Establishment of *in vitro* regeneration and transformation protocol in tomato (*Lycopersicon esculentum* Miller)"

A DISSERTATION SUBMITTED TO THE BRAC UNIVERSITY OF DHAKA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTERS OF SCIENCE IN BIOTECHNOLOGY.

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Certificate

*This is to certify that the research work embodying the results reported in this thesis entitled "**Establishment of in vitro regeneration and transformation protocol in tomato (Lycopersicon esculentum Miller)**" submitted by Mrs. **Jebunnesa Chowdhury**, has been carried out under my supervision in the Plant Biotechnology Laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka. It is further certified that the research work presented here is original and suitable for submission for the partial fulfillment of the degree of Master of Science in Biotechnology, BRAC University, Dhaka.*

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Certificate

This is to certify that the research work embodying the results reported in this thesis entitled ***"Establishment of in vitro regeneration and transformation protocol in tomato (Lycopersicon esculentum Miller)"*** *submitted by Mrs.* ***Jebunnesa Chowdhury****, has been carried out under my supervision in the Plant Biotechnology Laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka. It is further certified that the research work presented here is original and suitable for submission for the partial fulfillment of the degree of Master of Science in Biotechnology, BRAC University,Dhaka.*

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JEBUNNESA CHOWDHURY

ABBREVIATIONS

The following abbreviations have been used throughout the text.

A.	*Agrobacterium*
B-3	Bina tomato-3
B-5	Bina tomato-5
BAP	6-Benzylaminopurine.
BINA	Bangladesh Institute of Nuclear Agricultural
BR	Bahar
CaMV	Cauliflower Mosaic Virus
GUS	β-glucuronidase
IAA	Indole-3 acetic acid
IBA	Indole-3 butyric acid
Kan	Kanamycin
Kbp	Kilo base pair
Kn	Kinetin (6-furfuryl amino purine)
LB	Luria Broth
MS	Murashige and Skoog (1962) medium
Na_2-FeEDTA	Sodium salt of ferric ethylene diamine tetra acetate
NaOH	Sodium hydroxide

NAA	α- naphthalene acetic acid
NOS	Nopaline synthase
nptII	Neomycin phosphotransferase II
OD	Optical density
PR	Pusa Rubi
t.	*tumefaciens*
T-DNA	Transfer DNA
Ti	Tumour inducing
Vir	Virulence region
X-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
YEP	Yeast Extract Peptone
YMB	Yeast extract Mannitol Broth

Tomato (*Lycopersicon esculentum* Miller) is one of the most important and popular winter vegetable of Bangladesh. But its yield is hampered by various biotic and abiotic factors. To make it resistance towards these factors different transgenic approaches have been reported. In the present study, an efficient transformation method was developed with three locally grown tomato varieties, namely Bahar (BR), Bina tomato 5 (B-5), Bina tomato 3 (B-3) and one Indian commercial variety, Pusa Ruby (PR). A reliable and reproducible *in vitro* regeneration protocol for these varieties was obtained as a prerequisite for transformation. Cotyledonary leaf explants of tomato were collected from 8-10 days old *in vitro* germinated seedling. To increase germination, agitation of seeds following sterilization was found to be effective. Different concentrations and combinations of growth regulators were added to MS media to observe shoot initiation and root induction. MS media containing 2 mg/l BAP showed best shoot regeneration with maximum number of shoots (~6 shoots/ explant) for all four varieties and obtained 91%, 86%, 83% and 93% regeneration percentage in BR, B-5, B-3 and PR variety respectively. Rooting was best in half strength MS media supplemented with 0.2 mg/l IAA. The regenerated plantlets successfully acclimatized in soil, where they flowered and formed fruits. Seeds collected from these fruits were found to be viable in germination tests. For transformation, a genetically engineered *Agrobacterium* strain LBA4404 containing binary vector pBI121 was used to transform cotyledonary leaf explant of all the four tomato varieties and found to be susceptible towards it. Further studies showed that an OD_{600} of 0.8 with 10-15 mins of incubation and 3 days of co-cultivation period was the best to achieve maximum transformation ability. B-3 showed highest transformation ability (96.7%), whereas BR, B-5 and PR showed 83.3%, 93.3% and 93.3% as confirmed by transient GUS assay. Carbenicillin and cefotaxime were used as bacteriostatic antibiotics while kanamycin was used for the selection of transformed explants. Immediate selection pressure with 200 mg/l kanamycin in the regeneration media following co-cultivation was found to be the best selection condition to obtain transformed shoots. Putative transformed shoots that survived under selection pressure were transferred to ¼ strength of MS media containing 0.5 mg/l IBA and reduced concentration of bacteriostatic antibiotics to obtain rooting avoiding callus formation.

