianum*, *L. chilense* and *L. hirsutum* or interspecific hydrids like *L. esculentum* cv. VS36 × *L. penellii*.

Various strains of *Agrobacterium tumifaciens* including EHA105, LBA4404, C58, EHA105, EHA101 were used for the gene transfer. However, LBA4404 was found to be the most commonly used strain. Bombardment by gold particles was also used by researcher for gene transfer (66). Transformation frequency ranged from 6% in Pusa Ruby to as high as 49.5% in MicroTom variety (67) (68).

In these studies, cotyledons or leaves have been used for *Agrobacterium*-mediated tomato transformation. The efficiency of *Agrobacterium*-mediated gene transfer to tomato cells is influenced by many factors, the most important are: variety/genotype, explants type and orientation, plant growth regulators, selection system, addition of feeder cells or acetosyringone, *Agrobacterium* density, duration of infection, and effect of antibiotics on regeneration of tomato (69). Therefore, there is no universal

protocol for tomato transformation and regeneration thus subject of interest for many researchers.

Some of the aims of tomato plant transformations are:

- Characterization of gene function
- Salinity and drought tolerant plants
- Production of insect and disease-resistant plants
- Herbicide tolerance
- Improved fruit quality
- Delay in fruit ripening
- Production of foreign proteins

1.5. Expression of functional genes to improve tomato plant

To introduce foreign genes into plant cells and the successive development of transgenic plants, *Agrobacterium*-mediated transformation is the most functional method (70) for the biotic and abiotic stresses tolerance improvement.

1.5.1. Improvement of biotic stress tolerance

Insect resistant tomato was developed by *A. tumefaciens* (strain LBA4404) mediated insect control protein gene *WGA* (Wheat Germ Agglutinin) transfer (71). WGA protein is a glycoprotein with molecular weight of 34000 Da. Toxic effect appears to be mediated through binding of the lectins (*WGA*) to glycoproteins in the insect leading to the disruption of gut epithelial cells and those are believed to be "natures own insecticides". Successful transformation was confirmed by PCR analysis.

El-Siddig *et al.* (72) transformed tomato plants that can express a defensin gene (*AFP*) that code for antifungal protein. Exposure of cotyledonary explants (cv. Summer set) to *Agrobacterium* inoculums of 0.8 $OD_{600\ nm}$ for 30 min, subsequent regeneration on

MS medium supplemented with 2.5 mg/l BA and 1.0 mg/l IAA resulted in a transformation efficiency of 7%. Selection medium was supplemented with 25 mg/l hygromycin.

Another plant defensin gene wasabi defensin (*WD*) isolated from *Wasabia japonica* was introduced in Reiyo variety tomato plant (73). Use of Multi auto-transformation (MAT) vector system resulted in marker-free transgenic plants. Analysis of the developed shoots confirmed the integration of gene of interest (*WD*) and excision of selection marker isopentenyltransferase (*ipt*) gene. Transgenic plants exhibited greater resistance against grey mold (*Botrytis cinerea*), early blight (*Alternaria solani*), Fusarium wilt (*Fusarium oxysporum*) and powdery mildew (*Erysiphe lycopersici*).

Tomato plants partially resistant to *Botrytis cinerea* was developed by the introduction of inhibitor of virus-replication (*IVR*) gene (74). *A. tumefaciens* strain EHA 105 was used to transform Tomato cv. VF36 cotyledons. Lesion induced by fungus showed significant reduction in size in transgenic plant as compared to non-transgenic plant. However, the resistance was weakened when plants were kept at higher temperature (32°C).

Further investigation also revealed that the transgenic tomato plants carrying *IVR* gene showed partial resistance against seedling infection by *A. alternata*, *P. aphanidermatum* and *R. solani*, damping-off in soil (*P. aphanidermatum* and *R. solani*), early blight (*A. solani*) on plants grown in a screen-house and powdery mildew (*O. neolycopersici*) on plants grown in growth chambers (75).

Plant defensin gene (*MsDef*1) of *Medicago sativa* was also inserted in tomato (76). As a result, both the tested T_1 and T_2 young seedlings of transformed tomato plants were found to be more resistant than non transgenic tomato plant towards Fusarium wilt, a fungal pathogen that affects tomato cultivation. Here, Fusarium-susceptible tomato cultivar Castle Rock was

chosen for the transformation study. Biolistic gene delivery system was used for the transformation of excised hypocotyledonary sections.

Transformed tomato resistant to Gray mold caused by *Botrytis cinerea* Pers. Fr. was developed by the introduction of rice chitinase gene (*CHI*) and alfalfa (*alfAFP*) and their bivalent gene (*CHI-AFP*). *A. tumefaciens* strain EHA105 was used to transfer the gene in tomato variety Micro-Tom. The extent of resistance towards the pathogen was found to be proportional to the level of transgene expression (77).

Transgenic tomato plants were developed that can express huaman *β*-amyloid (A*β*). Alzheimer's disease is a neurodegenerative disease that has been linked with the increased accumulation *β*-amyloid in the brain. *β*-amyloid is a toxic protein, accumulation of which results in neuronal death. In previous studies vaccination with *β*-amyloid in mice has been found to produce antibody against the 42 amino acid containing protein A*β*. In the present study, mice immunized orally with total soluble extracts from the transgenic tomato plants elicited an immune response after receiving a booster (78).

A transformation protocol was established for BINA Tomato 3, BINA Tomato 5, Bahar and Pusa Ruby varieties with pBI121, OD_{600} 0.8 and infection time 10-15 minutes were found optimum for maximum transformed shoot regeneration on selection media containing 200 mg/l. Highest transformation ability (96.7%) was showed by BINA Tomato 3 in transient GUS expression and regeneration frequency (11.11%) of transformed shoot was observed for the same variety in stable GUS expression (79).

1.5.2. Improvement of abiotic stress tolerance

Salt and water stress resistant tomato was developed by transformation of tomato with bacterial *codA* gene (80). Transgenic plant carrying *codA* gene has the ability to synthesize glycinebetaine, a highly efficient compatible solute helping the plant grow in saline and dry environment. The *codA*-transgenic tomato plants revealed a much higher frequency of seed germination and faster growth of young seedlings under salt and water stress conditions than non-transformed tomato plants.

Drought and salt tolerant tomato plant was developed (81**)** through the insertion of two wheat genes encoding sodium antiporter and a vacuolar pyrophosphatase. Instead of cotyledons, primary leaves of Rio Grande tomato variety was used for the transformation. Transgenic T1 tomato plants exhibited a better appearance than their non transgenic counterparts when subjected to salt stress provided by a 200 mM NaCl solution.

Agrobacterium mediated gene encoding boiling stable proteins (*bspA*) was introduced to develop dessication tolerant tomato plant cv. Pusa Ruby (82). Boiling stable protein is a novel 66 kDa protein that is expressed in *Populus tremula* in response to water stress protecting proteins in membranes and cytosol. Stable integration of the T-DNA in to nuclear genome was confirmed by PCR and southern hybridization. Transgenic plants showed slightly increased tolerance to water stress compared to non-transgenics.

Transgenic tomato with improved salt tolerance was developed by the insertion of *HAL1* gene from *Saccharomyces cerevisiae* (83). Overexpression of *HAL1* gene provides tolerance against salt stress by maintaining a high internal K^+ concentration

and decreasing intracellular Na^+ during salt stress. A moderate level of salt tolerance was observed both in vitro and *in vivo* in transgenic tomato plants.

1.5.3. Quality improvement

First commercial transgenic tomato was the Flavr Savr tomato (84) transformed with an antisense polygalacturonase construct for development. This technique was used for introducing large fragment of DNA (up to 150 kb) into tomato genome (85).

As reported by Tanase *et al*. (86), the taste-modifying protein miraculin (MIR) was produced in tomato through the expression of *MIR* gene. However, the concentration of miraculin was less than that of miracle fruit. Therefore, a synthetic gene encoding *MIR* protein (*sMIR*) was designed to optimize its codon usage for tomato which resulted in higher accumulation of miraculin in tomato.

With a view to develop an easy, rapid and efficient tomato transformation protocol, *Arabidopsis* early flowering gene *AP1* was introduced in tomato through the infiltration of tomato fruits with *A. tumefaciens* EHA105 carrying engineered plasmid (87). As high as transformation efficiency 54-68% was observed in the seeds of the infiltrated fruits. PCR analysis of the transgenic plant confirmed the insertion of gene of interest and therefore established a novel method for *A. tumefaciens* mediated transformation.

Qiu *et al*. (88) reported an improved protocol for the incorporation of carotenoid biosynthetic genes *CsZCD* (Crocus zeaxanthin 7, 8-cleavage dioxygenase) in tomato variety Micro Tom. Tomato cotyledons used as the explants were kept for 1 day on the medium with zeatin 2 mg/l, IAA 0.1 mg/l, submerged in *Agrobacterium* (OD_{600} = 0.2) for 20 min and co-cultivated for 3 days on the same medium. Cotyledons were shifted to pre-selection medium with 500 mg/l cefotaxime for 3 days and shifted to

selection medium with 100 mg/l kanamycin and 500 mg/l carbenicillin for 6-8 weeks. 20% transformation efficiency was observed.

HBsAg (hepatitis B surface antigen) gene was successfully introduced into tomato plants (89). The *A. tumefaciens* strains LBA4404, EHA105 and C58 were transformed with the recombinant binary vector pBRSAg via the freeze-thaw method. Transformed cotyledon explants were cocultivated on MS medium with 6-BA (2 mg/l) and IAA (0.2 mg/l), timetin (200 mg/l) and hygromycin (7 mg/l). However this work demands further investigation to determine the amount of *HBsAg* protein produced and the inheritance of transgenes in successive generations (T_1, T_2). This report has opened up the opportunity to produce edible vaccines in tomato fruits. Similar investigation is reported by Baesi *et al*. (90). PCR analysis proved the presence of *HBsAg* gene in the transgenic plant.

1.6. Soil salinity and its effect in crop production

A saline soil is generally defined as one in which the electrical conductivity (EC) of the saturation extract in the root zone exceeds 4 dSm^{-1} at 25°C and has an exchangeable sodium percentage of 15 (91). Salinity is an increasingly important environmental constraint to crop production worldwide. Regardless of the cause (ion toxicity, water deficit, and/or nutritional imbalance), high salinity in the root zone severely impedes normal plant growth and development, resulting in reduced crop productivity or crop failure (92).

14 billion lands are available on earth; of which 6.5 ha are arid semi-arid. About 1 billion soils are saline of these lands (93). About 20% of cultivated lands and 33% of irrigated agricultural lands worldwide are badly affected by high salinity (94) (95). Salinization has already afflicted a large portion of irrigated land in Asia (96).

Furthermore, the increasing rate of salinized soil is 10% annually. Increasing soil salinity play a major role in low precipitation, high surface evaporation, weathering of native rocks, irrigation with saline water, and poor cultural practices (97).

The effect of salinity can affect plant differently based on the extent of salinity, age of plant and plant genotype. Some varieties are more prone to salinity than others. Salinity hinders plant growth for decrease of water accessibility to the plants and increase of salt or ion (98).

1.7. Salinity in Bangladesh

In 1990, Inter-governmental Panel on Climate Change (IPCC) estimates that with a business-as-usual scenario of greenhouse gas emission, the world would be 3.3°C warmer by the end of the next century, with a range of uncertainty of 2.2 to 4.9°C (99). With rise in temperature, sea level will also rise because of thermal expansion and ice melt. Bangladesh is thought to be one of the most vulnerable countries of the world to the climate change and sea level rise (CCSLR). One tenth of the total cultivable lands of Bangladesh are affected by salinity (100).

IPCC predicts that the climate change of Bangladesh may result in rising sea level by 14cm by 2025, 32cm by 2050 and 88 cm by 2100. It is revealed that the groundwater levels have gone down below the mean sea level ranging from 0 to 52 meters in many areas of this country. Connection between some of these areas and to the Bay of Bengal causes favorable conditions for saline water interruption (101). The area remains waterlogged and salinity increases because of drainage congestion (102).

1.8. Tomato and salinity

Most commercial cultivars of tomato are moderately sensitive to salinity at all stages of plant development, including seed germination, vegetative growth and

reproduction, and, as a result, their economic yield is substantially reduced under salt stress. At moderate to high levels of salt (EC $\geq$ 6 dSm^{-1}), osmotic effects and ion toxicities contribute to reduced growth (103).

It was reported that there is no correlation between salt tolerance of young and mature plants in tomato (104). Ranking of salt-tolerant genotypes based on vegetative characteristics in mature plants differed from the ranking based on fruit yield (105). Increased salt concentration is also associated with reduced root growth and may be due to cell growth restriction, because of the low water potential of external medium, interference of the saline ions with the plant's nutrition or the toxicity of accumulated ions leading to cell death. Salinity slows the shoot growth in tomato plant and the shoot is much more affected by salinity than the root. The adaptability to increased salt level is higher in older plants and a flowering and fruiting plant can withstand saline level high enough to kill at its seedling stage (106). Increased salinity also decreases dry leaf weight compared to plants growing in normal condition. The decrease in leaf weight is not caused by decrease in number of leaves but due to a decrease in the surface area of the leaves (107). Tomato yield can be reduced by decreased average fruit weight and lowering in the number of fruits produced by the plant (108) (109).

Wild species of tomato, such as *L. cheesmanii*, *L. peruvianum* and *L. pennellii* are comparatively more salt tolerant than the cultivated tomato plant *L. esculentum* (110). Some other wild species of *Lycopersicon* represents useful genes for salt tolerant breed but there are a few variations in cultivated species. Some Bangladeshi BARI tomato varieties, namely, BARI Tomato 2, BARI 14 and BARI Hybrid Tomato 5 showed greater adaptation ability under saline condition as they consistently produce more root dry matter at moderate saline condition (4.1-8.0 dS/m). All parameters of

tested varieties were reduced at that condition while these three varieties consistently showed superior biological activity (111).

Salt tolerance ability of tomato is increased with the plant age, this phenomenon seen in barley, corn, rice and wheat. Furthermore, various genes control salt tolerance during seed germination and vegetative stage in tomato (112) (113). Total soluble solids (TSS) determine the quality of tomato fruits increase with increase in salt concentration of 3-6 dS/m provided that it is also associated with a gradual yield reduction (114). Sucrose content from salt-stressed plant is higher from tomato of non salt-stressed plant which may be due to inactivation of cell wall invertase due to pH changes in apoplast or to Na^+ accumulation in the cell wall (115). Organic acid content and higher tritrable acidity is also associated with tomato fruits grown under salt stress than fruits grown with fresh water (116). Fruit shelf life (117) and fruit firmness (118) are lowered at salinities above 100 mM NaCl whereas with a less saline treatment (50 mM NaCl) both fruit shelf life and firmness remain unchanged (119). Babu *et al.* (120) reported that an increasing concentration of NaCl can decline fruit number and plant height of tomato cultivar PKM1 by limiting the biomass and photosynthetic yield.

1.9. General mechanisms of salt tolerance in plants

In general, two types of salinity-response mechanisms are in plants:

- Control of the salts entry into the plant (salt exclusion or glycophytic response) (121)
- Control of the salt's concentration in the cytoplasm through compartmentalization (sequestering salt in the cell's vacuole) (122).

In glycophytes, osmotic adjustment is mainly proficient by control of ion uptake (at the root or shoot level) and cellular synthesis of organic solutes (e.g., sugars and

amino acids), which are used as osmotica. With increasing salt stress, the ion elimination mechanism may fail and ionic stress resulting imbalances in metabolic processes due to excessive ion ingestion (123).

In halophytes, in contrast, osmotic adjustment is mostly attained by inorganic ions uptake from the soil and sequestering them in the cell vacuoles of the leaves or other plant organs (124). Most of the salt-tolerant genotypes within the cultivated tomato and the closely-related wild species *L. pimpinellifolium* generally exhibit a glycophytic response to salinity (125) (126) (127) (128). In contrast, salt-tolerant accessions within the tomato wild species *L. pennellii*, *L. cheesmanii*, and *L. peruvianum* generally exhibit a halophytic response (129) (130) (131).

1.10. Increased salt tolerance through genetic transformation

Plant's response to salt stress involves the functions of many genes that lead to a wide variety of biochemical and physiological changes. These include, for example,

- Expression of genes that facilitate compartmentalization of toxic ions in the vacuole.
- Activation of detoxifying enzymes.
- Synthesis of late-embryogenesis-abundant (LEA) proteins.
- Accumulation of compatible solutes (also known as osmolytes).

Genetic transformation is appeared more remarkable than traditional plant breeding to develop stress-tolerant plants since these are genetically inherited with moderate to low heritability (132).

Genetic engineering approaches have been used in producing transgenic plants during quite a few years. This transformation took place to make various stress (including

salt stress) tolerant plants by over-expression of genes controlling different tolerance-related physiological mechanisms (133) (134) (135).

For example:

- *Compatible solutes:* Plants engineered with genes encoding enzymes enhanced the synthesis of compatible solutes such as mannitol, glycine betaine, proline and polyamines (136) (137) (138) (139). This controls osmotic adjustment and improved plant stress tolerance (140) (141). Compatible solutes may also control stress tolerance through other functions such as enzyme protection and membrane structure etc (142) (143).
- *Antiport proteins:* Transgenic plants have been developed by over-expression of different vacuolar antiports proteins, which eliminates the toxic ions from the cell cytosol (144) (145).
- *Detoxification enzymes:* transgenic plants have been produced with increased expression of detoxification enzymes, which decreases oxidative stress (146).

In tomato, developments of plants by enhancing salt tolerance with transgenic approaches have been very low (147). However, a significant improvement was the progress in transgenic tomato plants by over-expressing *AtNHX1*, a single-gene controlling vacuolar Na^+/H^+ antiporter gene from *Arabidopsis thaliana* (148). Only transgenic plants were able to grow, set flower and produced fruit in the presence of 200 mM NaCl in greenhouse hydroponics. The transgenic plants acquired a halophytic response to salt stress, accumulating salts in the cell and sequestering them in the vacuole. This is unlike the normal response of the cultivated tomato to salinity stress, which is the exclusion of salts from the cell in the root and/or shoot, a glycophytic response whereas the non-transgenic (control) plants did not survive in the saline conditions.

According to this report, very high concentrations of Na^+ and Cl^- were accumulated in the leaves of transgenic tomato plants while salinity conditions were elevated. The ability of the transgenic plants was enhanced due to overproduction of the vacuolar Na^+/H^+ antiport protein. Therefore, it sequestered Na^+ in their vacuole while averting its toxic effects in the cell cytosol. Na^+ maintained osmotic balance to drive water into the cell. And the salty water expands cells and growth occurs. In addition, there were only minimum increases in Na^+ and Cl^- concentrations in the fruit. Thus, these transgenic plants could be produced commercially as a horticultural advantage (149).

1.11. Caution in tomato transformation for increased salt tolerance

Tomato salinity tolerance is regulated developmentally which is stage-specific. Tolerance at growth stages is genetically independent from one-to-other. Thus, when salt tolerance is improving, each development stage of the transgenic plants needs to be evaluated separately (150).

1.12. Objectives of the current study

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

- Establishment of a tomato regeneration protocol, which include:
  - Determination of the optimum concentration of growth factors in the culture medium.
  - Determination of the optimum spacing of tomato cotyledonary leaves explants in the culture medium.
- Determination of effect of hygromycin and kanamycin concentrations in the selection medium on tomato regeneration.
- Effect of salinity in tomato seed germination.
- Determination of factors influencing transformation efficiency of different tomato varieties by pBI121.
- Tomato plant infection with pH7WG2_OsNHX1_1.6 containing Na^+/H^+ antiporter gene (*OsNHX1*) in order to get salinity tolerant tomato plants.

Chapter 2

MATERIALS AND METHODS

Experiments of tomato tissue culture and transformation were carried out in Biochemistry and Molecular Biology lab, University of Dhaka and Plant Tissue culture lab, Department of MNS (Biotechnology Program), BRAC University.

2.1. Tissue culture

2.1.1. Seed collection

Several tomato (*Lycopersicon esculentum* Mill, Family: Solanaceae) varieties developed by Bangladeshi research organizations were used for tissue culture in the present study. Seeds were collected from Bangladesh Agricultural Research Institute (BARI) and Bangladesh Institute of Nuclear Agriculture (BINA) namely, BARI Tomato 2, BARI Tomato 3, BARI Tomato14, BARI Tomato 15, and BINA Tomato 3. A brief description of the selected tomato varieties are represented in **Table 2.1**.