With respect to transgenic B-3 plantlets which rooted in soil, efficiency of transformation was found to be 11.11%. Following rooting GUS histochemical assay was showed stable incorporation of GUS gene in these putatively transformed plantlets. Among the four varieties B-3 was chosen as it showed the highest transformation ability. Transformed B-3 plantlets were successfully acclimatized in soil and flowered normally. Different parts of the flower were found to be GUS positive and the flowers produced fruit. Seeds from the fruit have been collected for checking inheritance of the antibiotic resistant and GUS genes.

Contents

CHAPTER 1:
INTRODUCTION

Tomato (*Lycopersicon esculentum* Miller) is one of the most important winter vegetable crops of Bangladesh. It belongs to a large family Solanaceae, which contains many important food crops, like potato, egg plant, capsicum etc. The center of origin of tomato is said to be tropical America (Kalloo, 1986), particularly in Peru, Ecuador, Bolivia and Andes (Salunkhe et al. 1987). It was domesticated in Mexico over 1,500 years ago from South America. At present leading tomato producing countries include China, the USA, Turkey, Russia, Italy, Egypt, India, Spain and Mexico (Mark, 2002). Tomato is widely grown in many countries of the world including Bangladesh for its good taste and high nutritional value, and also for its adaptability to wide range of soil and climate (Ahmed, 1976).

The cultivated tomato is a short-lived diploid (2n =2x = 24) dicotyledonous annual plant, typically growing to 1-3 m in height, with a weak woody stem that usually scrambles over other plants. The leaves are 10-12 cm long, pinnate, with 5-9 leaflets, each leaflet up to 8 cm long, with a serrated margin, both stem and leaves are densely glandular-hairy. The flowers are 1-2 cm across, yellow, with five pointed lobes on the corolla; they are borne in a cyme of 3-12 flowers together. The fruit is an edible, bright colored (usually red, from the pigment lycopene) berry, 1-2 cm diameter in wild plants, commonly much larger in cultivated forms (Islam, 2007)

Due to increasing popularity and high food value, the production of tomatoes is increasing worldwide. The annual worldwide production of tomato in 2003 has been estimated at 110 million tons with a total production area of about 4.2 million ha (FAO, 2004).

In 2004, Mexico was world's leading fresh tomato exporter according to data from the Global Trade Atlas (GTA) and Food and Agriculture Organization of the United Nations (FAO), and The Foreign Agricultural

Service (FAS) attaché reports. The United States was the leading importer of fresh tomatoes in 2004. Other notable importers include Russia, EU and Canada. According to the GTA, China is the world's largest tomato paste and purée exporter. In 2004, China exported 438,192 tons of tomato and continues to make inroads in the world market.

In Bangladesh, the demand of tomato is increasing day-by-day in the agro. and food industries. The average yield of tomato in our country is 6.69 tons/ha (BBS, 2000) which is quite low as compared to other leading tomato producing countries of the world like China and the USA where per hector yield was reported as 30.36 tons and 59.30 tons, respectively (FAO, 2001). According to Bangladesh Bureau of Statistics, in the year 2003-2004 tomatoes was cultivated in about 44,000 acres and total production was about 120,000 metric tons with the rate of 2,709 kg per acre (BBS, 2005).

Tomato is a major vegetable crop that has achieves tremendous popularity. It is popular not only for its nutritive value, but also for its diverse uses (Bose, 1986). Ripe tomato is used mostly in salad and other forms of food products, such as jam, ketchup, pickle, sauce etc. It is estimated that unripe tomato contains 93.1 gm water, 0.7 gm fiber, 3.5 gm carbohydrates, 23 KCal energy, 500-1500 IU vitamin A, 0.01 mg vitamin B, 31 mg vitamin C, 20 mg Ca, 1.8 mg Fe and 192 gm carotene per 100 gm fresh weight (Bose, 1986). Tomatoes, aside from being testy, are very healthy as they are a good source of vitamin A and C. In our country, where every year about 30 thousand people become night blind, get more than 7% of total vitamin A from tomato as a cheap source of vitamin (Begum and Mia, 1993).

To meet the increasing demand of tomato in Bangladesh, it is necessary to develop good varieties with nutritional quality, higher yield potential and wide adaptability. Even after developing new mega varieties, the production

of tomatoes are severely decreased due to disease infestation. In total, there are more than 200 pathogens that infect tomato crop (Watterson, 1986). Tomato production in our country is hindered by various pathogens including fungi, virus and bacteria. Many recognized cultivars has gradually become decreased in vigor and cropping capacity largely due to one or more viruses. The major tomato viruses are Tomato Mosaic Virus (ToMV), Tomato Leaf Curl Virus (TLCV), Curly Top Virus (CTV), Tomato Yellow Top Virus (TYTV) and Cucumber Mosaic Virus (CMV). Viral diseases reduce plant vigor and yield potential of fruit to a considerable extent. The presence of two or more viruses may happen within a plant simultaneously. The wide host ranges of both the vactor and the virus complicate diseases management (Jones et al., 1991).

The bacterial diseases of tomato include bacterial canker caused by *Corynbacterium michiganense,* bacterial speck caused by *Pseudomonoas syringae* and bacterial wilt caused by *Ralstoni solanacearu.* The main fungal disease that affects the production of crop include (*Fusarium oxysporum* f. *lycopersici*) and Verticillium (*Verticillium dahlia*) wilts and powdery mildew caused by *Leveilulla taurica*, early and late blights caused by *Alternaria solani* and *Phytopthora infestans*, respectively, anthracnose caused by *Collitotrichum phomoides.* Tomatoes are subjected to a large number of pests from the time of first emergence till harvest. Aphids, flea beetles, leaf miners, and spider mites are a problem to plant bed tomatoes. Flea beetles, aphids, leaf miners, stink bugs and fruit worms cause foliage damage in the field. But their fruit damage and disease spreading problems can be very serious (Raj et al., 2005). Disease infestation is mainly controlled through application of chemicals which sometimes reaches the level of toxicity. So now it is evident that improvement of this crop is an essential task to overcome the constraints of tomato production.

To improve upon the characters of agronomic importance in tomato, conventional breeding methods were tried. But they were not very successful due to high degree of self pollination and non-availability of suitable wild germplasm. Moreover, conventional breeding programme is time consuming, extending over seven to eight years involving crossing and selection of desirable traits. In addition to this, genetic incompatibilities restrict many potentially important gene transfers by inter-specific cross.

Beside sexual breeding techniques, there are many ways of creating variability including induction of somaclonal variation through tissue culture, somatic hybridization and genetic engineering. Scowcroft and his colleagues suggested that, tissue culture techniques can play a significant role for enrichment of genetic variability by creating variation (somaclonal variation) or mutation (by applying radiation or chemical mutagens to *in-vitro* cultured plant materials) at an unexpectedly high rate and may be novel sources of genetic variability in many plant species (Scowcroft et al., 1987). But these were found to have limited application in many crop species (Islam, 1998).

The regeneration of plant by tissue culture technique is an important and essential component of biotechnological research, and required for the genetic manipulation of the plants. The totipotential capacity of a cell or tissue opens up several new contingencies in plant breeding programmes that provide gene manipulation and selection of desirable character. Tissue culture techniques have several advantages over traditional propagation methods. The application of *in vitro* techniques provides unique possibilities for overcoming the barriers of incompatibility existing between remote species and has facilitated rapid introduction of new varieties.

It may be mention here that several attempts have been taken to establish *in vitro* regeneration protocol for tomato. During these attempts a wide variety of explants have been used with the application of several growth regulators to regenerate plantlets with or without intervention of callus. For tomato, plant regeneration protocol has been established from various explants, such as leaf disk, cotyledon and protoplasts. The success of *in vitro* regeneration from tomato explants has been limited (Kut, 1982). As tomato is a predominantly inbreeding species, the genetic variation tends to decrease even without selection. For this reason, application of conventional breeding for improvement shows its limitation. To overcome such problems, "genetic transformation" technology has been evolved. Before applying this particular technology in improving any crop plant, a reliable genotype independent regeneration protocol needs to be established. This technology offers the potential for the introduction of specific genes from any source (related or unrelated plant species or even from animals) into existing elite plant lines (Gardner, 1993). Thus genetic transformation accelerates the development of new plant varieties, which is not possible through breeding or tissue culture alone.