2.1.2. Seed disinfection and germination

2.1.2.1. Seed sterilization

Under the environment of laminar air flow hood, the tomato seeds were first surface sterilized by immersing in 70% ethanol for 3 min. Then 30% Clorox and two drops of Tween-20 were added to the seeds and shaked by hands for 5 minutes (151). The seeds were then rinsed well with sterile distilled water for three times to remove any trace of sterilant. Finally the seeds were kept in a rotatory shaker (Model: WIS-20, Korea) at 180 rpm for overnight to remove the gelatinous layer around the seeds.

2.1.2.2. Seed germination

After overnight shaking sterilized seeds were directly transferred on to germination media and incubated at 25°C ±2 with 16 h photoperiod. Time required for seed germination and seedling development was recorded.

Table 2.1. List of tomato varieties used (Chowdhury, 2009; Dutta et al, 2004; BARI website; BINA website)

Tomato variety	Crop duration	Developed by	Release year	Yield (ton/ha)	Identifying characteristics	Fruit size and color	Sowing time
BARI Tomato 2 (Ratan)	120-130 days (DAT)	Olericulture Division, HRC, Gazipur	1986	85-90	High yielder and tolerant to BW, Fruits are round and red in color, Average fruit weight 85-90 g, Good shelf life	Round, red	September-October
BARI Tomato 3	120-130 days (DAT)	Olericulture Division, HRC, Gazipur	1996	85-90	Fruits are fleshy, semi-globe and red in color, Number of fruits/plant is about 28-30 and average fruit weight is about 85-90 g.	Semi-globe, red	September-October
BARI Tomato 14	130-160 days (DAT)	Olericulture Division, HRC, Gazipur	2008	85-90	Recommended for early and late winter, Large round fruit with attractive red flesh colour 90-95g av. fr. Wt, Prolonged harvesting period (80-90 days), Very good shelf life, Tolerant to bacterial wilt and TYLCV	Semi globe, red	September-October
BARI Tomato 15	110-120 days (DAT)	Olericulture Division, HRC, Gazipur	2009	80-85	Resistant to Tomato Yellow Leaf Curl Virus (TYLCV), Thick skin and edible flesh having very good self life, Obovoid fruit shape Less seeded fruits each of 65-70g in weight, Attractive red flesh color with brix value more than 4.0, Determinate type growth habit	Ovoid, red	October
BINA Tomato 3	60-65 days (DAT)	Bangladesh Institute of Nuclear Agriculture, Mymensingh	1997	48	Heat resistant, but have less fruit production, average fruit weight is 82 g, not tasty	Oval, red	May-June

2.1.3. Stock solutions preparation in MS media

In the present study, Murashige and Skoog (MS) medium (1962) was used for tomato tissue culture. Different components were required for the preparation of stock solution in MS media (**Table 2.2**).

Table 2.2. 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	6.2
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.6
$Na_2MoO_4 \cdot 2H_2O$	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

2.1.3.1. Macro nutrients stock solution preparation

The components of macro-nutrients (mentioned in **Table 2.2**) 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) and stored in a refrigerator at 4ºC for several weeks. This stock solution was made 10 times the concentration of the full medium.

2.1.3.2. Micro nutrients stock solution preparation

The components of micronutrients (mentioned in **Table 2.2**) 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. The solution was made 100 times of their full strength.

2.1.3.3. Iron EDTA stock solution preparation

$FeSO_4.7H_2O$ (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.

2.1.3.4. Organic stock solution preparation

The stock solution was made 100 times of their full strength. Components (mentioned in **Table 2.2**) were added one by one and stirred some more time before adding next. Then it was stored at 4ºC.

2.1.4. Stock solutions of growth hormones

2.1.4.1. BAP stock solution preparation (10mg/100ml)

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 stock solution was then filtered, labeled and stored at 4ºC for up to 2 months.

2.1.4.2. IAA stock solution preparation (1 mg/ml)

First, 200 mg of IAA (Sigma) was dissolved with 1 drop of 'ethanol absolute.' The total volume 200 ml was made by using double-distilled water. Finally it was filtered and labeled and was stored at -20°C for several months.

2.1.5. Preparation of MS medium

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

Table 2.3. Different components for preparation of MS media

Components (stock conc.)	Amount (for 100 ml)
Macronutrients (10x)	10 ml
Micronutrients (100x)	1 ml
Vitamin/Organic (100x)	1 ml
Fe- EDTA (100x)	1 ml
Myo-inositol	0.01 g
Sucrose	3 g

All components (**Table 2.4**) were added to a conical flask and volume up to 100 ml with ddH_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.

2.1.6. Germination media preparation

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

2.1.7. Shoot regeneration media preparation

Different concentrations and combinations of growth hormones were prepared for plant regeneration. To determine the effect of phytohormones on shoot regeneration MS media was supplemented with BAP (1, 2, 5 and 7 mg/l) alone or in combination with IAA (0.1, 0.2, 0.5, 0.7 and 1 mg/l).

2.1.8. Subculture media preparation

Regenerated explants need to subculture every 3 to 4 weeks. Media with same hormonal supplementation was used for the explants.

2.1.9. Rooting media preparation

Half strength MS medium was used as basal medium for rooting. The medium was supplemented by various IAA. For solidification, 0.3% (w/v) phytagel (Sigma) was used instead of agar in root formation media. Five different concentrations (0.1, 0.2, 0.5, 0.7, 1.0 mg/l) of IAA were tested for rooting response.

2.1.10. Explant culture and shoot regeneration

Cotyledonary leaves from seedlings were collected as explants and cut on to small pieces and then placed to the medium for regeneration. The explants were placed with the both upper and lower surface in contact with medium in different studies. Results were recorded according to the presence of shoot after 45-60 days of inoculation.

2.1.11. Effect of spacing on the shoot formation of the explants

Explants compete with each other for their nutrient source during culture on media (152) (153). Therefore, a minimum distance between the explants should be maintained. Beyond a certain distance, an increase of distance will impart a little or no effect in the competition for nutrition. In the present study, three different distances, *i.e.*, 1.0, 1.5, 2.0 cm distances were chosen. Effects of the given distances were determined for the following parameters: fresh and dry weight of the explants, number of shoot and chlorophyll content. The results were compared between three distances maintained in different varieties.

2.1.11.1. Determination of chlorophyll content

Total chlorophyll content was determined according to the method as described by Curtis and Shetty (154) with some modifications (155). Fresh leaves were weighed and 50 mg was taken in 3 ml of methanol and kept in dark for 2 h at 23°C to allow the chlorophyll dissolved into the solvent. Absorbance was determined at 665 nm and 650 nm. Total chlorophyll content was calculated according to Arnon's equation (156) and expressed as "µg chlorophyll/g fresh tissue" (157) (158).

2.1.12. Plant hardening procedure

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

- The regenerated plants were carefully removed from the rooting media using a forcep when the roots were 3 to 4 cm long. The agar attached to their root part was gently washed with running water. It was to make sure that the entire agar was removed completely to avoid any contamination.

- Then the plants were transferred in a pot containing autoclaved soil. Perforated plastic bags were taken to cover the potted plantlets. The inside of the bags were sprayed with water to maintain the humidity and to prevent moisture shock. Plantlets were kept inside the culture room for 15 days. During these 15 days the moisture inside the bags were maintained constantly.
- 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.
- On the eighth week, the plants were exposed to direct sunlight for 2 hours a day. This treatment was continued for 2 more weeks. Lastly the plants were placed in natural environment. 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.

2.1.13. Analysis of reproductive response of the regenerated plantlets

Following acclimatization of regenerated plantlets survivability, flowering and fruiting response in natural environment were assessed by fruit weight, number of fruits per plant etc. Seeds were collected and germination was tested as **section 2.1.2.2**.

2.2. Tomato transformation

Agrobacterium-mediated transformation of five tomato varieties was carried out in the present study.

2.2.1. Plant material

8-10 days old seedling of five tomato varieties namely, BARI Tomato 2, BARI Tomato 3, BARI Tomato14, BARI Tomato 15, and BINA Tomato 3, were used to

perform the transformation. Cotyledonary leaves of these five varieties were used as explants source.

2.2.2. *Agrobacterium* strain and plasmid vectors

Agrobacterium tumefaciense strain LBA4404 with plasmids constructs, pBI121 and pH7WG2_OsNHX1_1.6 were used for transformation.

pBI121: The total size of pBI121 is 12.8 kb (**Fig. 1 A**) according to its construction map (159). The Ti plasmid contains a plant selectable marker gene neomycin phosphotransferase II (*npt* II) conferring resistance to kanamycin (160) 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.

pH7WG2_OsNHX1_1.6: The Na^+/H^+ antiporter gene (*OsNHX1_1.6*) cloned from rice was immobilized to Gateway vector, pH7WG2_OsNHX1_1.6 (161). This final construct pH7WG2_OsNHX1_1.6 (**Fig. 1 B**) 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.

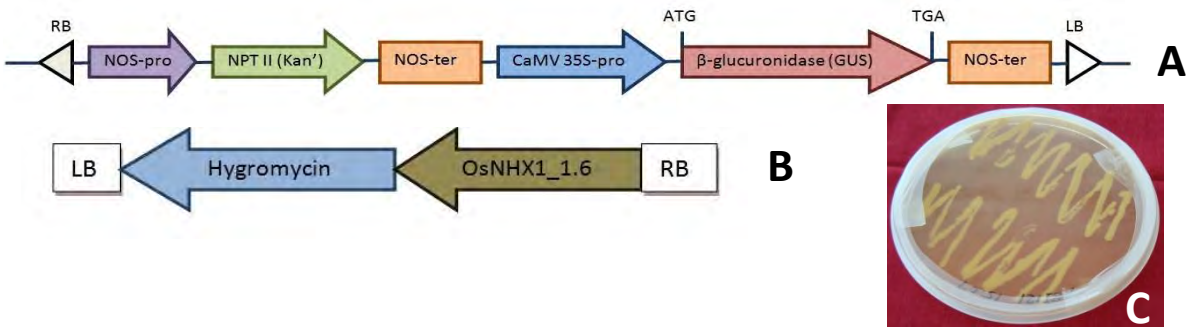

Fig. 1. Schematic diagram of **A.** the T-DNA region of the binary pBI121, **B.** Constructed vector pH7WG2-OsNHX1-1.6 **C.** Pure culture of *Agrobacterum* strain LBA4404 on YMB medium.

2.2.3. Antibiotics used in tomato transformation

Two antibiotics, namely, hygromycin and kanamycin (Duchefa Bioc) were used as selectable agaents. For *Agrobacterium* culture, three antibiotics (kanamycin for *Agrobacterium* strain LBA4404 containing pBI121, and streptomycin and spectinomycin ((Duchefa Bioc)) for *Agrobacterium* strain LBA4404 containing pH7WG2_OsNHX1_1.6) were used. Cefotaxime (Duchefa Bioc) was used after co-cultivation as bacteriostatic against *Agrobacteria*.

2.2.4. Antibiotics stock solution preparation (25 mg/ml)

Kanamycin sulfate, hygromycin, streptomycin, spectinomycin and cefotaxime stock solutions were prepared. 1 g of either one 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.

2.2.5. *Agrobacterium tumefaciense* culture media preparation

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

Table 2.4. 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 media.

2.2.6. Co-cultivation media preparation

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

2.2.7. Media for kanamycin or hygromycin sensitivity test

Regeneration media with different concentrations of kanamycin or hygromycin was used for plant sensitivity tests.

2.2.8. Selection media preparation

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

2.2.9. Determination of baseline saline tolerance level of tomato seedlings

In the present study, the effect of salinity on germination of tomato seeds was investigated as it is the first stage towards salinity tolerant transgenic tomato production. Therefore, five local varieties, namely, BARI Tomato 2, BARI Tomato 3, BARI Tomato 14, BARI Tomato 15 and BINA Tomato 3 were selected for the study. To represent various salinity conditions, different NaCl concentrations ranging from 5-200 mM, which in turn stands for 0.5-20 dS/m were chosen for the study. MS media was prepared with different amount (5 mM, 10 mM, 20 mM, 50 mM and 100 mM) of NaCl in each 100 ml of media.

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

	NaCl concentration in MS media (mM)	Amount of NaCl added in MS Media (g)
For 100 ml MS media	5	0.029
	10	0.058
	20	0.117
	50	0.292
	100	0.585

Seeds were placed in these media and the result was recorded after a week to get their germination response in different salt concentrations.

2.2.10. 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* (transformed with 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 in 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 (OD_{600} nm) of the overnight grown culture taken with autoclaved fresh liquid YMB media were ready to be used as blank by using spectrophotometer. The zero time absorbance of culture density was obtained from the blank. The Petri-dish with filter paper is soaked with liquid MS media and then the Petri-dish was used to cut explants. Explants were dipped in bacterial suspension for 30 to 60 minutes infection and then placed on co-cultivation media 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 antibiotic containing MS media. If there is any bacterial overgrowth shown on explants, then those explants were washed with cefotaxime and transferred to cefotaxime containing MS media.

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.

2.2.11. Determination of antibiotic concentration for selection medium

Antibiotics impart inhibitory effect against the growth of the cells and tissues. Plants can resist the inhibitory effect of antibiotics up to a certain concentration. Above that concentration plant cells cannot survive. In transformation protocols antibiotics are

used in selection media to differentiate transformed cells from the non-transformed ones. Therefore, optimum concentration just to inhibit the explants regeneration is necessary to establish a transformation protocol. Antibiotic resistant gene within the transformed cells protects the cells of the explants from the toxic effect of antibiotic. Such selection process has a growth penalty because this process requires a great deal of energy which in turn affects the growth of the explants (162) (163). Determination of the antibiotic concentration optimum for selection of transformed cells can be obtained by applying a range of a given antibiotic to the explants through plant regeneration media. The optimum concentration of antibiotic for selection varies with any given antibiotic and plant varieties. This is due to the fact that all the antibiotics do not behave the same way in terms of mode of action or the extent of toxicity (164). Also genotype of plant species and varieties considerably influences its response toward a given antibiotics (165) (166). The minimum concentration killing the explants is the concentration to be used in selection media. In the present study different concentrations of hygromycin and kanamycin were used to determine their concentration in selection media for tomato transformation.

2.2.11.1. Methodology of kanamycin sensitivity test

Different concentrations (0, 50, 100, 150 and 200 mg/l) of kanamycin were added to autoclaved regeneration media after cooling down. The explants were placed in those media to check their regeneration response. The result was recorded after 45 days of inoculation of cotyledonary leaf explants in BARI Tomato 2, BARI Tomato 14 and BINA Tomato 3.

2.2.11.2. Methodology of hygromycin sensitivity test

To study the effect of hygromycin on the growth of tomato cotyledonary explants, regeneration media containing various concentrations (0 mg/l, 1 mg/l, 3 mg/l, 4 mg/l, 5 mg/l, 10 mg/l, 20 mg/l and 30 mg/l) of hygromycin was prepared. After autoclaving, hygromycin was added to the media inside laminar air flow hood and then it was separated into Petri-dishes. Ten explants were subjected to each concentration of hygromycin. Cotyledonary leaf explants from all three varieties viz. BARI Tomato 15, BARI Tomato 14 and BINA Tomato 3 were tested. Cotyledonary leaf explants without any physical damages of the varieties were placed in the prepared media while maintaining the aseptic condition. A control was also maintained which contained no hygromycin. Survivability and regeneration response of the explants were recorded.

2.1.12. Precaution to maintain aseptic condition

To maintain aseptic condition, all inoculation was carried out under the laminar air flow hood (SCV-$AI, Singapore) UV light of laminar hood was on for 30 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. 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.

2.2.13. Preparation of reagents for performing histochemical GUS assay

2.2.13.1. Preparation of MES buffer

2.44 g MES were weighed into a clean dry beaker. 20 ml ddH_2O was added and mixed well to dissolve MES completely. pH was adjusted to 5.6 with 5 M KOH. Final volume was made up to 25 ml and stored at room temperature.

2.2.13.2. Preparation of fixation solution

Table 2.6. 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 ddH_2O was added to make final volume upto 100 ml. Stored at room temperature for next three months or until precipitate appears.

2.2.13.3. 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

2.2.13.4. 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) 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.

2.2.14. Histochemical GUS assay

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

2.2.15. Plant DNA Isolation

2.2.15.1. Preparation of stock solution of DEB (DNA Extraction Buffer)

Table 2.7. Components for preparation of DEB

Components	Concentration	To make 1000ml
1 M Tris-HCl, pH 8.0 (autoclaved)	100 mM	100 ml
500 mM EDTA, pH 8.0 (autoclaved)	5 mM	200 ml
5 M NaCl	200 mM	29.22 g
20% SDS	0.2 %	62.5 ml
Sodium bisulphate	-----	3.8 g
DDH_2O	-----	637.5 ml

All components (mentioned above in **Table 2.7**) were added to ddH_2O and volume was made upto 1000 ml. Finally the pH 7.8-8.0 was set by using NaOH.

2.2.15.2. Plant genomic DNA isolation procedure

- Plant tissue was crushed in liquid Nitrogen and collected in 1.5 ml eppendorf tubes. 800 µl pre-heated DNA extraction buffer (mentioned in **section 2.2.15.1**) was added and mixed well by vortexing and inverting.
- Those tubes were placed in 65°C water bath for 20 minutes (after 10 minutes mix by inverting and return to the water bath).
- The tubes were removed, mixed by inverting and brought to a chemical fumehood. 700 µl chloroform mix (24:1) mixture of chloroform and isoamyl alcohol) was added. Tubes were tightly closed and invert repeatedly for 2-3 minutes.
- The tubes were centrifuged for 8 minutes at 11,000 rpm in a micro centrifuge. 300-500 µl of the upper aqueous layer was taken to a new 1.5 ml tube (being careful not to pipette near the dirty layer). 1000 µl cold ethanol was added and mixed by inverting. Then centrifuged for 12mins at max speed (13,200 rpm).
- Solution was decanted by pouring into a beaker and then touched the tip of the tube to a tissue to remove excess solution while keeping the DNA pellet undisturbed at the bottom of the tubes. 1000 µl cold 70% ethanol was added to all tubes and spin for 3-5 minutes at 13,200 rpm.
- The 70% ethanol was poured off into a beaker from all tubes. Then the remaining liquid was removed with p-200. The pellet was dried out by inverting the tubes on a bench top on top of tissue for 45 minutes to 1 hour. The pellet was then resuspended in 100 µl TE buffer and dissolved by warming in a 65°C water bath for 1 hour.

- DNase free RNase was added to final concentration of (10 µg/ml) and incubated for 30 minute at 37°C. Equal volume phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged 10 minute 10,000 rpm.
- Equal volume phenol: chloroform: isoamyl alcohol (25:24:1) was added again and then centrifuged 10 minute 10000 rpm. Aqueous phase was taken and 3M Na-acetate was added at $^{1}/_{10}$ volume of the aqueous solvent and double volume of the total solvent 99% ice cold ethanol were added.
- This mixture was kept on ice for 30 minute and centrifuged for 10 minute 10000 rpm. The supernatant was discarded, washed with 3-5ml 70% ice cold Ethanol and centrifuged for 10 minute 10000 rpm.
- The supernatant was discarded and the pellet was dried out completely. Then it was dissolve in minimum TE solution and stored at -20°C. After that the DNA was analyzed by Agarose gel (0.8%) electrophoresis.

2.2.16. PCR protocol

2.2.16.1. Components for a 15 µl reaction

1) 1µl of 80 ng/µl DNA
2) 1.5 µl of 10X PCR buffer
3) 1 µl of 25 mM $MgCl_2$
4) 1.5 µl of 1 mM dNTP
5) 0.5 µl OsNHX1_F_1.6
6) 0.5 µl OsNHX1_R_1.6
7) 0.5 µl Taq Polymerase
8) 2 µl DMSO
9) 6.5 µl PCR H_2O

The reagents were kept on ice. Taq polymerase was added last. Prior to placing the samples all the mixture were mixed properly.

2.2.16.2. PCR cycle

Step 1: 95°C for 5 min

Step 2: 95°C for 1 min

Step 3: 61.4°C for 1 min

Step 4: 72°C for 1 min

Step 5: Step 2 to 4 were repeated for 35 cycles

Step 6: 72ºC for 7 min (final extension)

A typical PCR reaction of 15 µl contained 1 µl of 80 ng/µl extracted DNA as template, 10 mM Tris-HCl, (pH 8.3; at room temperature) 50 mM KCl, 0.5-3.5 mM $MgCl_2$ solution, 1.5 µl of 1 mM dNTPs, 0.5 µl of Taq polymerase, and 0.5 µl of each of primers. Pre-PCR denaturing at 95°C for 5 min, annealing at 61.4°C for 1 minutes followed by extension at 72°C for 1 minutes followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 61.4°C for 1 min and extension at 72°C for 1 minutes with a final extension cycle of 7 minutes at 72°C.