Generally in the process of genetic transformation, the delivery of the desired foreign gene into the host genome is mediated through successful utilization of a soil bacterium, called *Agrobacterium tumefaciens*. This soil bacterium has capability to form gall at the wound sites of many dicotyledonous plants (Gustavo, 1998). The introduction of tumor is due to the presence of a large Ti (tumor inducing) plasmid in the virulent strains of *Agrobacterium.* Part of this Ti plasmid is "transfer DNA" (T-DNA) which is transfer to the plant nuclear genome during transformation (Gelvin, 2003). In all transformation experiments specific reporter genes are required to be incorporated into the plants prior to the integration of gene/genes of interest. These reporter genes

can be recognized in plant tissue culture with the help of selectable agents, confirming the transformation of the plant tissue. However, relatively small number of individual cells incorporates the new DNA into their chromosomes and so become transgenic. For successful transformation experiment, regeneration of plant is essential from these transgenic cells by culturing through suitable *in vitro* method (Gardner, 1993). Thus this technology depends on in one hand, in the one hand on *in vitro* regeneration techniques for the generation of transgenic plants, and in the other hand, selection of transformed cell to ensure incorporation of desired trait(s).

Regeneration protocol has been established in various commercial and un-released tomato varieties worldwide. Le et al.(1991) stated that, cotyledons of tomato cv. Bony Best produced loose, yellow and rapid growth of callus on MS medium with low levels of BAP and high levels of 2,4-D whereas low levels of 2,4-D gave better results when hypocotyls were used as explants. Bookout et al. (1987) reported that the presence of IAA in shoot inducing medium containing 2 mg/l zeatin was better while they were working with leaf explants of tomato on various modifications of MS medium. However, use of zeatin for *in vitro* regeneration is predominant in some report (Ye et al., 1994; Costa et al., 2000). Sizes of explants were also reported to have influence on regeneration capacity (Schuetze and Wieezorrek, 1987). While working with Bangladeshi tomato varieties, good callus was obtained by Begum and Miah using leaf explants of two (E-6 and S-1) strains of tomato on MS medium supplemented with 2 mg/l IAA and 2 mg/l kinetin. For regeneration of plants calli were transferred onto MS medium supplemented with 4 mg/l IAA and 4 mg/l kinetin (Begum and Miah, 1993). But using Indian variety (*Lycopersicon esculentum* cv. PKM. 1), Jawahar et al., (1997) induced callus from hypocotyls of tomato on MS medium supplemented with IAA 2 mg/l and BAP 1 mg/l and after subculture

in the same medium they got better shoot proliferation. Dwivedi et al., (1990) found that direct shoot bud differentiation was induced in tomato leaf segments by culturing them in the medium containing 0.5 mg/l BAP and 0.25 mg/l NAA for 7 days. Shoot bud grew into shoots by subculturing in the medium supplemented with 0.25 mg/l BAP and 0.01 mg/l NAA. Yassen et al., (1998) cultured the excised tomato cotyledons and hypocotyls explants on MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l IAA. Regenerated shoots rooted on MS medium containing 1 mg/l IBA and 50 mg/l kanamycin after transformation. A more detailed work was done by Costa et al., (2000). They compared cotyledonary explants of two tomato cultivars (IPA-5 and IPA-6) for *in vitro* regeneration in five different culture media with different combinations of growth regulators. They reported both the varieties exhibited higher shoot regeneration when cultured on MS medium supplemented with 1 mg/l zeatin and 0.1 mg/l IAA or 2.5 mg/l BAP and 0.2 mg/l IAA.

For rooting of *in vitro* shoots various strength of MS medium with or without auxin supplementation has been reported. Sheeja and his team reported, plain half strength of MS as the best media for rooting when addition of IAA (0.1 mg/l) was found essential to induce longer roots (Sheeja et al., 2004). However, IBA (0.1-0.5 mg/l) was found to be the best hormonal supplementation on half strength of MS by Vekatachalam et al., (2000). Liu et al., (2003) cultured shoot of tomato cv. Peral for root formation in MS medium supplemented with 0.2 mg/l IAA or 0.1 mg/l IAA and 0.1 mg/l NAA. IAA at 0.2 mg/l resulted in high rooting and in the production of thick and strong roots.

Tomato is also considered as a genetic model for improving other dicotyledonous crop plants (Ling, 1998). The first report of tomato transformation was by McCormick et al., (1986). However, tomato is still

considered more difficult to transform than other species, such as *Petunia hybida* and *Nicotiana tabacom,* and can show widely different success rates, possibly depending on cultivar, *Agrobacterium* strain, antibiotic selection and /or the personnel performing experiment (McCormick, 1991).