2.2.16.3. Agarose gel electrophoresis

0.8% agarose gel electrophoresis was done to visualize the amplified PCR product and 1 kb plus ladder marker DNA was used. The purity and concentration of amplified DNA was checked from the band in agarose gel by using known concentrations of bacteriophage lambda DNA (50ng/µl).

2.2.16.4. Gel electrophoresis procedure

Agarose gel preparation

- 50x TAE buffer was measured in a conical flask and diluted by adding ddH_2O to attain required volume. Then the agarose (0.8% w/v) was melted in a microwave for 2 to 3 minutes. The solution was cooled until the temperature is below 50-55°C.
- The combs were placed in the gel casting tray and the cooled solution was poured into it. The gel was kept there until solid.
- The combs were gently removed and the gel was then placed in the electrophoresis chamber with 1x TAE buffer. Enough TAE buffer was added to cover the gel so that buffer remains 2-3 mm over the gel.

Agarose gel loading

- The DNA samples with sample loading buffer were carefully loaded on the gel and consequently for size standards lambda DNA was loaded in one row on each.

Agarose gel running

- The electrophoresis chamber was covered with the lid and the power supply was turned on.
- Then 100V was applied. The electrophoresis was done till the blue dye was found three fourth of the length of the gel, then the power supply was turned off and the lid was removed.
- The gel with tray was removed carefully by using gloves.

Agarose gel staining

- The gel was removed from the casting tray and was stained for 15 minutes as it was dipped in water containing Ethidium bromide (0.5 g/ml).
- Finally the gel was photographed under UV light and the data was recorded.

Chapter 3

RESULTS

In the present study, regeneration and transformation response of five Bangladeshi farmer popular tomato varieties, namely BARI Tomato 2, BARI Tomato 3, BARI Tomato 14, BARI Tomato 15 and BINA Tomato 3, were studied. This chapter captures the results of establishing *in vitro* regeneration protocol (**section 3.1**) and transformation protocol and putative transgenic regeneration (**section 3.2**) of these five varieties.

3.1. *In vitro* regeneration

Cotyledonary leaves collected from aseptically germinated seedlings were used as explants to perform *in vitro* regeneration. Successfully regenerated shoots were placed for root formation. Those plantlets were then transformed to the soil for flowering and fruit setting. Viability of seeds from mature fruits of these *in vitro* regenerated plants was tested.

3.1.1. Time requirement for seed germination initiation

All the tomato varieties included in this study showed germination when the seeds were added in germination medium. Responsive seeds of BARI Tomato 2, BARI Tomato 3 and BINA Tomato 3 took 3-4 days for germination initiation, while the rest took 2-3 days after placing in germination media (**Table 3.1**).

3.1.2. Seed germination rate

For seed germination, a total of 50 seeds of each variety were taken and this experiment was done in triplicate. Data on germination rate are presented in Table 3.2. Germination rate varied among different tomato varieties. Highest germination rate was observed in BARI Tomato 14 (94%) followed by BARI Tomato 15 (84%), and the lowest for BARI Tomato 3 (75.2%). BINA Tomato 3, the only BINA variety included in this study showed a moderate germination rate of 82%. In all the cases,

the standard errors were within the acceptable statistical limit. Germinated seedlings of different varieties are shown in **Fig. 2 A-F**.

Table 3.1. Seed germination rate in five tomato varieties

Tomato varieties	**Total no of seeds inoculated**	**Percentage of germinated seeds**	**Mean no. of germinated seeds ±SE***	**Days required for germination initiation**
BARI Tomato 2	50	83.2	41.6±2.2	3-4
BARI Tomato 3	50	75.2	37.6±2.1	3-4
BARI Tomato 14	50	94	47±1.4	2-3
BARI Tomato 15	50	84	42±1.8	2-3
BINA Tomato 3	50	82	41±1.8	3-4

*Mean values are from 3 replications. Data was taken after a week of inoculation.

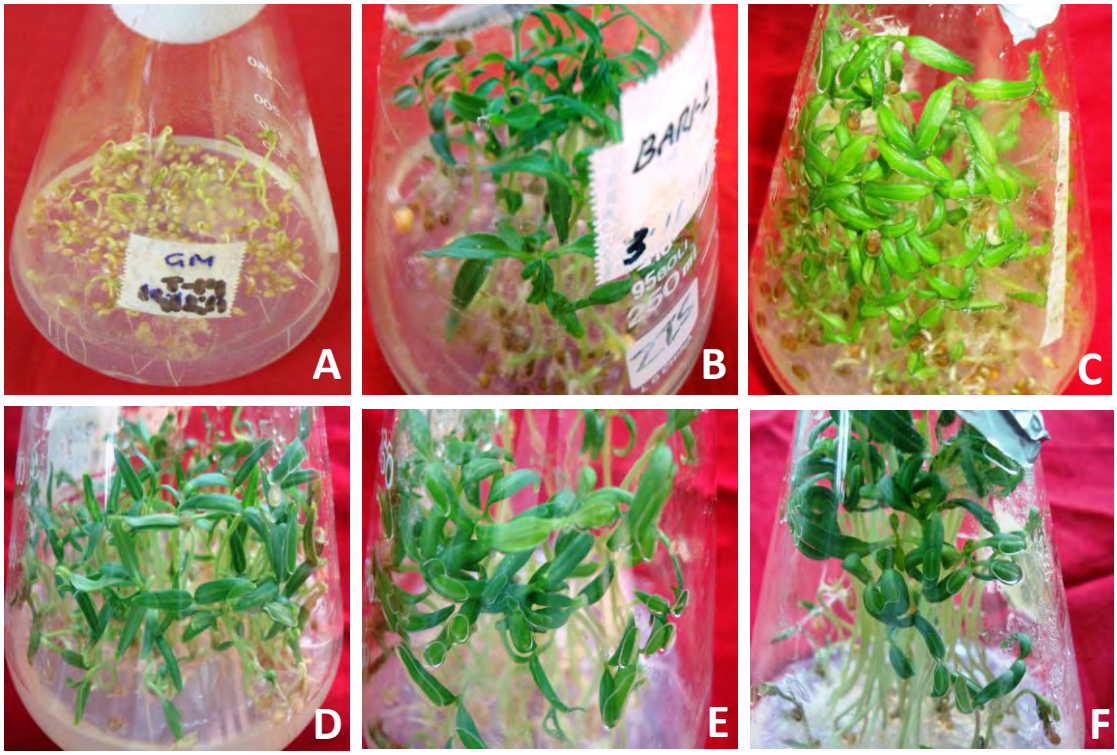

Fig. 2. Germinated seedlings of tomato varieties **A.** germination initiation in BARI Tomato 14 after 4 days of inoculation, **B.** 7 days old seedlings of BARI Tomato 2, **C.** 12 days old seedlings of BARI Tomato 3, **D.** 10 days old seedlings of BARI Tomato 14, **E.** 7 days old seedlings of BARI Tomato 15 and **F.** 10 days seedlings of BINA Tomato 3

3.1.3. Effect of explant orientation on regeneration

Explants positioned in abaxial (lower surface facing down) orientation showed maximum response in shoot formation (**Fig 3. A**) compared to the explants inoculated in adaxial (upper surface facing down) orientation (**Fig 3. B**). And the regeneration started from one or both the cut ends of cotyledonary leaf explants (**Fig 3. C-D**).

3.1.4. Effect of BAP concentrations on regeneration and shoot formation

Regeneration initiation from explants of all varieties took place within 15 to 20 days depending on the hormonal supplementation in the regeneration media. Shoot formation was also found depending on BAP concentrations. Along with supplementation of growth regulators in media, genotype was also found to influence the regeneration response.

Explants enlargement was observed before regeneration started (**Fig. 3 E**). Low concentration of BAP led to callus formation wherefrom shoots appeared in the later stage (**Fig. 3 F-G**). When the concentration of BAP was high, shoots directly came out without forming callus (**Fig. 3 H**) in the same varieties. Similar response was observed in BARI Tomato 3, BARI Tomato 14 and BINA Tomato 3. Variation was observed in BARI Tomato 15 where low BAP initiated indirect regeneration, but higher BAP supplementation failed to attain any regenerative response.

Regeneration response of various varieties is presented in **Table 3.2**. Among the five varieties, highest regeneration response was observed in two varieties, namely BARI Tomato 2 and BARI Tomato 15. In BARI Tomato 15, highest response was observed in MS medium supplemented with 2 mg/l BAP. Interestingly in BARI Tomato 2,

similar response was triggered by both low (2 mg/l) and high (7 mg/l) BAP containing media.

In most of the cases, highest number of shoots was found in low BAP supplementation. For BARI Tomato 3, BARI Tomato 14, BARI Tomato 15 and BINA Tomato 3, 2 mg/l BAP supplementation was observed to be enough for highest shoot formation. In BARI Tomato 3, BARI Tomato 14 and BINA Tomato 3, shoot number decreased when BAP was 5 mg/l or more in the regeneration media. In addition to this, abnormal morphology such as abnormal leaf formation was shown in these conditions (**Fig. 3 I**). But surprisingly, for BARI Tomato 2, highest number of shoots was formed in 7 mg/l BAP concentration (**Fig. 3 J**) which is contrasting to other varieties. Multiple shoots were mostly found in 1 mg/l and 2 mg/l BAP concentrations in all varieties (**Fig. 3 K**) except BARI Tomato 15 (**Fig. 3 L**).

The regeneration percentages of all varieties were analyzed by ANOVA. The results were statistically significant as found from one way ANOVA analysis where F (3.3) was greater than F crit (3.0) and the P value was statistically significant (<0.05) (**Table 3.3**).

Table 3.2. Effect of BAP concentrations on shoot regeneration in all tomato varieties

Tomato varieties	BAP conc. (mg/l)	Time required for regeneration initiation (days)	Total no. of explants	Percentage of regeneration	Mean shoot no. ± SE*	Time required for shoot development (days)
BARI Tomato 2	1	22	25	84	4.3±0.4	14
	2	18	22	**96**	7.6±0.4	15
	5	19	29	90	6.0±1.8	16
	7	19	22	**96**	**8.0±0.7**	16
BARI Tomato 3	1	17	12	75	6.6±1.0	17
	2	18	11	**82**	**7.0±0.7**	17
	5	18	21	76	5.7±0.4	18
	7	18	19	73	2.6±0.4	18
BARI Tomato 14	1	11	30	78	8.3±0.4	12
	2	12	22	**87**	**8.6±1.0**	14
	5	12	20	75	5.0±0.7	14
	7	14	27	74	5.3±0.8	14
BARI Tomato 15	1	11	22	91	7.6±0.4	16
	2	12	22	**96**	**8.0±1.4**	17
	5	14	23	87	7.0±1.8	16
	7	14	23	73	0	N/A
BINA Tomato 3	1	15	18	84	8.6±0.4	15
	2	15	20	**90**	**9.0±0.7**	15
	5	17	33	76	4.3±0.8	16
	7	17	24	75	3.3±0.4	16

*Data was taken after 60 days after regeneration initiation

Table 3.3. One way ANOVA analysis for the effect of BAP concentrations on regeneration

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	607.3	4	151.825	3.288628	0.040058	3.055568
Within Groups	692.5	15	46.16667			
Total	1299.8	19				

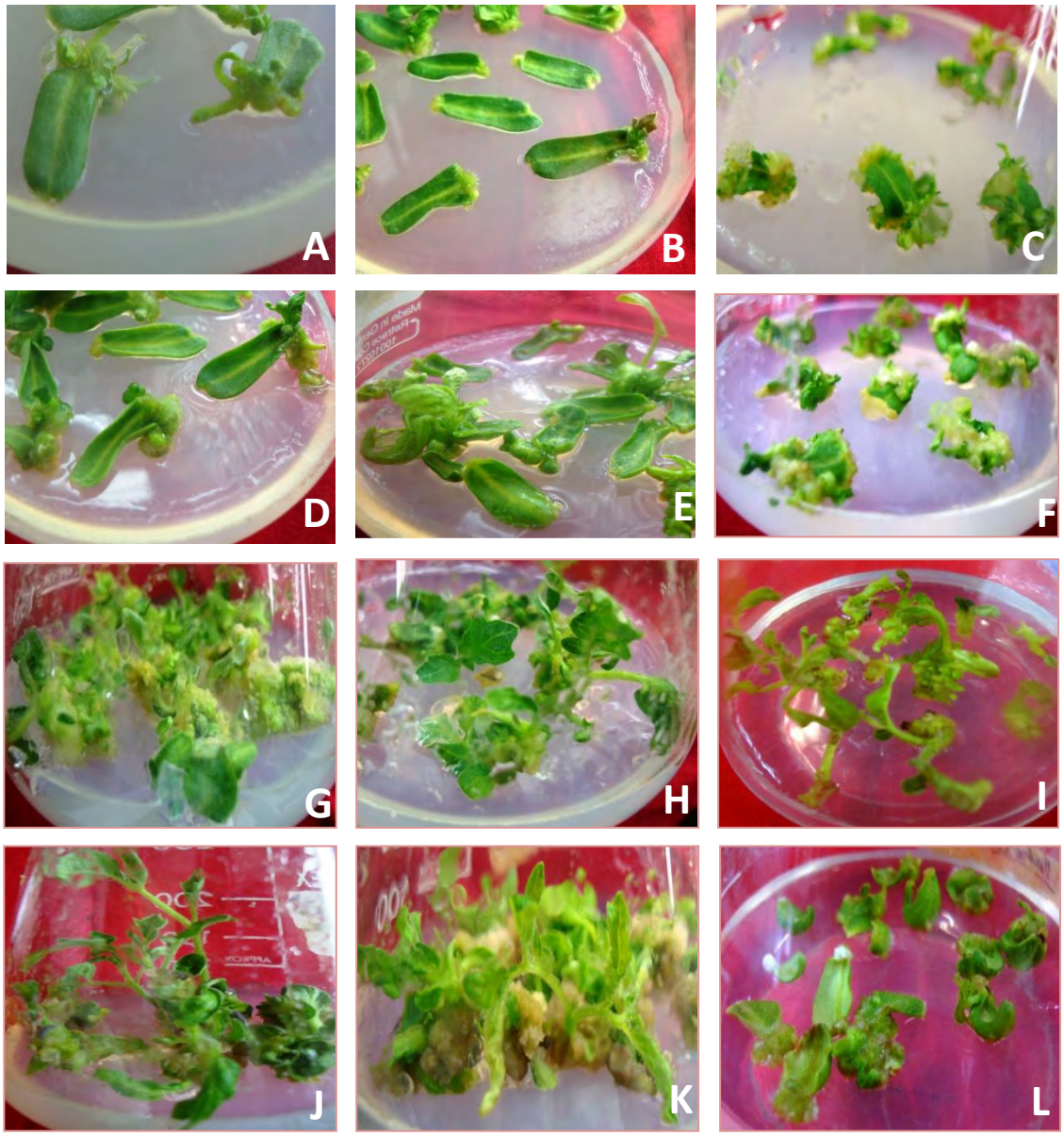

Fig. 3. Effect of BAP on regeneration of tomato varieties **A.** explants in media positioned in abaxial and **B.** adaxial orientation **C.** explants regeneration from both cut ends and **D.** one end **E.** explants increase three times the original size before regeneration takes place, **F.** BARI Tomato 2 on regeneration media containing 1 mg/l BAP after 30 days of their inoculation, **G.** same variety at same concentration after 60 days of their inoculation, **H.** BARI Tomato 2 on media supplemented with 7 mg/l after 30 days of their inoculation, **I**. Abnormal morphology of BARI Tomato 3 explants in BAP 7 mg/l after 30 days of inoculation, **J.** BARI Tomato 2 on media supplemented with 7 mg/l BAP after 60 days of their inoculation, **K.** Multiple shoots of BARI Tomato 3 and **L.** BARI Tomato 15 in 7 mg/l BAP concentration after 60 days of inoculation.

3.1.5. Effect of BAP and IAA combinations on regeneration of different tomato varieties

In this study, regeneration response was also studied with BAP and IAA combination. The results are presented in Tables 3.4-3.8. While studying the effects of the combinations of different concentrations of BAP and IAA, highest regeneration was observed in all five varieties at BAP concentration 2 mg/l with IAA concentrations ranging from 0.1 to 1.0 mg/l. Most of the combinations tested resulted in good regeneration and shoot formation, although for some varieties, the regeneration and shoot formation fell with the increase in BAP concentrations (5 mg/l and 7 mg/l) irrespective of IAA supplementation.

Among the growth hormone supplementation in media tested, MS medium supplemented with 2 mg/l BAP + 0.1 IAA gave the best multiple shoots response in BARI Tomato 2 and BARI Tomato 14 (**Fig. 4 A-B**). For BARI Tomato 15, highest number of shoots was obtained in two combinations, 2 mg/l BAP+ 0.1 mg/l IAA (**Fig. 4 C**) and 2 mg/l BAP+ 0.2 mg/l IAA (**Fig. 4 D**).

Among the varieties, highest number of shoot formation (7.00 ± 0.58) was found in BARI Tomato 2 and BARI Tomato 15 in MS media supplemented with 2 mg/l BAP+0.1 mg/l IAA (**Table 3.6**). While the lowest number of shoot was obtained in BARI Tomato 3 at 7 mg/l BAP+0.7 mg/l IAA containing MS media (**Fig. 4 E**).

One way ANOVA analysis showed that the results obtained for different concentration groups significantly differed from each other as the F (2.11) was greater than F crit (1.71) and the P value was statistically significant (<0.05) (**Table 3.9**).

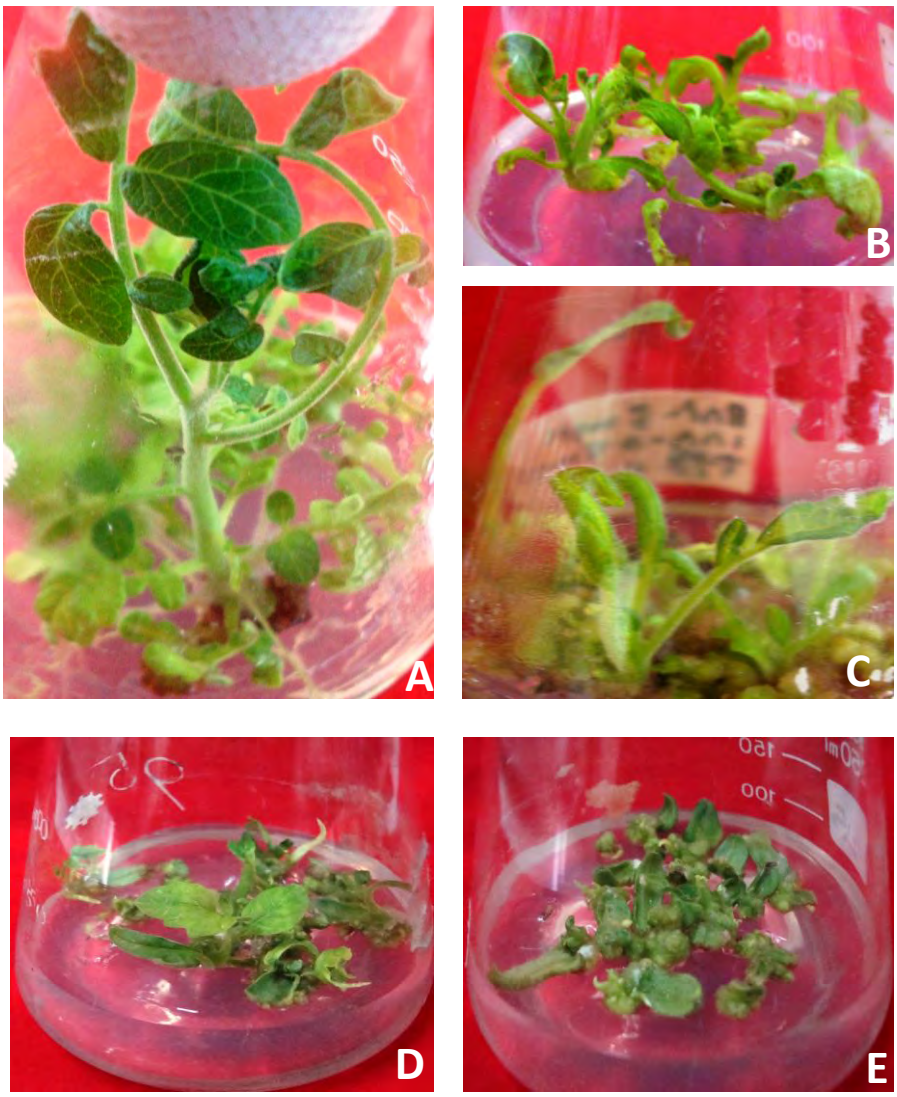

Fig. 4. Effect of BAP and IAA combinations on regeneration **A.** Regenerated BARI Tomato 14 and **B.** BARI Tomato 2 on regeneration media supplemented with 2 mg/l BAP+ 0.1 mg/l IAA, **C.** BARI Tomato 15 on regeneration media containing 2 mg/l BAP +0.2 mg/l IAA **D.** BARI Tomato 15 on regeneration media containing 2 mg/l BAP +0.1 mg/l IAA **E.** BARI Tomato 3 on regeneration media with 7 mg/l BAP and 0.7 mg/l IAA supplementation

Table 3.4. Effect of BAP+ IAA combinations on regeneration of BARI Tomato 2

BAP conc. (mg/l)	IAA conc. (mg/l)	Total no. of explants	No. of explants regenerated	Percentage of regeneration	Mean shoot no. ± SE*	Time required for shoot development (days)
	0.1	10	9	90	**6.33 ± 0.33**	14
	0.2	10	9	90	5.00 ± 0.71	15
1	0.5	11	10	**91**	5.33 ± 0.67	16
	0.7	11	9	81	4.67 ± 0.33	16
	1	10	7	70	5.00 ± 0.00	17
	0.1	10	10	**100**	**8.33 ± 0.40**	17
	0.2	11	9	**100**	7.00 ± 0.58	16
2	0.5	10	9	90	5.67 ± 0.33	15
	0.7	10	8	80	3.33 ± 0.33	16
	1	10	10	**100**	2.33 ± 0.33	16
	0.1	10	7	70	2.33 ± 0.33	15
	0.2	12	8	67	**5.67 ± 0.67**	16
5	0.5	10	8	80	4.00 ± 0.00	15
	0.7	10	7	70	4.33 ± 0.33	15
	1	10	10	**100**	3.67 ± 0.67	15
	0.1	12	10	84	4.34 ± 0.33	17
	0.2	10	9	**90**	**5.67 ± 0.67**	17
7	0.5	11	9	81	4.00 ± 0.00	16
	0.7	10	8	80	3.33 ± 0.75	16
	1	10	7	70	4.67 ± 0.33	16

*Data was taken after 60 days after regeneration initiation

Table 3.5. Effect of BAP+IAA combinations on regeneration of BARI Tomato 3

BAP conc. (mg/l)	IAA conc. (mg/l)	Total no. of explants	No. of explants regenerated	Percentage of regeneration	Mean shoot no. ± SE*	Time required for shoot development (days)
	0.1	10	8	80	5.00 ± 0.58	16
	0.2	10	10	**100**	**7.00 ± 0.58**	15
1	0.5	10	9	90	6.33 ± 0.33	16
	0.7	9	9	**100**	4.00 ± 0.33	16
	1	10	9	90	3.67 ± 0.33	15
	0.1	10	10	**100**	**6.67 ± 0.88**	17
	0.2	9	9	**100**	5.33 ± 0.67	18
2	0.5	10	10	**100**	5.00 ± 0.33	18
	0.7	10	10	**100**	6.33 ± 0.33	12
	1	10	10	**100**	4.00 ± 0.33	14
	0.1	10	7	70	**3.67 ± 0.88**	14
	0.2	9	9	**100**	3.00 ± 0.00	15
5	0.5	9	7	78	2.00 ± 0.58	15
	0.7	10	8	80	2.33 ± 0.33	16
	1	10	7	70	1.00 ± 0.58	17
	0.1	10	8	80	2.00 ± 0.00	18
	0.2	10	7	70	**3.00 ± 0.58**	18
7	0.5	10	7	70	2.33 ± 0.33	16
	0.7	9	9	90	0.67 ± 0.33	15
	1	10	8	80	1.33 ± 0.67	16

*Data was taken after 60 days after regeneration initiation

Table 3.6. Effect of BAP+IAA combinations on regeneration of BARI Tomato 14

BAP conc. (mg/l)	IAA conc. (mg/l)	Total no. of explants	No. of explants regenerated	Percentage of regeneration	Mean shoot no. ± SE*	Time required for shoot development (days)
	0.1	10	10	**100**	4.67 ± 0.33	14
	0.2	10	9	90	5.67 ± 0.67	15
1	0.5	10	10	**100**	**6.33 ± 0.67**	16
	0.7	10	8	80	4.00 ± 0.33	16
	1	10	8	80	3.67 ± 0.33	16
	0.1	10	10	**100**	**6.33 ± 0.33**	17
	0.2	10	9	90	4.00 ± 0.33	17
2	0.5	10	9	90	5.67 ± 0.67	16
	0.7	10	10	**100**	4.33 ± 0.88	16
	1	10	10	**100**	6.33 ± 0.88	17
	0.1	10	7	70	1.00 ± 0.58	14
	0.2	10	9	**90**	**4.00 ± 0.33**	14
5	0.5	10	8	80	1.00 ± 0.00	16
	0.7	10	9	**90**	2.33 ± 0.33	17
	1	10	9	**90**	1.67 ± 0.67	16
	0.1	10	9	90	1.00 ± 0.58	16
	0.2	10	10	**100**	**3.67 ± 0.88**	17
7	0.5	10	9	90	1.00 ± 0.00	17
	0.7	10	8	80	1.00 ± 0.58	15
	1	10	6	60	2.33 ± 0.33	15

*Data was taken after 60 days after regeneration initiation

Table 3.7. Effect of BAP+ IAA combinations on regeneration of BARI Tomato 15

BAP conc. (mg/l)	IAA conc. (mg/l)	Total no. of explants	No. of explants regenerated	Percentage of regeneration	Mean shoot no. ± SE*	Time required for shoot development (days)
1	0.1	10	10	**100**	4.66 ± 0.67	14
	0.2	10	10	**100**	**5.67 ± 0.33**	14
	0.5	10	9	90	4.00 ± 0.33	15
	0.7	10	10	**100**	3.67 ± 0.33	15
	1	10	10	**100**	5.00 ± 0.33	15
2	0.1	10	10	**100**	**8.33 ± 0.40**	17
	0.2	10	10	**100**	8.0 ± 0.40	16
	0.5	10	10	**100**	3.00 ± 0.00	15
	0.7	10	9	90	3.67 ± 0.33	15
	1	10	9	90	4.33 ± 0.33	14
5	0.1	10	8	80	3.00 ± 0.00	14
	0.2	9	7	78	2.00 ± 0.58	14
	0.5	11	10	**91**	1.34 ± 0.33	16
	0.7	10	9	90	**3.67 ± 0.33**	17
	1	11	10	**91**	3.00 ± 0.58	16
7	0.1	10	8	80	1.00 ± 0.58	15
	0.2	10	10	**100**	1.00 ± 0.00	15
	0.5	11	10	91	**3.33 ± 0.33**	14
	0.7	10	9	90	**3.33 ± 0.33**	14
	1	10	10	**100**	2.00 ± 0.58	14

*Data was taken after 60 days after regeneration initiation

Table 3.8. Effect of BAP+IAA combinations on regeneration of BINA Tomato 3

BAP conc. (mg/l)	IAA conc. (mg/l)	Total no. of explants	No. of explants regenerated	Percentage of regeneration	Mean shoot no. ± SE*	Time required for shoot development (days)
1	0.1	11	8	73	3.00 ± 0.58	15
	0.2	12	12	**100**	4.33 ± 0.33	15
	0.5	13	12	92	4.00 ± 0.00	16
	0.7	12	12	**100**	4.33 ± 0.33	16
	1	13	13	**100**	**6.33 ± 0.33**	15
2	0.1	10	10	**100**	4.67 ± 0.33	15
	0.2	11	10	91	3.67 ± 0.33	15
	0.5	12	11	92	4.33 ± 0.33	16
	0.7	11	10	91	3.33 ± 0.33	16
	1	10	9	90	**6.00 ± 0.58**	16
5	0.1	10	8	80	**2.33 ± 0.88**	16
	0.2	10	8	80	2.33 ± 0.33	15
	0.5	10	9	90	2.00 ± 0.00	16
	0.7	11	11	**100**	1.00 ± 0.58	17
	1	10	10	**100**	0.67 ± 0.33	16
7	0.1	10	10	**100**	1.00 ± 0.00	15
	0.2	11	11	**100**	2.00 ± 0.58	15
	0.5	13	11	85	1.34 ± 0.33	15
	0.7	11	10	91	**2.67 ± 0.67**	16
	1	12	12	**100**	1.33 ± 0.33	16

*Data was taken after 60 days after regeneration initiation

Table 3.9. One way ANOVA analysis for the effect of BAP+IAA on regeneration

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	3650.16	19	192.1137	2.114509	0.011082	1.718026
Within Groups	7268.4	80	90.855			
Total	10918.56	99				

3.1.6. Effect of explants spacing on the shoot formation and chlorophyll content of the explants

Cotyledonary leaf explants of BARI Tomato 3 and BARI Tomato 15 were placed on regeneration media at 1 cm, 1.5 cm and 2 cm distances apart (**Fig. 5 A-C**) to see their effect on regeneration and shoot formation. This experiment was done to get an understanding of regeneration response in relation to nutrient thus survivability.

Fresh weight and dry weight of explants were measured. In this study, highest fresh weight and dry weight were found in both BARI Tomato 3 and BARI Tomato 15 varieties placed 2 cm apart (**Table 3.10**). For both the varieties, lowest fresh weight and dry weight were found when explants were placed only at 1 cm away from each other. Regeneration and shoot formation were observed in both the varieties (**Fig. 5 D-I**).

Highest shoot number was obtained for both BARI Tomato 15 and BARI Tomato 3 at 1.5 cm distance apart (**Table 3.11**). When chlorophyll content was analyzed, the best results were obtained at 1.5 cm for both BARI Tomato 3 (**Table 3.12**) and BARI Tomato 15 (**Table 3.13**) varieties.

Table 3.10. Fresh weight and dry weight of explants cultured at 3 different spacing

Tomato varieties	Space between explants (cm)	Average fresh weight (g) ±SE*	Average dry weight (g) ±SE*
BARI Tomato 3	1	0.0803±0.005	0.0068±0.006
	1.5	0.0883±0.011	0.0091±0.001
	2	0.1092±0.012	0.0092±0.001
BARI Tomato 15	1	0.079±0.015	0.0068±0.0005
	1.5	0.086±0.016	0.0065±0.001
	2	0.1006±0.016	0.0073±0.0012

*Average values are from 6 replications

Table 3.11. Effect of different spacing on shoot formation

Tomato varieties	Spacing (cm)	No of explants	Mean no. of shoot ± SE*
BARI Tomato 3	1	12	9.6±0.4
	1.5	12	10±1.4
	2	12	7.3±1.0
BARI Tomato 15	1	12	8.5±0.3
	1.5	12	10±0.7
	2	12	5.1±1.2

*Average values are from 3 replications

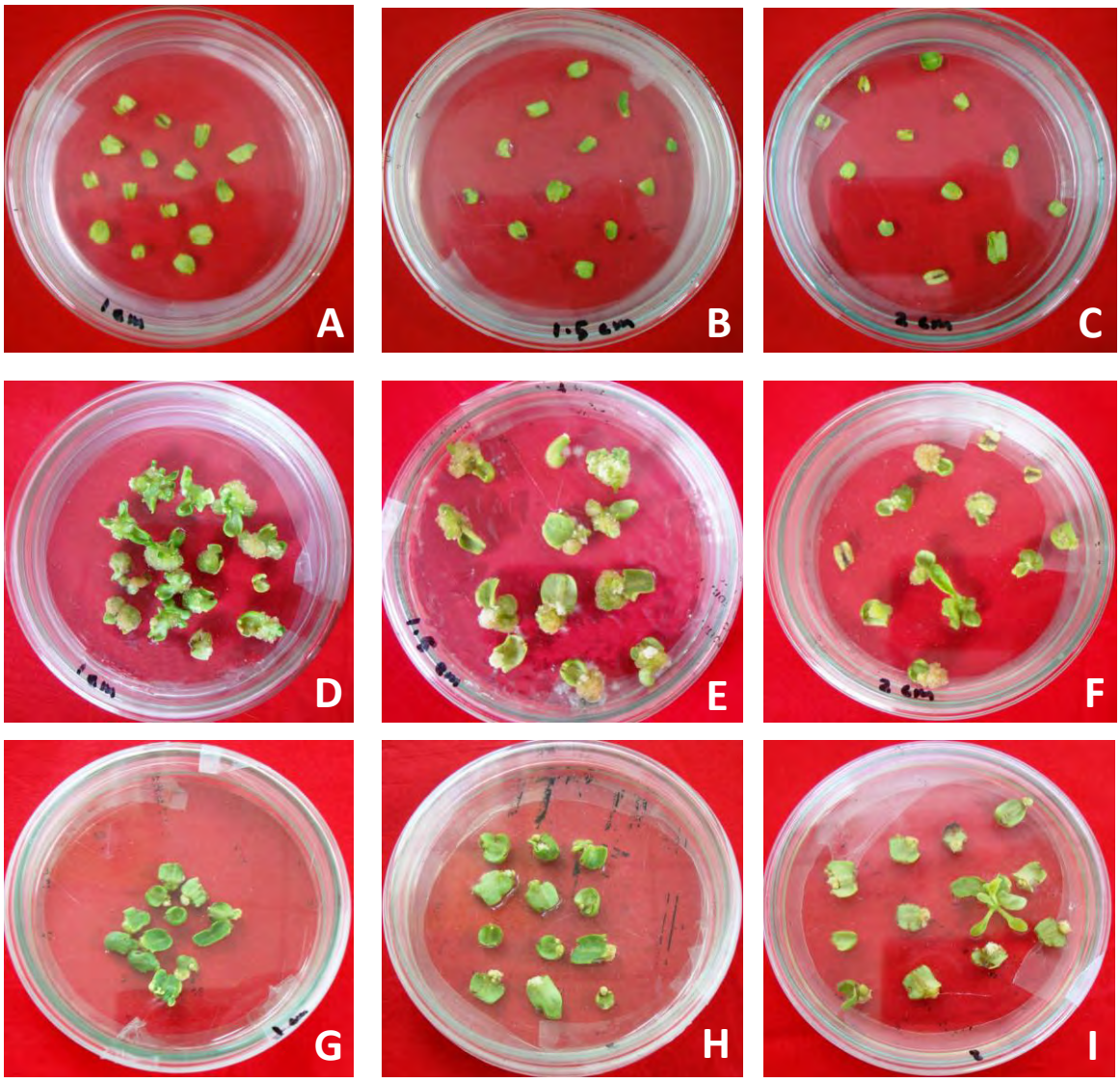

Fig. 5. Effect of explants cultured at different spacing **A.** Explants are placed on regeneration media at 1 cm distance, **B.** 1.5 cm distance, **C.** and 2 cm distance (These Photos were taken 3 days after placing them on media)

Regenerated explants of BARI Tomato 15 at **D.** 1 cm distance, **E.** 1.5 cm distance, and **F.** 2 cm distance and regenerated explants of BARI Tomato 3 at **G.** 1 cm distance, **H.** 1.5 cm distance and **I.** at 2 cm distance (These photographs were taken 60 days after inoculation)

Table 3.12. Effect of spacing between tomato explants of BARI Tomato 3 on chlorophyll content

Spacing between explants (cm)	Absorbance in methanol		Chlorophyll content (µg/g fresh tissue)					
	665 nm	650 nm	Chlor *a*	Chlor *a* (mean±SE)	Chlor *b*	Chlor *b* (mean±SE)	Total chlorophyll content	Total chlorophyll content (mean±SE)
	0.271	0.155	0.074		0.136		0.318	
1.0	0.278	0.160	0.077	0.078±0.003	0.141	0.144±0.006	0.327	0.330±0.009
	0.285	0.171	0.084		0.154		0.344	
	0.466	0.291	0.146		0.268		0.576	
1.5	0.460	0.282	0.140	0.152±0.012	0.258	0.279±0.02	0.563	0.588±0.022
	0.479	0.325	0.170		0.312		0.624	
	0.301	0.185	0.092		0.169		0.369	
2.0	0.309	0.196	0.099	0.1±0.006	0.182	0.184±0.011	0.386	0.388±0.014
	0.321	0.212	0.109		0.201		0.411	

Table 3.13. Effect of spacing between BARI Tomato 15 tomato explants on chlorophyll content

Spacing between explants (cm)	Absorbance in methanol		Chlorophyll content (µg/g fresh tissue)					
	665 nm	650 nm	Chlor *a*	Chlor *a* (mean±SE)	Chlor *b*	Chlor *b* (mean±SE)	Total chlorophyll content	Total chlorophyll content (mean±SE)
1.0	0.185	0.061	0.016	0.015±0.0004	0.031	0.03±0.0007	0.162	0.16±0.001
	0.184	0.059	0.015		0.029		0.160	
	0.180	0.059	0.015		0.03		0.158	
1.5	0.543	0.237	0.092	0.094±0.0008	0.173	0.175±0.001	0.548	0.55±0.001
	0.541	0.239	0.094		0.176		0.549	
	0.545	0.24	0.094		0.176		0.553	
2.0	0.388	0.159	0.058	0.057±0.0007	0.109	0.107±0.001	0.379	0.379±0.0004
	0.389	0.158	0.057		0.107		0.378	
	0.393	0.157	0.056		0.105		0.379	

3.1.7. Subculture of regenerated shoots

In the BAP and BAP with IAA supplemented media, shoots initiation was found to be non-synchronized. In all the varieties some shoots were found to attain 3-4 cm in length within 4 weeks of culture. For remain shoots the culture were placed in fresh media containing same hormonal supplement for proper shoot growth and elongation (**Fig. 6 A-C**). Elongated shoots were taken to next step where rooting was attempted. Vitrification was observed in BARI Tomato 2 variety when sub-cultured on media containing higher concentration (7 mg/l) of BAP (**Fig. 6 D**).

3.1.8. Effect of IAA on root formation in different tomato varieties

Well elongated shoots (3 - 4 cm) were excised and placed to ½MS media supplemented with IAA for root induction. Rooting response of regenerated shoots of five tomato varieties are expressed in **Table 3.14**. Among the varieties BARI Tomato 14, BARI Tomato 15 and BINA tomato 3 showed highest rooting response while the number of root formation was relatively low in BARI Tomato 2 and BARI Tomato 3. In rooting media, plants formed fibrous root and tap root depending on the supplemented IAA concentration. Tap roots were observed in 0.1 mg/l and 0.2 mg/l IAA supplemented ½MS media (**Fig. 7 A, D, F & I**). The BARI varieties mostly produced fibrous roots in media containing 0.5 mg/l, 0.7 mg/l and 1 mg/l IAA (**Fig. 7 B, C & H**). Interestingly, BINA Tomato 3 formed long and slender roots in all concentrations of IAA (**Fig. 7 E**). Auxin was needed for root formation however some shoots start forming tap-roots in media containing only BAP (**Fig. 7 J**).

One way ANOVA analysis indicated that the responses for different IAA concentrations differenced were highly significant as F (7.91) was much greater than F crit (2.86) and the P value was statistically significant (<0.05) (**Table 3.15**).