The transformation efficiency of cultivated tomato (*Lycopersicon esculentum* cv. UC82) using *Agrobacterium tumefaciens* was improved from 14% to 25% by Phillips et al., (2001). They evaluated several variables potentially involved in the improvement of the transformation efficiency including enhancements in the regeneration system, antibiotics used for *Agrobacterium* overgrowth control, and method of applying kanamycin for selection. To reduce the complexity of *Agrobacterium*-mediated gene transfer in tomatoes, effects of various parameters, such as shoot regeneration medium (SRM), wounding type, infection method, pre-culture and co-cultivation temperature, have been evaluated by Ahsan et al., (2007) who found that transformation frequency was highly depend on the wound type of the explants, infection method and co-cultivation temperature. On the other hand, a commonly used pre-incubation method did not show any significant improvement regarding transformation frequency.

Transgenic tomato engineered with an antisense poly-galacturonase gene for increased shelf-life called "Flavor Savor" tomato was the first commercial product of recombinant DNA technology (Christou, 1996). This improvement was done considering the popularity of this tasty crop. Plastid transformation of tomato could be useful in bio-farming like production of vaccines, antibodies etc. Using tomato plastid (*Lycopersicon esculentum* var. IAC- Santa clara) high foreign protein (>40% of the total soluble protein) accumulation rate could be achieved in this system (Stephanie et al., 2001). Youm et al., (2008) developed transgenic tomatoes expressing human beta-

amyloid for use as a vaccine against Alzheimer's disease using *Agrobacterium*–mediated nuclear transformation.

But according to Park et al., (2003) tomato transformation is neither routine nor reliable (Ling, 1998). Nonetheless, there are success stories of transgenic tomato production. Therefore, the development of an efficient and genotype independent tomato transformation method is crucial.

Very little studies have been attempted in Bangladesh on *in vitro* regeneration protocol development and transformation on different crops (Islam et al., 2000; Sarker et al., 2005, 2008). In addition to this there is very few reports available on Bangladeshi tomato varieties (Begum and Mia, 1993; Islam 2007).

Objectives of the present study

Considering the importance and potential of tomato production in Bangladesh and to overcome the obstacles for improvement of this crop, the aim and objective of the study were –

1. To develop a reliable and reproducible *in vitro* regeneration protocol for four locally grown popular tomato verities.
2. To analysis various factors effecting transformation efficiency to establish an *Agrobacterium*-mediated transformation protocol.
3. To regenerate putative transgenic tomato plants using *Agrobacterium*-mediated genetic transformation protocol. The transformation experiment was carried out using GUS and *nptII* marker genes.

CHAPTER 2:
METHODS AND MATERIALS

2.1. MATERIALS

2.1.1. Plant materials

Seeds of four varieties of tomato (*Lycopersicon esculentum* Mill.) were use in this study. They are Bahar (BR), Bina tomato 5(B-5), Bina tomato 3(B-3), and Pusa Ruby (PR). Among these Bina tomato 3 (B-3) is a summer variety, Bina tomato 5 (B-5) is a winter variety and Bahar (BR) is the mutant one. All varieties were collected from Bangladesh Institute of Nuclear Agriculture (BINA). The remaining variety, Pusa Ruby, is an Indian variety available in our local market. Important characteristics of all the varieties are described below:

2.1.1.1. Bahar (BR)

Bahar (BR) is a hybrid tomato raised by breeding between "Oxheart" and "Anobic" variety. This is a high yielding winter variety, released in 1992. Plants are determinate in habit. It requires 90-100 days to mature after transplantation. Fruits are large, fleshy, more tasty and contain less number of seeds. Average fruit weight is 110 gms. Vitamin C content is 21.19 6mg/100gms. Maximum fruit yield is 75.0 tons/ha (av.65 tons/ha) (Dutta, 2004).

2.1.1.2. Bina tomato 5 (B-5)

In 2005, a high yielding winter tomato variety was released by National Seed Board from BINA named Bina tomato 5 (B-5). This was developed by mutating a Taiwanese variety through gama-Ray, and 6-7 years field trials at Mynmangingh, Comilla, Rangpur, Dinajpur, Jooshore, Magura, Kustia. It does not require any artificial hormone for its fruit setting. Leaf color is light green and curled. Fruits are long, smooth and average weight is 50-65 gms. It requires 90-95 days to mature after transplantation. Maximum fruit yield is 69.0 tons/ha (av.60 tons/ha) (Dutta, 2004).