Fig. 6 Shoot elongation and vitrification **A.** Multiple shoots formation by BARI Tomato 14 followed by subculture, **B.** Longer shoots of BARI Tomato 15 within a month in where subculture was not necessary, **C.** shoot formation of BARI Tomato 3, in where subculture was needed **D.** Vitrification was observed in BARI Tomato 2 while sub-cultured on media containing higher BAP concentration (7 mg/l) [Photos were taken after 75 days of inoculation]

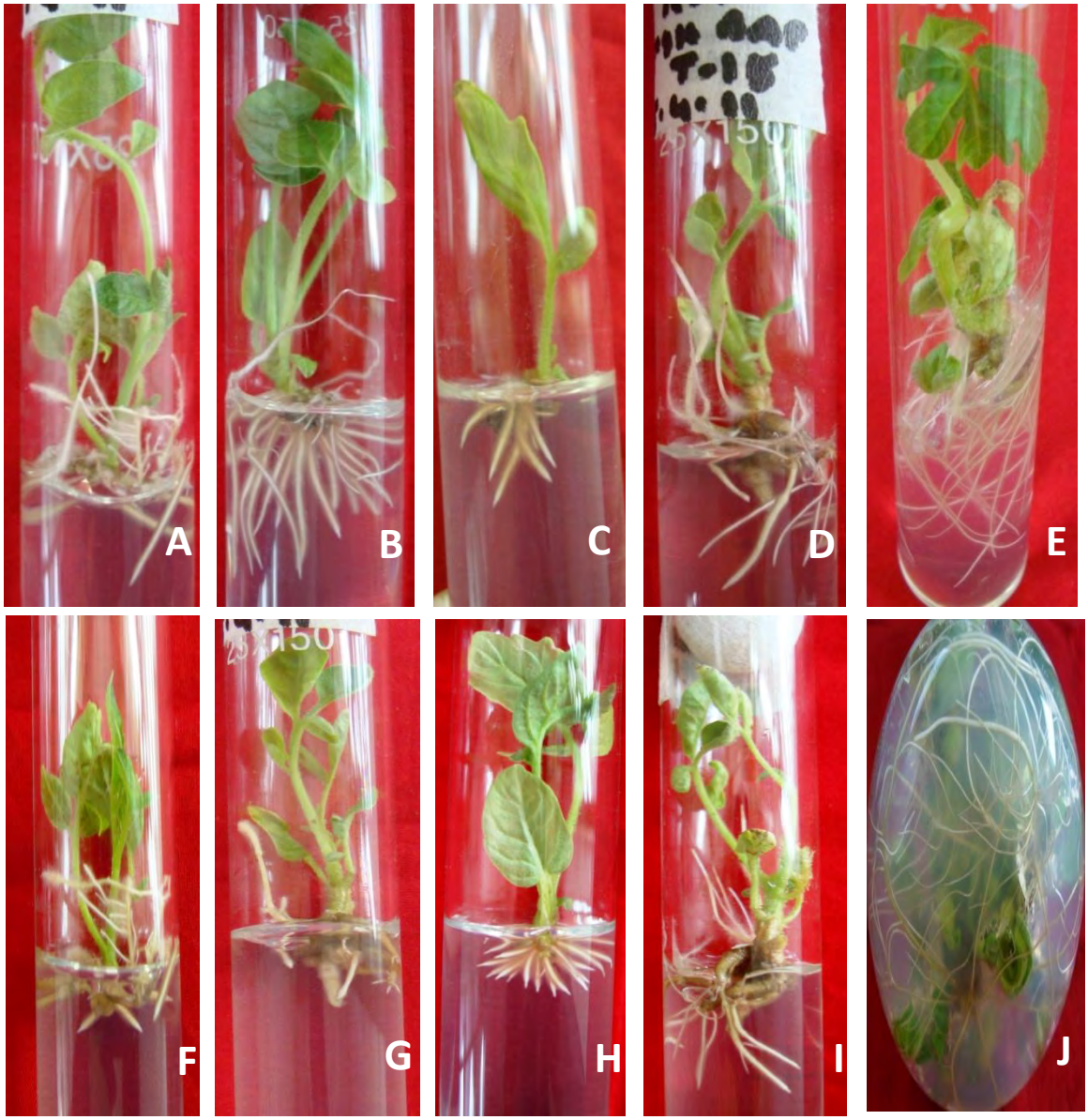

Fig. 7. Effect of IAA on root formation **A.** Root formation of BARI Tomato 14 in ½MS+0.1 mg/l IAA, **B.** BARI Tomato 2 in ½MS+0.5 mg/l IAA, **C.** BARI Tomato 3 in ½MS +0.7 mg/l IAA, **D.** BARI Tomato 15 and **E.** BINA Tomato 3 both in ½MS+0.2mg/l IAA concentration, **F.** BARI Tomato 2 and **G.** BARI Tomato 14 in ½MS +0.1 mg/l IAA, **H.** BARI Tomato 15 in ½MS + 1 mg/l IAA, I. BARI Tomato 3 in ½ MS + 0.1 mg/l IAA**, J.** Tap roots are formed in 2 mg/l BAP and no IAA concentration in BARI Tomato 15.

Table 3.14. Effect of IAA on root production in different tomato varieties

Tomato varieties	Conc. of IAA (mg/l)	No. of shoot inoculated	Percentage of shoot producing root	Days required for root initiation	Type of root	Roots/ shoot
	0.1	9	88	9	Tap root	8
	0.2	10	**90**	9	Tap root	9
BARI	0.5	10	80	8	Fibrous	9
Tomato 2	0.7	10	70	9	Fibrous	10
	1.0	10	80	8	Fibrous	10
	0.1	10	60	8	Tap root	5
	0.2	9	56	8	Tap root	6
BARI	0.5	8	**75**	8	Fibrous	6
Tomato 3	0.7	10	60	9	Fibrous	7
	1.0	10	70	8	Fibrous	6
	0.1	10	80	9	Tap root	14
	0.2	10	**100**	9	Tap root	13
BARI	0.5	10	90	10	Fibrous	15
Tomato	0.7	9	88	9	Fibrous	16
14	1.0	10	80	8	Fibrous	12
	0.1	10	90	10	Tap root	8
	0.2	10	80	9	Tap root	10
BARI	0.5	10	80	9	Fibrous	11
Tomato	0.7	9	**100**	10	Fibrous	12
15	1.0	10	**100**	9	Fibrous	14
	0.1	8	75	7	Slender, Long	12
	0.2	9	**100**	7	Slender, Long	12
BINA	0.5	10	90	7	Slender, Long	14
Tomato 3	0.7	10	**100**	8	Slender, Long	15
	1.0	9	**100**	8	Slender, Long	20

Table 3.15. One way ANOVA for the results of effect of IAA on root formation

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2625.84	4	656.46	7.91297	0.00054	2.866081
Within Groups	1659.2	20	82.96			
Total	4285.04	24				

3.1.9. Transplantation of regenerated plantlets and survivability in natural environment

Mature rooted plantlets were transferred to the soil in small pots and covered with perforated poly bag for hardening procedure (**Fig. 8 A**). During hardening maximum success was obtained in BARI Tomato 3 (100%) and the lowest 70% in BARI Tomato 14 (**Table 3.16**). The survived plants were then transferred to larger pots and shifted to net house (**Fig. 8 B-F**). In the natural environment, survival rate of all varieties were 100%.

Table 3.16. Survival of regenerated tomato plantlets of different varieties in soil

Tomato varieties	No of plantlets taken for hardening	Successfully hardened plantlets	Percentage of success	No of plantlets transferred to the soil	Percentage of survived plants in natural environment
BARI Tomato 2	8	7	87.5	7	100
BARI Tomato 3	9	9	100	9	100
BARI Tomato 14	10	7	70	7	100
BARI Tomato 15	12	11	91.6	11	100
BINA Tomato 3	10	9	90	9	100

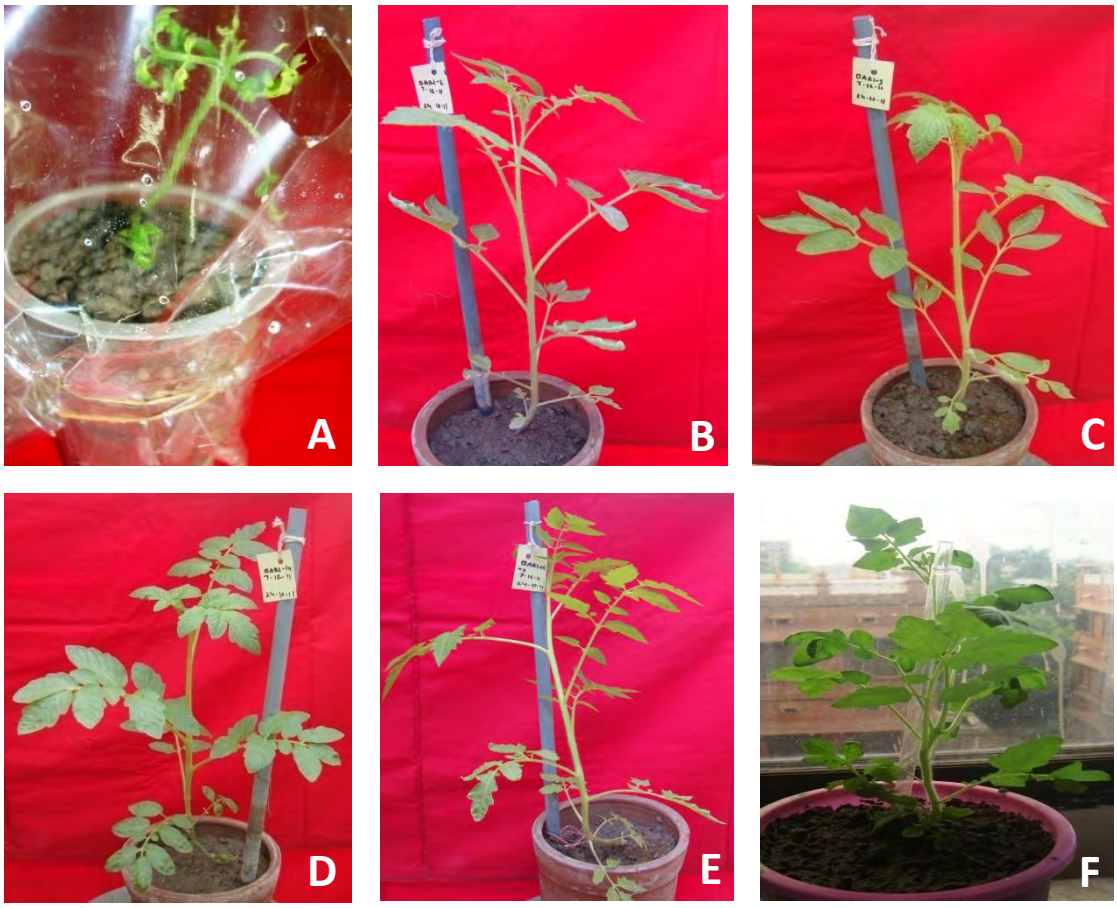

Fig. 8. Hardening and acclimatization of regenerated plantlets **A.** hardening of plantlets **B.** Regenerated plants BARI Tomato 2, **C.** BARI Tomato 3, **D.** BARI Tomato 14, **E.** BARI Tomato 15 and **F.** BARI Tomato 3 growing in pots.

3.1.10. Analysis of reproductive response of the regenerated plants

Transplanted plants flowered in natural environment but time required for flowering varied considerably depending on season. Plantlets transferred to nature in the month of April-May took 3 to 4 months to flower (**Fig. 9 A-E**). However plantlets transferred in September-October flowered in 3-4 weeks. All the plants set fruits 15 to 20 days after flowering (**Fig. 9 F - J**) and 4 to 5 weeks were needed for maturation (**Fig. 9 K**). Data on time required in flowering, fruit setting and fruit maturation and analysis of mature fruits are presented in **Table 3.17**. Maximum number of fruits was found in BARI Tomato 14 and highest number of seeds was produced by BARI Tomato 3 (**Table 3.18**).

Table 3.17. Time required for flowering and fruit setting of the regenerated plantlets

Tomato varieties	Flowering after Transplantation (months)*	Fruit setting (days)	Fruit maturation (weeks)
BARI Tomato 2	3-4	15-20	4-5
BARI Tomato 3	3-4	15-20	4-5
BARI Tomato 14	3-4	15-20	4-5
BARI Tomato 15	3-4	15-20	4-5
BINA Tomato 3	4-5	12-15	4-5

***Transplantation took place in April-May**

Table 3.18. Mature fruits analysis of regenerated plants of different varieties

Tomato varieties	Fruits/plants	Average fruits weight (g)	Average seed no. /fruits	Fruit shape
BARI Tomato 2	8	65	44	Round
BARI Tomato 3	6	75	46	Semi-globe
BARI Tomato 14	9	80	50	Semi globe
BARI Tomato 15	5	65	25	Ovoid
BINA Tomato 3	7	70	35	Oval

3.1.11. Viability test of seeds from mature fruits produced by regenerated plants

Seeds were collected from ripen fruits and their viability was tested (**Fig. 9 L**). All varieties showed good response in seed germination test. BARI Tomato 14 showed the highest germination rate (92%) while BARI Tomato 3 showed the lowest (75%) (**Table 3.19**).

Table 3.19. Seed germination test of different tomato varieties

Tomato varieties	**Total no of seeds inoculated**	**Percentage of germinated seeds**	**Mean no. of germinated seeds ±SE***	**Days required for germination initiation**
BARI Tomato 2	50	84	42±1.8	3-4
BARI Tomato 3	50	74.6	37.3±1.7	3-4
BARI Tomato 14	50	92	46±1.0	2-3
BARI Tomato 15	50	86.6	43.3±1.0	2-3
BINA Tomato 3	50	82	41±1.7	3-4

*Mean values are from 3 replications. Data was taken after a week of inoculation.

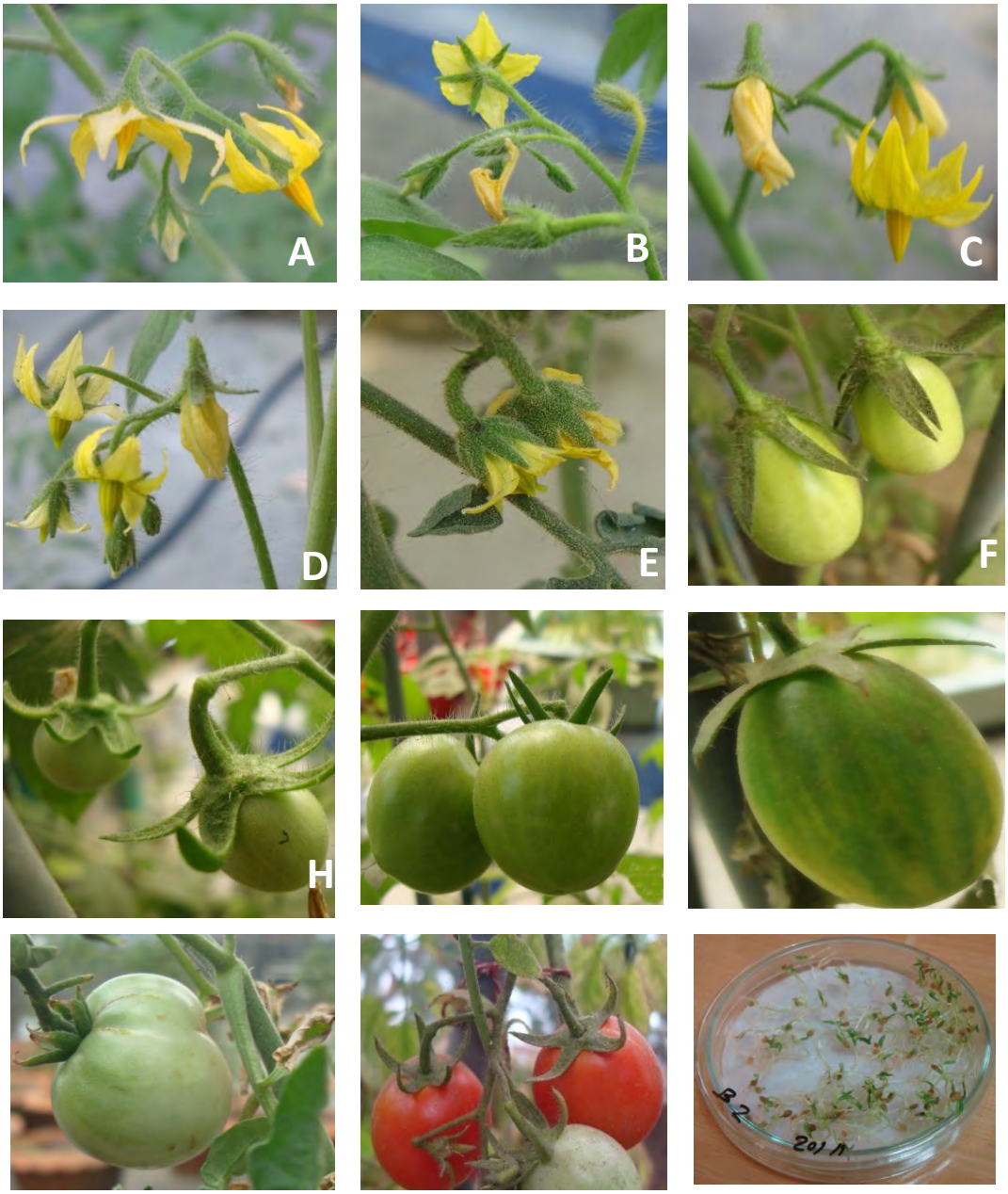

Fig. 9. Flowering and fruiting response of regenerated plantlets **A.** BARI Tomato 2**,** **B.** BARI Tomato 3, **C.** BARI Tomato 14, **D.** BARI Tomato 15, **E.** BINA Tomato 3, **F.** fruits of BARI Tomato 2, **G.** BARI Tomato 3, **H.** BARI Tomato 14 **I.** BARI Tomato 15, **J.** BINA Tomato 3, **K.** mature fruits of BARI Tomato 3, and **L**. seed germination test from mature fruits of BARI Tomato 2.

3.1.12. Determination of growth hormone affecting regeneration response

Best hormonal supplementation in media was determined. MS media supplemented with 2 mg/l BAP found the best for most of the cases among the hormonal supplementation tested. In some cases, both BAP and combination of BAP and IAA were gave quite similar result.

Among all five varieties, BARI Tomato 14 was given the best response on germination and regeneration. Results are further present in **Table 3.20** below.

Table 3.20. Best response among the five tomato varieties

Tomato varieties	Best hormonal supplementation in media		Highest number of shoots	Percentage of highest number of shoot producing root	Percentage of germinated seeds
	BAP conc.	BAP + IAA conc.			
BARI Tomato 2	_	2 mg/l BAP+ 0.2 mg/l IAA	8.33±0.40	90	84
BARI Tomato 3	2 mg/l	1 mg/l BAP+ 0.2 mg/l IAA	7.00 ± 0.7; 7.00 ± 0.58	75	74.6
BARI Tomato 14	2 mg/l	_	8.6±1.0	100	92
BARI Tomato 15	2 mg/l	2 mg/l BAP+ 0.1 mg/l	8.00 ± 1.4; 8.33 ± 0.4	100	86.6
BINA Tomato 3	2 mg/l	_	9.0±0.7	100	82

3.2. *Agrobacterium*-mediated genetic transformation of tomato varieties

The objective of the present study was to get salinity tolerant tomato varieties. For this the salinity tolerant gene needs to be incorporated into the tomato genome successfully. To achieve that target, an endeavor of establishing a suitable protocol for *Agrobacterium*-mediated genetic transformation took place.

At first, a genetically engineered *Agrobacterium* strain LBA4404 containing pBI121 containing selection marker *uidA* gene (β-glucuronidase) and *npt*II (neomycin conferring kanamycin resistance) was used to determine optimum conditions for *Agrobacterium*-mediated transformation. Five varieties, namely, BARI Tomato 2, BARI Tomato 3, BARI Tomato 14, BARI Tomato 15 and BINA Tomato 3 were treated with different parameters to achieve optimum condition for proper transformation. Using this proper condition genetically engineered *Agrobacterium* strain containing pH7WG2_OsNHX1_1.6 (*OsNHX1* antiporter gene, cloned from rice) will be incorporated in these tomato varieties to make them salinity tolerant. The putative transgenic tomato varieties were achieved by the end of this study.

3.2.1. Salinity stress tolerance test of tomato seedlings of different tomato varieties

Before doing any transformation, salinity stress tolerance level of these tomato varieties need to be assessed. In this study, effect of salinity on tomato seed germination was observed. Seedlings of BARI Tomato 14, BARI Tomato 15 and BINA Tomato 3 were placed on MS media with various concentrations (0 mM, 5 mM, 10 mM, 20 mM, 50 mM and 100 mM) of NaCl and data were collected after 10 day (**Table 3.21**). The highest number of seed observed to germinate in MS media supplemented with 0 mM NaCl (**Fig. 10 A-B**). The germination rate was decreased with the increase of NaCl amount in media (**Fig. 10 C-F**). Germination rate was

below 50% when the NaCl amount was 20 mM in the media (**Fig. 10 G-H**). In addition to this, time requirement was also found to be influenced by the presence of NaCl. The number of days needed for germination drastically increased at 50 mM NaCl (**Fig. 10 I-J**) in compare to 20 mM NaCl. And in media containing 100 mM of NaCl found to have 1.6% germination (**Fig. 10 K-L**).

Table 3.21. Effect of salinity on seed germination of tomato varieties

NaCl concentration into germination media	**No. of seeds inoculated**	**Percentage of germinated seeds**	**Mean no of germinated seeds±SE***
0 mM	20	81.5	16.3±1.4
5 mM	20	73	14.6±2.2
10 mM	20	56.5	11.3±2.4
20 mM	20	46.5	9.3±0.8
50 mM	20	36.5	7.3±0.8
100 mM	20	1.5	0.3±0.4

*Mean values are from 3 replications. Data was taken after 12 days of inoculation.

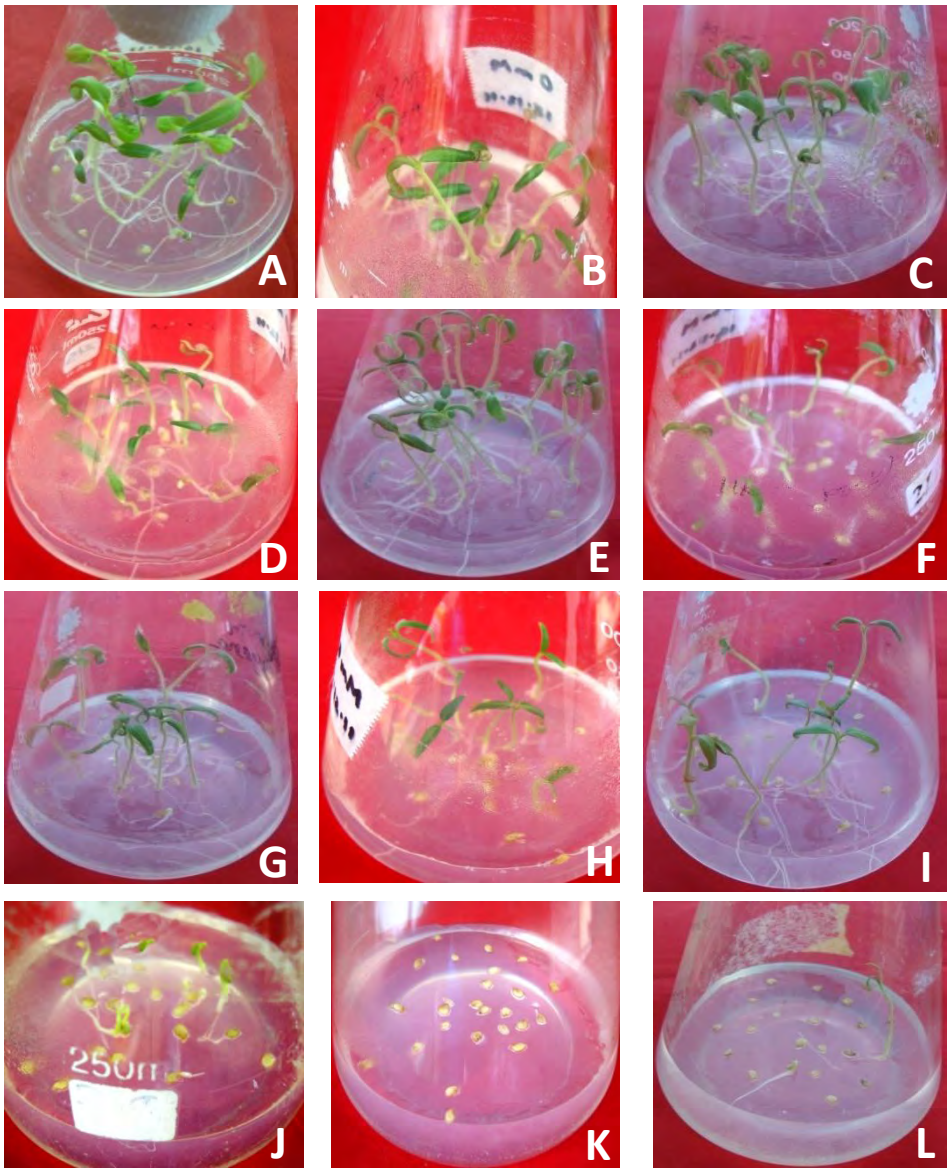

Fig. 10. Seed germination in presence different amount of NaCl **A-B.** 0 mM, **C-D.** 5 mM, **E-F.** 10 mM, **G-H.** 20 mM, **I-J.** 50 mM and **K-L.** 100 mM.

3.2.2. Determination of factors affecting transformation efficiency of five tomato varieties

Agrobacterium strain LBA4404 harboring pBI121 was used to check its compatibility with five different varieties of tomato. In this study GUS histochemical assay was done to observe the transfer of marker gene *uidA* (*β*-glucuronidase).

3.2.2.1. Effect of bacterial culture density on transformation efficiency

Bacterial cultures of different optical densities (O.D.) were used in this study to learn its effect on transformation efficiency on the five tomato varieties. Maximum GUS positive explants were found at OD_{600} 0.68. BARI Tomato 3, BARI Tomato 14, and BINA Tomato 3 explants were found to give 100% positive transient GUS expression while BARI Tomato 15 and BARI Tomato 2 gave 80% GUS positive expressions in GUS assay at this OD_{600}.

For OD_{600} 0.45, highest number of GUS positive explants (80%) was obtained in BINA Tomato 3 and the expression ranged between 60-80% for rest of the varieties (**Table 3.22**).

3.2.2.2. Effect of incubation period on transformation efficiency

In the previous experiment OD_{600} 0.68 was found optimum for transformation but time requirement for each of these Agrobacterial culture density might vary. To determine the effect of incubation period on transformation efficiency two different incubation periods namely 30 minutes and 60 minutes against two OD_{600} were tested. In most of the cases, higher culture density (OD_{600} 0.68) gave better transformation in short incubation period (30 min). On the other hand longer incubation period (60 min) gives higher transformation when culture density of bacterial suspension was low. In addition to this, high OD_{600} 0.68 gave low transformation frequency in long infection

period (**Fig. 11**). Nonetheless, cent percent transformation was observed at OD_{600} 0.68 with 30 minutes of infection period in BARI Tomato 3, BARI Tomato 14 and BINA Tomato 3.

3.2.2.3. Effect of pre-culture on transformation efficiency

Interestingly this factor did not influence transformation efficiency but had a positive effect on regeneration initiation. Pre-cultured explants started to regenerate faster followed by transformation than the non-pre-cultured explants (**Fig. 12 A-C**). It was found that the higher number of shoots formed by pre-cultured explants while the non pre-cultured formed less number of shoots (**Table 3.23**).

3.2.2.4. Effect of co-cultivation period on transformation efficiency

Co-cultivation period of 48 hours were found best for all five tomato varieties. Highest response of transient GUS assay was obtained by both BARI Tomato 3 and BARI Tomato 14. The percentage of positive GUS expression was decreased with the decrease of co-cultivation period (**Table 3.24**). Explants having co-cultivation period of 3 or more days showed overgrowth of bacteria (**Fig. 12 D**). Thus they failed to regenerate and finally necrosis was found (**Fig. 12 E-F**).

It was found by doing the GUS assay that all five varieties were compatible with the strain to transfer transgene as they gave blue color at the cut ends and within the tissue of the cotyledonary leaf explants (**Fig. 12 G-L**). Control treatment did not give the blue color.

Table 3.22. Effect of Optical Density (OD_{600}) and incubation period of *Agrobacterium* suspension on transformation efficiency of five tomato varieties

Tomato Varieties	OD_{600}	Incubation period (min)*	No. of explants used in GUS assay	Percentage of GUS positive explants
BARI Tomato 2	0.45	30 min	10	80
		60 min	12	87
	0.68	30 min	10	80
		60 min	11	65
BARI Tomato 3	0.45	30 min	10	40
		60 min	10	60
	0.68	30 min	10	100
		60 min	10	80
BARI Tomato 14	0.45	30 min	9	75
		60 min	10	80
	0.68	30 min	13	100
		60 min	11	93
BARI Tomato 15	0.45	30 min	10	51
		60 min	10	65
	0.68	30 min	10	65
		60 min	10	60
BINA Tomato 3	0.45	30 min	10	80
		60 min	10	90
	0.68	30 min	10	100
		60 min	13	77

* *Agrobacterium* strain LBA4404 containing pBI121

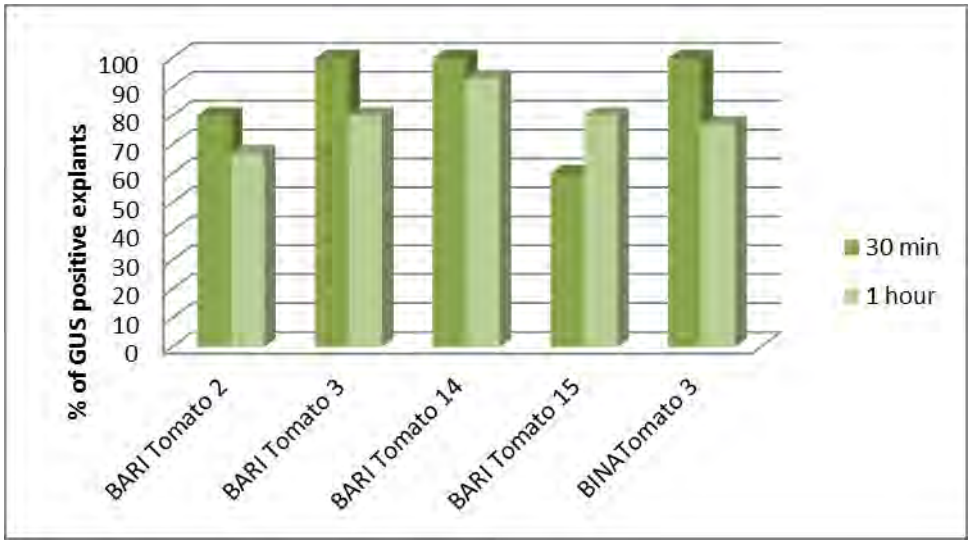

Fig. 11. Effect of incubation periods on transformation in all five varieties

Table 3.23. Effect of pre-culture on transformation efficiency in five tomato varieties

Tomato varieties	Infected explants*	Percentage of GUS positive explants	Days required for regeneration initiation	Mean no. of shoot ± SE (followed by transformation)
BARI Tomato 2	Pre-cultured	80	16	4.33±0.40
	Non pre-cultured	82	20	3.33±0.40
BARI Tomato 3	Pre-cultured	90	15	4.00±0.70
	Non pre-cultured	100	23	2.6±0.40
BARI Tomato 14	Pre-cultured	94	14	5.5±0.35
	Non pre-cultured	100	19	4.0±0.70
BARI Tomato 15	Pre-cultured	86	12	4.33±0.80
	Non pre-cultured	90	20	2.0±0.70
BINA Tomato 3	Pre-cultured	82	18	5.3±0.40
	Non pre-cultured	86	25	4.3±1.08

* *Agrobacterium* strain LBA4404 containing pBI121

Table 3.24. Effect of co-cultivation periods on transformation efficiency of different tomato varieties

Tomato Varieties	Co-cultivation period*	No. of explants assayed in GUS assay	Percentage of GUS positive explants
BARI Tomato 2	24 hours	7	86
	48 hours	8	88
BARI Tomato 3	24 hours	8	88
	48 hours	8	100
BARI Tomato 14	24 hours	9	89
	48 hours	8	100
BARI Tomato 15	24 hours	7	72
	48 hours	10	90
BINA Tomato 3	24 hours	9	67
	48 hours	7	86

* *Agrobacterium* strain LBA4404 containing pBI121

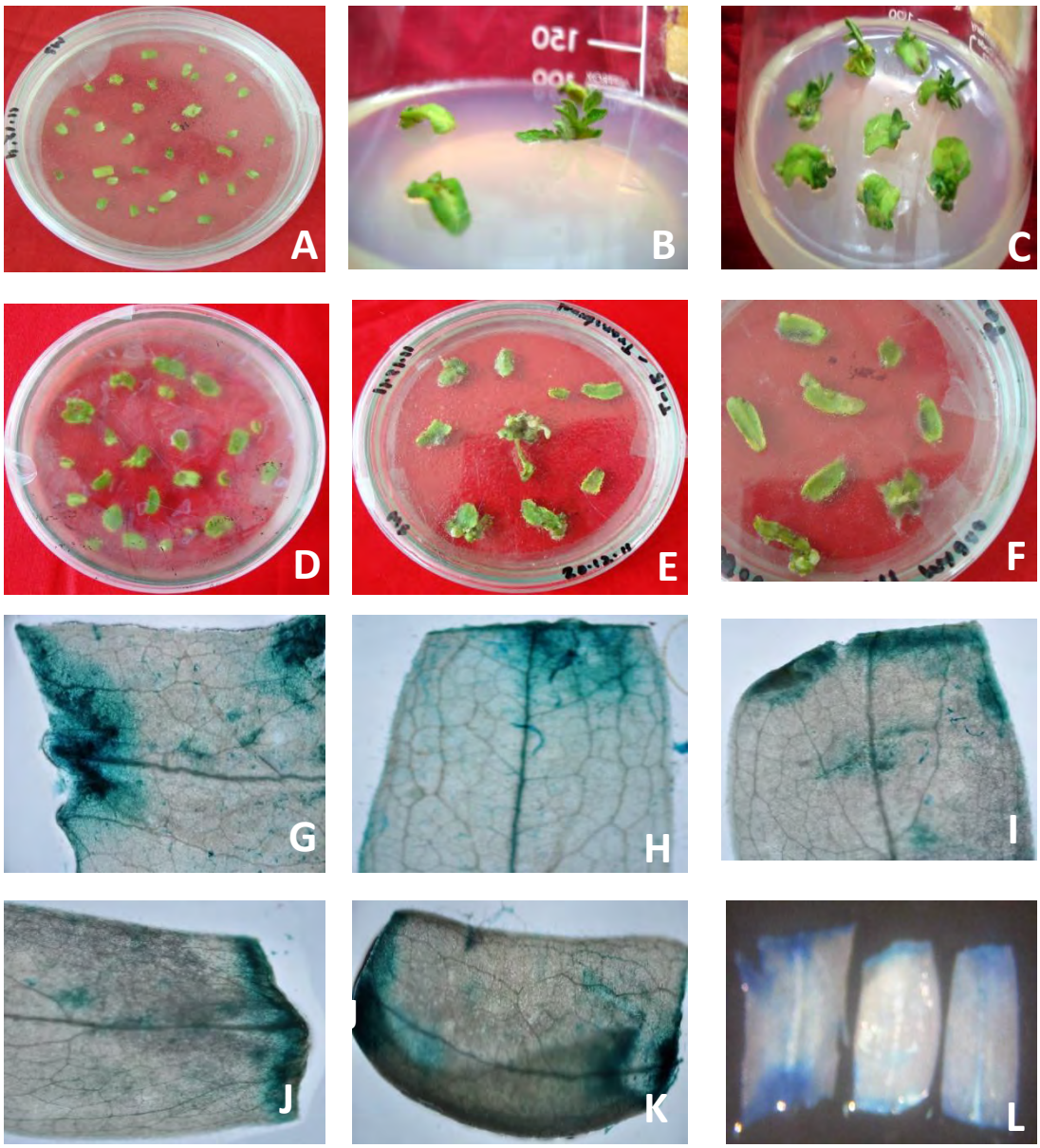

Fig. 12. Factors affecting transformation, blue color in GUS assay **A.** Pre-culture of explants **B.** non pre-cultured BARI Tomato 2 explants in regeneration media containing 150 mg/l kanamycin and **C.** pre-cultured BARI Tomato 2 explants in regeneration media containing 150 mg/l kanamycin after 60 days of inoculation followed by transformation. **D.** Agrobacterial overgrowth after 3 days of co-cultivation in BARI Tomato 15, **E-F.** Necrosis due to bacterial overgrowth **G-L.** Steriomicroscopic view of GUS activity within the tissue underneath the epidermis **L.** and at cut ends of cotyledonary leaf explants.

3.2.3. Determination of antibiotic concentration for selection medium

For antibiotics sensitivity test, cotyledonary leaf explants were tested with various concentrations of antibiotic (kanamycin and hygromycin). All the explants in the control treatment survived and led to regeneration. The appearance of all the explants found to be normal in the explants cultured on media without antibiotic supplementation.

3.2.3.1. Kanamycin sensitivity test

In selection medium containing kanamycin at five concentrations (0 mg/l, 50 mg/l, 100 mg/l, 150 mg/l and 200 mg/l) were chosen for this study (**Fig. 13 A-I**). None of the explants survived (**Table 3.25**).

So, none of concentrations do not allow the explants to regenerate and survive. The explants were found to become albino at 100 mg/l kanamycin. They became brown at 150 mg/l kanamycin concentration and finally died at 200 mg/l. So 150 mg/l kanamycin concentration used in media was optimum for selection and transformed explants were survived at this concentration for one month considered as transformed.

Table 3.25. Effect of various kanamycin concentrations on the regeneration of tomato cotyledonary leaf explants

Kanamycin concentration (mg/l)	Percentage of shoot formation*	Percentage of Survival*	Visual appearance[#]
0	60	100	Normal, green
50	0	0	Albino
100	0	0	Albino
150	0	0	Brown
200	0	0	Brown

*Out of ten cotyledons; [#]Visual appearance of the regenerated explants, which survived or died

[Data was collected after 45 days of inoculation and shoot formation rate was recorded after 60 days of inoculation]

3.2.3.2. Hygromycin sensitivity test

At first cotyledonary leaf explants were subjected to five different concentrations (5 mg/l, 8 mg/l, 10 mg/l, 20 mg/l and 30 mg/l) of hygromycin including a control experiment with 0 mg/l (**Fig. 14 A-L**). None of the explants survived in presence of hygromycin. To identify the minimum tolerance level toward hygromycin further experiment was done with (0 mg/l, 1 mg/l, 2 mg/l, 3 mg/l and 4 mg/l) hygromycin. The number of explants in selection medium containing 1 mg/l hygromycin found to have 41% survival rate, and in media containing 3 mg/l hygormycin 28% of explants were survived. The survival rate fallen to 0% when the hygromycin concentration was 4 mg/l in selection media which means no explants survived at this level (**Table 3.26**).

From this study, it is clear that hygromycin concentration above 3 mg/l do not allow the regeneration of tomato cotyledonary leaf explants. Explants became albino in media containing 4mg/l hygromycin and at 5 mg/l concentration explants eventually became brown and lethal dose was found to be 10 mg/l (**Fig. 14 K-N**).

Table 3.26. Effect of various hygromycin concentrations on the regeneration of tomato cotyledonary leaf explants

Hygromycin concentration	Percentage of shoot formation*	Percentage of Survival*	Visual appearance[#]
0	50	100	Green
1	20	41	Green
2	18	37	Green
3	10	28	Green
4	0	0	Albino
5	0	0	Albino
8	0	0	Brown
10	0	0	Brown
20	0	0	Brown
30	0	0	Brown

*Out of ten cotyledons; [#]Visual appearance of the regenerated explants, which survived or died

[Data was collected after 45 days and shoot formation rate was recorded after 60 days of inoculation of inoculation]

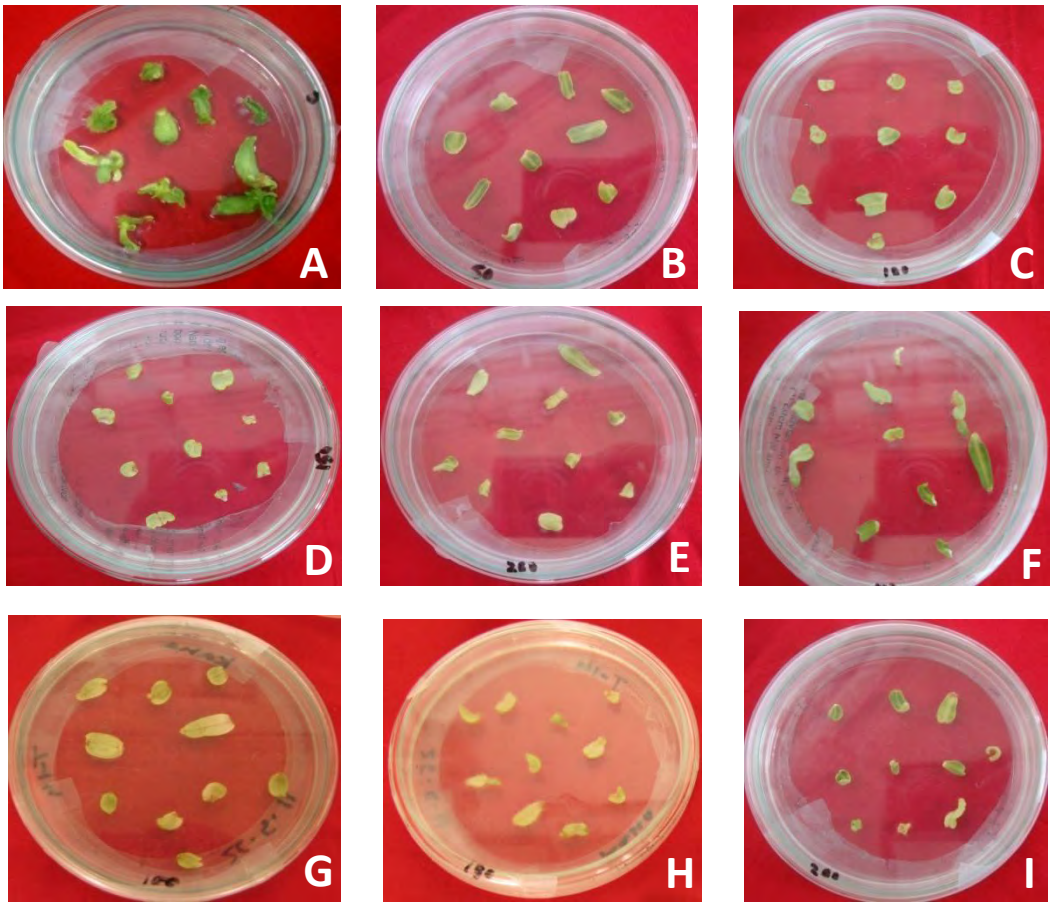

Fig. 13. Effect of various concentration of kanamycin on tomato cotyledonary explants of BARI Tomato 2, **A.** Control (0 mg/l kanamycin), **B.** 50mg/l, **C.** 100mg/l, **D.** 150mg/l and **E.** 200mg/l kanamycin respectively [Photos were taken after 45 days of inoculation] Explants of BARI Tomato on media containing 14 **F.** 50 mg/l, **G.** 100 mg/l, **H.** 150 mg/l and **I.** 200 mg/l kanamycin [Photos were taken after 60 days of inoculation]

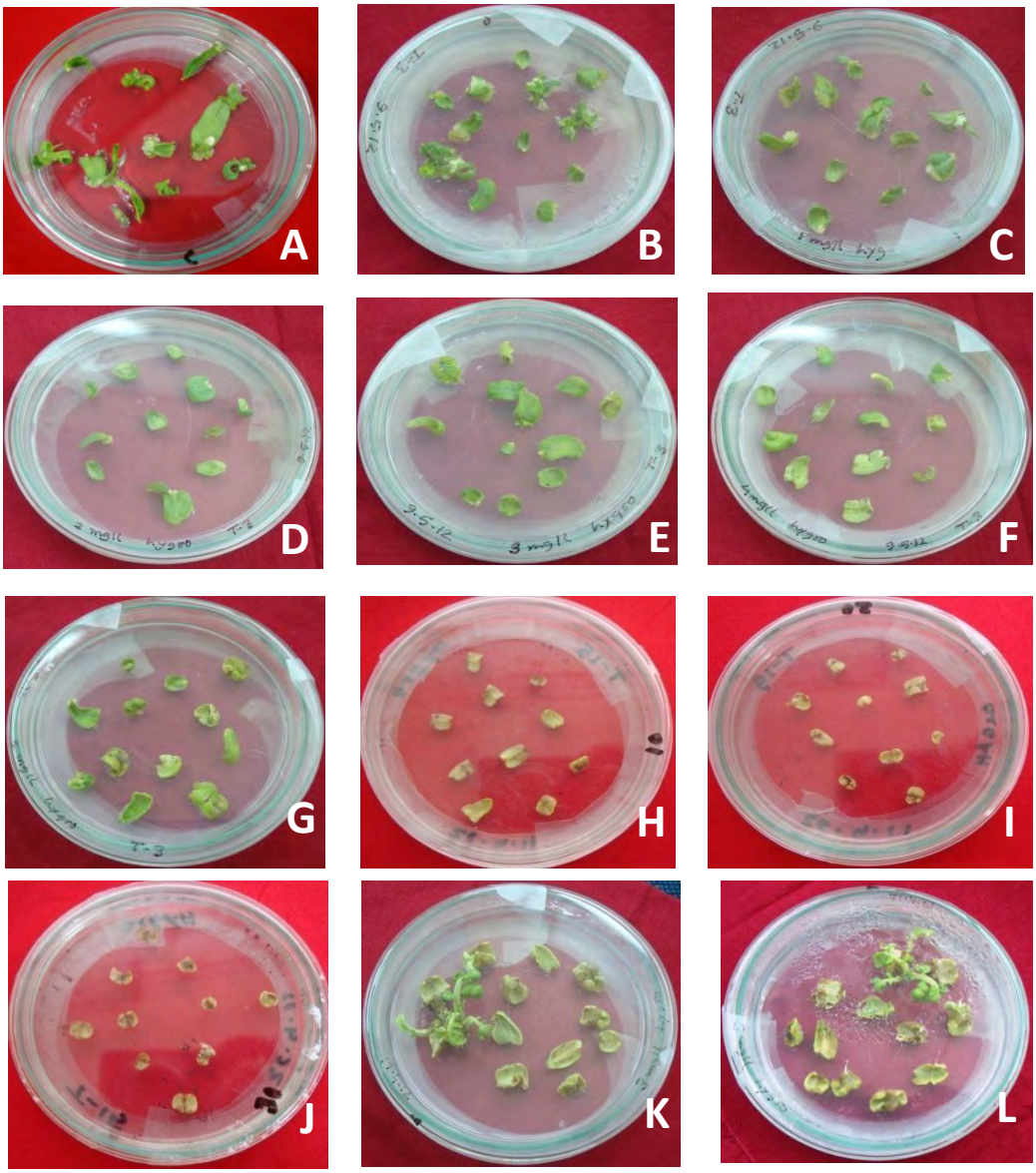

Fig. 14. Effect of various concentration of hygromycin on tomato cotyledonary explants of BARI Tomato15, **A.** Control (0 mg/l hygromycin,) **B.** 1mg/l, **C.** 2 mg/l, **D.** 3mg/l, **E.** 4mg/l, **F.** 5 mg/l **G.** 8 mg/l, **H.** 10mg/l, **I.** 20mg/l and **J.** 30 mg/l hygromycin respectively **K.** shoot formation in 3 mg/l hygromycin [These photographs were taken 45 days after inoculation.] **L.** explants becoming brown on media containing 3 mg/l hygromycin [photo was taken 60 days of their inoculation]

3.2.3. Transformation frequency of five tomato varieties (transformed with pBI121) based on regeneration on selection media

Following transformation explants were placed on media containing 200 mg/l cefotaxime and 50 mg/l kanamycin to control Agrobacterial overgrowth and to obtain regeneration from transformed explants. The survived explants were then placed on media containing 100 mg/l kanamycin after two week and the amount of kanamycin was eventually increased to 150 mg/l. Transformation efficiency was calculated by the number of regenerated putative transgenic shoots on selection. The control experiment (explants without infection) was found to have no regeneration at all (**Fig. 15 A**).

The highest average percentage of regeneration (47±1.7) was found in BARI Tomato 3 and the second highest (43±1.6) was obtained by BARI Tomato 14 at OD_{600} 0.72. BARI Tomato 3 and BARI Tomato 15 was found to have quite similar result (42±1.8 and 42±1.0, respectively) in regeneration response while in kanamycin supplemented media and the incubation period was 30 minutes for all of the varieties (**Fig. 15 B-D**). In BINA Tomato 3 the regeneration response was the lowest (27±1.4) with incubation period of 60 minutes at OD_{600} 0.72 (**Fig. 15 E**). Results were further presented in **Table 3.27**.

Next, these varieties will be studied in transformation with *Agrobacterium* strain LBA4404 containing pH7WG2_OsNHX1_1.6.

Table 3.27. Transformation frequency based on regeneration on media containing kanamycin

Varieties	Incubation period (min)*	Average transformation frequency (%±SE)#
BARI Tomato 2	30 min	34±1.4
	60 min	33±2.1
BARI Tomato 3	30 min	47±1.7
	60 min	42±1.8
BARI Tomato 14	30 min	43±1.6
	60 min	40±1.0
BARI Tomato 15	30 min	42±1.0
	60 min	38±1.7
BINA Tomato 3	30 min	37±1.0
	60 min	27±1.4

* *Agrobacterium* strain LBA4404 containing pBI121

Transformation frequency values represent the average percent ± Standard Error (SE) of regeneration percentage from transformed explants on regeneration media containing kanamycin. Values were presented from three independent experiments. Standard error is mentioned for the values of transformation efficiency.

3.2.4. *Arobacterium*-mediated transformation with pH7WG2_OsNHX1_1.6

Five varieties of tomato, namely, BARI Tomato 2, BARI Tomato 3, BARI Tomato 14, BARI Tomato 15 and BINA Tomato 3 were chosen for this experiment. This selection of the parameters was made based on the better transformation efficiencies obtained in previous study during transformation with construct pBI121. In the current study, all five tomato varieties were infected using higher OD_{600} and were incubated for 30 minutes.

3.2.4.1. Transformation frequency of five tomato varieties (transformed with pH7WG2_OsNHX1_1.6) based on regeneration on selection media

Regeneration media containing hygromycin (4 mg/l and 10 mg/l) was used to see the effect on regeneration following transformation of explants of five varieties (**Fig. 15 F-L**). BARI Tomato 3 attained the highest regeneration response (20.5±2.9) in OD_{600} 0.71 with 30 minutes of incubation period. The second best regeneration response (17.3±1.71) was obtained in BARI Tomato 14 in OD_{600} 0.69 when the incubation period was 30 minutes. The lowest regeneration response (13.5±2.9) was found to be in BARI Tomato 15 in OD_{600} 0.69 and 30 minutes of incubation period (**Table 3.28**).

Table 3.28. Transformation frequency based on regeneration on media containing hygromycin

Tomato Varieties	OD_{600}*	Incubation period (min)*	Average transformation frequency (%±SE)#
BARI Tomato 2	0.71	30 min	14.9±3.0
BARI Tomato 3	0.71	30 min	20.5±2.9
BARI Tomato 14	0.69	30 min	17.3±1.71
BARI Tomato 15	0.69	30 min	13.5±2.9
BINA Tomato 3	0.71	30 min	18.2±3.5

* *Agrobacterium* strain LBA4404 containing pH7WG2_OsNHX1_1.6

Values represent the average percent ± Standard Error (SE) of regeneration percentage from transformed explants on regeneration media containing hygromycin. Values were presented from three independent experiments.

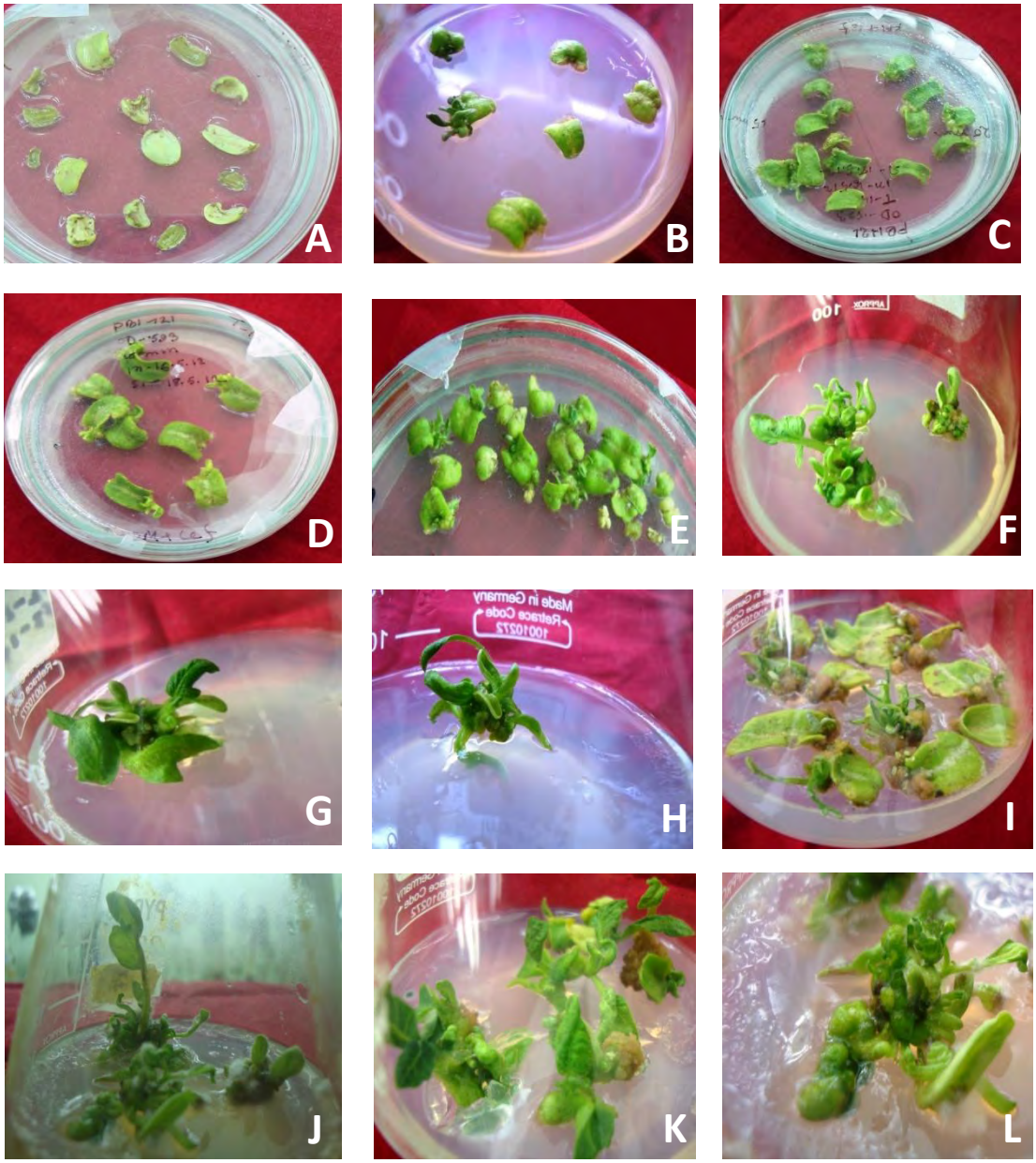

Fig. 15. Regeneration response on selection media **A.** no regeneration in control experiment (explants without infection but cultured on 100 mg/l kanamycin), **B-E.** explants on selection media containing 100 mg/l kanamycin **B.** BARI Tomato 3 **C.** BARI Tomato 14, **D.** BARI Tomato 15 and **E.** BINA Tomato 3 [Photos taken after 30 days of inoculation]

F. BARI Tomato 14, **G.** BARI Tomato 3, **H.** BARI Tomato 15 **I.** BINA Tomato 3 shoot formation on selection media containing 4 mg/l hygromycin

J. BARI Tomato 3, **K.** BARI Tomato 14 and **L.** BARI Tomato 15 putative plantlets on selection media containing 10 mg/l hygromycin [Photos taken after 60 days of inoculation]

3.2.5. Molecular analysis of regeneration of putative transformed shoots

Putative shoots (60 days old) regenerated on 4 mg/l hygromycin containing selection media was subjected to molecular analysis through PCR (**Fig. 16**). Through OsNHX1 specific primer expected 600 bp band was found in three regenerated shoots belonging to BARI Tomato 3 variety.

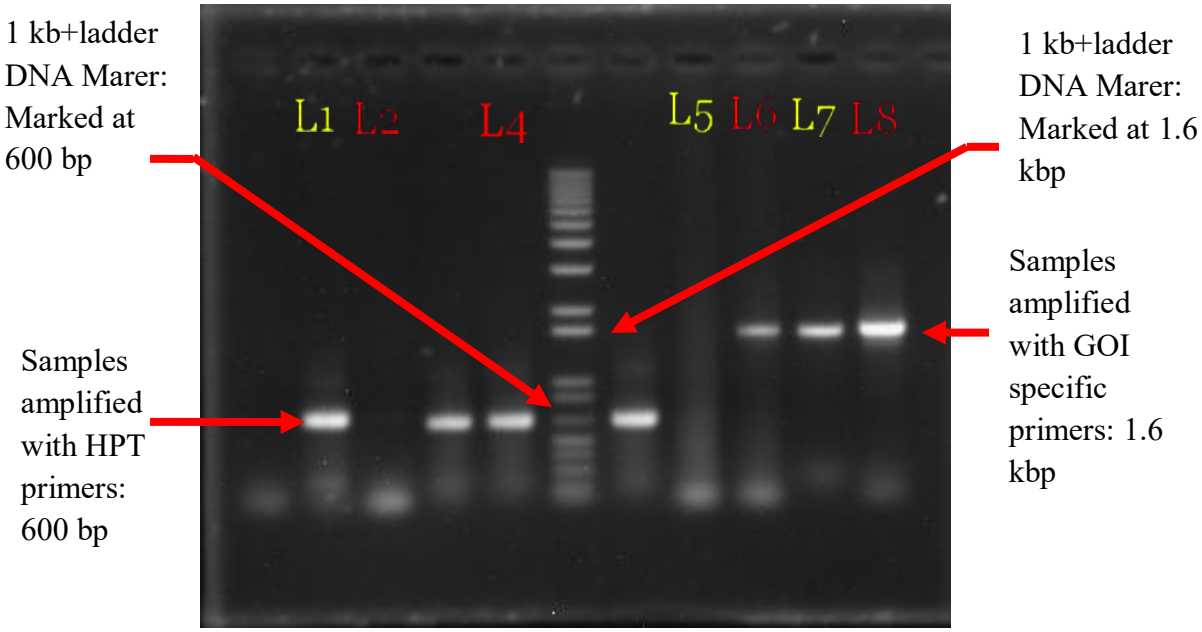

Fig. 16. PCR of putative transgenic shoot (60 days after infection) using HPT Primer: Primers for Hygromycin gene, GOI Specific Primer: Primers for coding sequence for OsNHX1

L1: Positive control; L2: Negative control; L3-L4: Samples amplified with HPT Primers

L5: Negative control; L6: Positive control; L7-L8: Samples amplified with GOI Primers.

Chapter 4

DISCUSSION

In the present study, suitable protocols for regeneration and *Agrobacterium*-mediated transformation were studied for five tomato varieties, namely, BARI Tomato 2, BARI Tomato 3, BARI Tomato 14, BARI Tomato 15 and BINA Tomato 3. In the first phase of this study, *in vitro* regeneration protocol was established as it is a prerequisite for transformation. In the second phase, transformation experiments were carried out with these five varieties using pBI121 (containing *nptII* marker gene and *uidA* gene) to determine the optimum conditions for transformation. In the third phase, transformation was attempted with pH7WG2_OsNHX1_1.6 construct (containing *OsNHX1*, Na+/H+ antiporter gene, cloned from rice) to obtain transgenic salt tolerant tomato varieties on the basis of optimum conditions determined at the second phase. Finally, analysis of these putative transgenic plants was done to confirm transgene incorporation.

4.1. Tomato Tissue culture

Regeneration ability of the explant is vital for the successful application of *in vitro* tissue culture techniques (167) and tomato has been reported to be highly compatible to *in vitro* culture previously (168).

4.1.1. Factors affecting *in vitro* regeneration

Some of the important factors influencing this regeneration potential are: genotype, explant, composition of the basal medium, growth regulators and gelling agent etc (169) (170) (171). Keeping these in mind, the present study was carried out to study the effects of growth regulators and genotype in tissue culture.

4.1.1.1. Explant type selection

In tomato, reports are available demonstrating genotype and explants type to influence *in vitro* shoot regeneration (172). Cotyledonary leaves were reported to be the best

responsive explants (173) (174) (175) (176) among all other explants sources, such as, leaves, hypocotyls, cotyledons, stem, meristem, node, petioles, anthers etc (177) (178) (179) (180) (181) (182) (183) (184) (185). For this reason, in the present study cotyledonary leaves were used to develop *in vitro* regeneration protocol of the tomato varieties.

4.1.1.2. Effect of explant orientation on regeneration

Position of the explants was also found to influence regeneration ability in tomato. In the present study, 20% reduction in regeneration rate was observed in adaxial orientation compared to explant placed in abaxial orientation. Similar approach was established earlier **(**186**)** (187). However, it was reported that no significant differences in average regeneration frequency and shoot number based on the position of the cotyledon segments (188).

4.1.1.3. Media selection for tomato tissue culture

MS media (189) was the most used media in '*in vitro*' regeneration of tomato (190) (191). In the present study, full strength and MS medium with various concentrations and combinations of growth hormones were applied to see their effects on tomato regeneration.

4.1.1.4. Effect of cytokinin (BAP) on regeneration

Cytokinin concentrations in a media are crucial for *in vitro* shoot regeneration (192) (193) and BAP is the most efficiently used among all cytokinin used in different studies of various plant species regeneration (194) (195) (196).

In this present investigation, higher concentration (5-7 mg/l) of BAP gave direct shoot regeneration while lower concentration (1-2 mg/l) induced indirect regeneration. Similar result was found in tomato regeneration (197). In the present

study, vitrification was found in BARI tomato 2 and BARI Tomato 3 while maintaining culture at higher BAP concentration. Similar observation was reported while working with Bangladeshi BARI Tomato 3 and Indian Pusa Ruby varieties (198). This might happen due to the availability of BAP both exogenously and endogenously (199) as BAP was reported to induce vitrification more frequently compared to other cytokinin (200) (201).

In the present study, all five varieties produced higher number of shoots while on media supplemented with 2mg/l BAP. Similarly, 2 mg/l BAP concentrations were reported to be the best in various tomato varieties (202) (203) (204). However, the use of TDZ (Thidiazuron) was reported instead of BAP for high frequency shoot regeneration in tomato tissue culture (205).

4.1.1.5. Effect of BAP and IAA combination on regeneration

Combination of phytohormones (cytokines and auxins) has been applied in tomato cultivars to obtain morphogenic response such as shoot organogenesis (206) (207). Regeneration response was studied with BAP and IAA combination in the present study. Highest number of shoots was found in media containing 2 mg/l BAP along with 0.1 mg/l and 0.2 mg/l IAA in regeneration of BARI Tomato 15 and BARI Tomato 2, respectively. Moreover, BARI Tomato 3 needed 1 mg/l BAP in combination with 0.2 mg/l IAA to obtain highest shoot number. Similar report came earlier in which highest multiple shoot regeneration was obtained in media containing 1 mg/l BAP and 0.1 mg/l IAA in BARI Tomato 3 and Pusa Ruby regeneration (208). In a different study, higher number of multiple shoots was found on MS medium supplemented with 2.0 mg/l BAP + 0.2 mg/l IAA in tomato cv Rio Grande regeneration (209). However, maximum regeneration was obtained on MS medium

with higher supplementation as 4 mg/l of BAP+ 0.5 mg/l IAA in Feston using hypocotyls and leaf disc explants (210).

4.1.1.6. Effect of spacing on regeneration

Effect of tissue density on *in vitro* regeneration of tomato was evaluated in the current study. Plant density is the main reason for nutrient competition, which has an effect on development and yield of many crops and fruits (211) (212) (213) (214) (215).

4.1.1.6.1. Dry and Fresh weight

At 2 cm distances, the tested varieties have higher fresh and dry weights. Similar results were reported in *Linum usitatissimum* **(**216**)**. It was reported that an increase in fresh and dry weight due to better nutrient and sucrose uptake (217) (218) (219) (220) (221).

4.1.1.6.2. Shoot regeneration

In the present study, in respect to shoot regeneration, response towards 1.5 cm were found to be the best and decreased significantly at 2 cm distances apart for the tested tomato varieties. Similar findings were also reported on shoot regeneration, competition between explants for the constant amount of nutrients and water uptake in growth medium at 1 cm distances apart but less number of shoot was found at 2 cm distances apart due to more water and nutrient that could lead to relief in explants **(**222**).**

4.1.1.6.3. Chlorophyll content measurement

The plant growth potential may be measured by estimation of chlorophyll content in leaves (223) as photosynthesis plays an important role in which leaf chlorophyll content was required in enough amounts (224) (225) (226) (227). The highest amount

of chlorophyll produced by the explants obtained the maximum chlorophyll due to a competition for light (228).

In present study, total chlorophyll content was higher with explants spacing at 1.5 cm for the varieties used. Similar result was found the best for *Linum usitatissimum* (229**)**. For explants growing at a distance of 2.0 did not require competing for sunlight and produced less chlorophyll. For explants growing at a distance of 1.0 cm were scarce of nutrients and therefore produced the minimum amount of chlorophyll among three groups. Considering overall response, 1.5 cm distance was followed in the successive experiments.

4.1.1.7. Subculture

For achieving shoot elongation and maximum shoot regeneration, subculture of the explants was essential. Otherwise, shoot regeneration found to be suppressed by callus formation at the base of the regenerated shoots. Similar approach was reported earlier in tomato regeneration **(**230**).**

4.1.1.8. Effect of IAA on rooting

Best response in rooting was found in the media supplemented with various concentration of auxin than the auxin free media (231). IAA has been reported to be more effective in producing healthy roots compared to NAA in tomato (232) (233) (234). For this reason only IAA was used in the present study. Among five varieties tested in this study, higher IAA concentration (0.7mg/l and 1 mg/l) was found optimum in BARI Tomato 15 and BINA Tomato 3 while lower (0.2 and 0.3 mg/l) was best for the rest of them. Similarly, 0.2 mg/l IAA (235), 0.3 mg/l IAA (236) and 1 mg/l IAA (237) were found optimum for different tomato varieties. In contrast, 100% rooting was found in *in vitro* regeneration of tomato explants on media without hormonal supplementation (238). This genotypic response is irrespective to hormonal

supplementation. It can be concluded that all the five concentrations of IAA were suitable for root formation but was greatly affected by the variety used.

4.1.2. Flowering and fruit setting

All rooted plants flowered and set fruits like the non regenerated control plants. However, plants took longer or shorter time to flower due to the seasonal variation. Fruits numbers obtained in the present study were differed from naturally produced fruits number mentioned in BARI and BINA websites. These differences might be due to seasonal variation for example, highest number of fruits were obtained in winter and reduced in summer in BINA Tomato 5 and BARI Tomato 7 (239) and other tomato varieties (240) (241). In the current study, fruits weights were found less than the fruits weight gained naturally according to BARI and BINA websites. The reason of these variations because there was a considerable difference between season and genotype was reported earlier (242) (243) (244).

Viability test was done with the seeds of these mature fruits which showed cent percent viability. It was seen that the germination response of these seeds were more or less the same as the seeds from naturally grown plants used from the beginning of this study. These demonstrate that the present protocol is efficient and reproducible for these farmer popular tomato varieties.

4.2. Tomato transformation

In present study, introduction of pH7WG2_OsNHX1_1.6 (containing *OsNHX1* antiporter gene) into tomato tissues was the main goal to attain salinity tolerant tomatoes. Before that, two analyses were done. First was the salinity test of tomato varieties and secondly evaluation was for determination of factors affecting

Agrobacterium-mediated transformation using screenable marker gene like GUS (*uidA*).

4.2.1. Determination of baseline salinity tolerance level

To determine a baseline salinity tolerance level of untransformed tomato plants, seeds were subjected to germinate on media containing different NaCl concentrations ranging from 5-100 mM (0.5-10 dS/m). In present study, germination rate of seedlings fall to 46.4% in media containing 20 mM (2.0 dS/m) NaCl which was 81.6% in control experiment. And serious reduction in germination rate (1.6%) was observed at 100 mM (10 dS/m) NaCl. Apart from germination rate, the time requirement for germination at 50 mM (5 dS/m) NaCl and above was also influenced by salinity. Similar report has been found that only a few genotypes were able to germinate at high salt concentration because it also increased the time for germination. For example, tomato seeds needed 50% and 100% additional days to germinate at 80 and 190 mM NaCl, respectively, than in a medium without salt (245). This indicated that salinity severely influence the plant physiology (246) (247) (248).

4.2.2. Factors affecting transformation

In present study, tomato varieties, namely BARI Tomato 2, BARI Tomato 3, BARI Tomato 14, BARI Tomato 15 and BINA Tomato 3 were tested with *Agrobacterium* strain LBA4404 containing pBI121 (containing *nptII* marker gene and *uidA* gene) for determination factors influencing transformation. Transformation rate was found to be proportional to the relationship between infected (transformed) explants and inoculation time, co-cultivation period, bacterial suspension concentration, and selection antibiotic concentration (249).

4.2.2.1. Bacterial culture density

Maximum transformation efficiency (100%) was observed by GUS assay at OD600 0.68 in BARI Tomato 3, BARI Tomato 14 and BINA Tomato 3 and least (51%) was at OD600 0.45 in BARI Tomato 15 used in present study. Similar result was reported in where maximum transformation (~95%) was observed at OD600 0.79 and minimum was at OD600 0.42 in Bahar, BINA tomato 3, BINA tomato 5 and Pusa Ruby (250).

Contrasting to these results, a low bacterial suspension concentration (OD600 0.2 and 0.5) showed the best result in tomato transformation with *Agrobacterium* strain EHA101 and EHA105 respectively (251) (252). This difference may be related to the use of different bacterial strains which are super-virulent because of the extra copy of *vir* gene present in the cell compared to moderately virulent LBA strains (253).

4.2.2.2. Inoculation period

The efficiency of the transformation system mediated by *Agrobacterium* was reported to be influenced by the inoculation period (254), and it differs among plant species (255). In the present study, 30 minutes of inoculation period gave higher transformation frequency (100%) in BARI Tomato 3, BARI Tomato 14 and BINA Tomato 3 during GUS assay compared to explants inoculated for 60 minutes at higher bacterial density OD600 0.68 for all five tomato varieties. Same Inoculation period (30 minutes) was reported to be optimum for tomato varieties Pusa Ruby, Arka Vikas and Sioux when transformed with *Agrobacterium tumefaciens* strain, AGL1, carring either pCTBE2L or pRINASE2L construct (256). This is in agreement with transformation of some other tomato varieties (257) (258).

In contrast to this, using same strain LBA4404, 50 minutes and 60 minutes was reported as optimum inoculation period (259) (260). On the other hand,

transformation efficiency reported to decline above 15 minutes of inoculation period using LBA4404 in Bahar, BINA tomato 3, BINA tomato 5 and Pusa Ruby transformation (261).

4.2.2.3. Pre-culture

In the present study, pre-cultured explants influenced regeneration of putative transgenic shoots. As maximum shoot number was obtained by the pre-cultured explants of tomato varieties compared to non pre-cultured. Highest shoot number was found in pre-cultured BARI Tomato 14 explants among the five varieties tested in the present study. Pre-culture enhances the regeneration percentage as explants are considerably swelled during this treatment which helps cell or tissue to overcome the stress followed by co-cultivation with *Agrobacterium* (262) thus improves the transformation frequency in tomato (263) (264) (265) (266). The transformation frequency was reported to be low (2–15.5 %) without a pre-culture in tomato transformation (267).

In contrast, pre-culture was reported to enhance tomato transformation frequency, but the regeneration of the transformed cells scored to be reduced **(**268**)**. These diverse observations may be due to variation of tomato genotypes (269).

4.2.2.4. Co-cultivation period

The co-cultivation period was one of the main factors affecting transformation as 'too long period' resulted bacterial overgrowth and 'too short period' resulted declination of transformation frequency indicating explants death on selection media (270) (271). Longer co-cultivation time also results into delay growth from transformed explants (272).

In the present study, co-cultivation time span of two days (48 hours) were found appropriate in transient GUS expression (86-100%) for BARI and BINA varieties

tested. Two days of co-cultivation period was ideal in tomato cvs. Megha (L 15), Pusa Ruby, Arka vikas transformed with *Agrobacterium* in various studies (273) (274) (275). However, one day was appropriate co-cultivation period for Micro-Tom tomatoes (276). The reason behind this may be related to the tomato genotype and the use of different plant tissue as explants, different Agrobacterium strain and genes that has been transformed (277) (278).

4.2.2.5. Bacteriostatic antibiotic use

For elimination of *A. tumefaciens* after co-cultivation, antibiotics are required to use in regeneration medium. A commonly used antibiotic for *A. tumefaciens* removal from plant tissues is cefotaxime which influences morphogenesis of the transformed tissue (279). In the present study, transformation efficiency was adversely affected by the *Agrobacterium* growth in the medium after two days of co-cultivation period. The same result was found in tomato cv. Riogrande transformation **(**280**)**. In the present study, 200 mg/l cefotaxime was used in selection media for tomato varieties which prevented bacterial overgrowth completely. Similar approach was also reported in tomato cv. Pusa Ruby transformation with *Sclerotium rolfsii* lectin gene (281).

However, a higher concentration (500 mg/l) of cefotaxime was in tomato cvs. Riogrande (282), Money maker (283), Pusa Ruby (284) (285) and Micro-Tom tomato (286) varieties led to complete control of *Agrbacterium* growth with maximum transformation events. This variation is because of variation in antibiotics sensitivity of plants is species and plants physiology and *Agrobacterium* strain (287) (288) (289).

4.2.2.6. Marker gene selection

In this experiment, at first, two selectable marker genes *nptII* (encoding resistance to kanamycin) in pBI121 and *hptII* (encoding resistance to hygromycin) in pH7WG2_OsNHX1_1.6 were used in transformation of tomato explants.

In *Agrobacterium*-mediated transformations in tomato, transformation frequencies are not only related to co-cultivation time, the inoculation time, bacterial suspension density and antibiotic concentrations but also correlated with the plasmid of transformation containing various selection marker genes which have an effect on genetic transformation (290).

4.2.3. Kanamycin sensitivity

Kanamycin was most preferable to obtain transgenic plants (291) (292) (293) (294) however it hinders growth and development of several species (295) (296) (297) (298). In present study, all inoculated explants survived in control experiments (regeneration media without antibiotic), but survival percentage declined in presence of kanamycin. Explants did not regenerate at 50 mg/l of kanamycin and became albino at 100 mg/l. The selection of kanamycin was maintained at 150 mg/l concentration for transgenic tomato shoot screening in the present study. Higher concentration was used to avoid high frequency of non-transformed 'escapes' or chimeric plant production (299) (300) (301). A Higher concentration (200 mg/l) of kanamycin was used for Bahar, BINA Tomato 3, BINA Tomato 5 and Pusa Ruby (302). Lower concentrations 100 mg/l (303) and 50 mg/l (304) were used in different studies for Pusa Ruby variety. Hence, this proves that the variation take place because of the genotypes of tomato cultivars (305).

4.2.4. Hygromycin sensitivity

After Kanamycin, hygromycin is the second most preferred used antibiotic for selection (306). In the present study, selection of transformed explants was 5 mg/l hygromycin which gradually increased to 10 mg/l hygromycin. All the un-transformed explants died at final concentration. For selection of transformed tomato explants cvs. Riogrande, Roma and Summer set, 25 mg/l hygromycin was standardized as a lethal dose (307) (308). However, further increase (40 mg/l of hygromycin) was reported during drought tolerant tomato cv. Pusa Ruby selection (309). And 50 mg/l of hygromycin was effectual for tomato cv. Riogrande selection (310).

Hygromycin selection system found to be a developed system for transgenic shoots with low frequency of selection 'escape' in soybean (311) (312).

4.2.5. Transformation frequency

More than a dozen of high demanding and agronomically important plants have been transformed and transgenic plants been regenerated but still the transformation frequencies of most of the plant species are low (313). A range of tomato cultivars was used in transformation according to different authors (314) and their transformation frequencies have ranged from 6% to 49-49.5% (315) (316) (317) (318).

In this present event, transformation of five tomato varieties with *Agrobacterium* strain containing pBI121, gave rise to higher transformation efficiencies by transient GUS expression than the frequencies calculated by using regeneration percentage of transformed shoots. It was reported earlier that a big difference between transformation frequencies obtained by transient GUS expression and by regeneration on selection media during transformation of tomato cv. Moneymaker with various

combinations of binary vectors and *Agrobacterium* helper strains (319). In case of transformation of tomato varieties performed by pH7WG2_OsNHX1_1.6, transformation frequency was calculated by only regeneration percentage on selection media. This is because pH7WG2_OsNHX1_1.6 does not contain any GUS (*uidA*) gene.

In present study highest transformation frequency 47% was obtained by calculating BARI Tomato 3 in transformation performed with pBI121 (containing *nptII* and *uidA* genes) and 20.5% by BARI Tomato 3 transformed with pH7WG2_OsNHX1_1.6 (containing *OsNHX1*, Na+/H+ antiporter gene) based on regeneration percentage of transgenic explants on selection media. Similar result was found that 49% of the tomato cv. Riogrande shoots were transformed with *Agrobacterium tumefaciens* strain EHA101 harboring pBI333 (320). Transformation efficiency 49.5% was achieved in tomato cv. Pusa Ruby shoots transformed with *Agrobacterium* containing TLCV-CP construct and regenerated T0-generation (putative transgenic plants) were screened by molecular analysis (321). However, transformation efficiency of tomato cv. Pusa Ruby was found to be 8% when transformed with *Agrobacterium tumefaciens* strain LBA4404 carrying the binary vector pBI121 (322). These results were differed from each other revealed may be due to differences in bacterial strain (323), plasmid construct (324), plant genotype (325) and transformation procedure (326) etc.

4.2.6. Molecular analysis

Till now, in some cases, transgenic explants selection was made after development of plantlets from transformed cells, and then the selection in transformation was confirmed using molecular analysis (327). In the present study, molecular analysis of putative transformed explants was done by PCR for confirmation of

pH7WG2_OsNHX1_1.6 (*OsNHX1*, Na+/H+ antiporter gene) incorporation in BARI Tomato 3. In the current study, BARI Tomato 3 variety was found to have highest transformation ability.

Chapter 5

CONCLUSION

Findings

- Efficient and reproducible regeneration protocol was established. Regeneration media with 2 mg/l BAP supplementation was found best for maximum number of shoot formation for all five varieties tested. Highest number of shoots was obtained by BINA Tomato 3 variety.
- In BARI Tomato 2, highest regeneration rate was found while on media supplemented with BAP and IAA compared to the media containing only BAP. In this case, regeneration media supplemented with 2 mg/l BAP+0.2 mg/l was optimum for highest shoot regeneration.
- Rooting media supplemented with 0.2 mg/l IAA was found optimum for BARI Tomato 2, BARI Tomato 14 and BINA Tomato 3. 100% shoot producing root was found in BARI Tomato 14, BARI Tomato 15 and BINA Tomato 3.
- Higher transformation efficiency (100%) was observed in transient GUS expression in BARI Tomato 3. Higher OD600 (0.68) with 30 minutes of incubation period and 48 hours of co-cultivation period was optimum for achieving maximum transformation frequency.
- Higher *A. tumefaciens* cultural density increases transient GUS expression but this was not associated with higher stable transformation frequency. For example BARI Tomato 3 gave 100% transformation efficiency observed in GUS assay but 47% transformation efficiency was found based on their regeneration on selection media.
- Transformation frequency of putative transgenic shoots was confirmed by GUS histochemical assay and also by Polymerase Chain Reaction (PCR).

Chapter 6

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APPENDICES

APPENDIX-1

Statistical formulas used in analysis

1. Arithmetic Mean ($\overline{X}$) = $\frac{\sum X}{n}$

Where, ΣX = Summation of Observed Value

n = No. of Observation

2. Standard Deviation (SD) = $\sqrt{\frac{\sum(X-\overline{X})^2}{n}}$

Where, X = individual Value

$\overline{X}$ = Mean Value

n = No. of Observation

3. Standard Error (SE) = $\frac{SD}{\sqrt{(n-1)}}$

Where, SD = Std. Deviation

n = No. of Observation

5. Degree of Freedom = $(n_1 - 1) + (n_2 - 1)$

Where, n_1 = Number of observation's of 1st sample

n_2 = Number of observation's of 2nd sample

6. % of Writhing = (Mean of Test / Mean of Control) X 100

7. SE for % Writhing = (SE / Control Mean) X 100

APPENDIX-2

Equation used to calculate regeneration frequency

$$\text{Regeneration frequency (\%)} = \frac{\text{Number of shoot produced by explants}}{\text{Number of explants inoculated/ Petri-dish}} \times 100$$

Equation used to calculate transformation frequency

$$\text{Transformation frequency (\%)} = \frac{\text{Number of shoot initiation by explants (On selection media)}}{\text{Number of explants inoculated/ Petri-dish}} \times 100$$

Equations used to calculate chlorophyll content:

Chlorophyll *a* = (ml solvent) [(0.0127 × Absorbance 665) - (0.00269 × Absorbance 650)]/ g leaf

Chlorophyll *b* = (ml solvent) [(0.0229 × Absorbance 645) - (0.00468 × Absorbance 665)]/ g leaf

Total chlorophyll content = (ml solvent) [(0.0202 × Absorbance 650) + (0.00802 × Absorbance 665)]/ g leaf