2.1.1.3. Bina tomato 3 (B-3)

This summer tomato variety Bina tomato 3 (B-3) was released in 1997 by the National Seed Board from BINA. It was developed through the selection after performing the cross between Bahar and S-1 mutant (Hamid, 2004). The S-1 mutant variety was developed through gama ray. It was a summer variety but the production was found to be less, fruit size was small and not tasty. However the hybrid B-3 variety does not require any artificial hormone for its fruit setting. Leaf color is light green and they are slightly curled. Fruits are oval and average weight is 82 gms. It requires 60-65 days maturing after transplantation. Vitamin C content is 19.5 mg/100 gms. Maximum fruit yield is 48.0 tons/ha (av.40 tons/ha) (Dutta, 2004).

All these high yielding varieties suffer from Nematode attack and stem rot (Hamid, 2004).

2.1.1.4. Pusa Ruby

This is an exotic variety grown in Bangladesh. This variety released by International Agricultural Research Institute (IARI), New Delhi, India. It is an early growing cultivar. Plants are erect in nature and inflorescence is of determinate type. Fruits are medium-sized, uniform red, oblate and yellow stem end, slightly furrowed with uniform ripening. This variety is suitable for sowing both in spring–summer and autumn-winter seasons. It comes to fruiting in about 60-65 days after planting. Crop duration of the variety is about 130-135 days, with 32.5 tons/ha yields.

All these tomatoes were also grown and maintained in the Botanical Gardens of the Department of Botany, University of Dhaka.

2.1.2. *Agrobacterium* strain and vector plasmid

Genetically engineered *Agrobacterium tumefaciens* strain LBA4404 were used for infection in the transformation experiments (Fig. 1.). It contains

plasmid pBI121 of 14 KDa (binary vector). This binary vactor contains following genes within the right border (RB) and left border (LB) region of the construct:

1. The *udiA* gene (Jefferson, 1986) encoding GUS (β-glucuronidase), driven by CaMV promoter and NOS terminator. This reporter gene can be used to assess the efficiency of transformation through the GUS histochemical assay.
2. The *nptII* gene (Herrera, 1983) encoding neomycin phosphotransferase II conferring kanamycin resistance, driven by NOS promoter and NOS terminator.

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

2.1.3. Different culture media used

Different culture media used in the present investigation for various purposes were as follows:

2.1.3.1. Seed germination and seedling development media

For seed germination MS basal (Murashige and Skoog, 1962) solidified with phytogel were used.

2.1.3.2. Regeneration initiation and shoot differentiation media

For regeneration initiation cotyledonery explants were cultured on modified MS media supplemented with different concentrations and combinations of various growth regulators. After shoot initiation same media was used for shoot elongation.

2.1.3.3. Root induction media

For induction of roots from the *in-vitro* grown multiple shoots, half strength of MS basal medium supplemented with different auxins, namely, NAA, IAA and IBA with different concentrations were tried.

2.1.3.4. *Agrobacterium* culture media

Two states of bacterial culture media, YMB (Yeast Extract Mannitol Broth) were used for fresh culture and maintaining of genetically engineered *Agrobacterium tumefaciens*, with appropriate concentrations of antibiotics. Liquid YMB medium was used to grow the genetically engineered *Agrobacterium tumefaciens* strain LBA4404. This bacterial suspension was used as working culture for infection. Agar solidified YMB medium were used as maintenance media.

2.1.3.5. Bacterial resuspension media

Before infection, the freshly cultured *Agrobacterium sp.* was pelleted and resuspended in liquid MS basal medium.

2.1.3.6. Co-culture media

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

2.1.3.7. Media for Kanamycin sensitivity test

Sensitivity towards various concentrations of kanamycin was tested for cotyledonary explants of tomato to determine the selection concentration of kanamycin. Different concentrations of kanamycin were added to the best shoot regeneration medium.

2.1.3.8. Selection media

Appropriate concentration of antibiotics such as carbenicillin and cefotaxime along with adjusted concentrations of kanamycin was used with the best regeneration media as the selection media.

2.1.4. Materials used for histochemical GUS assay

2.1.4.1. 0.5 M morphinoethane sulphonic acid (MPS); pH- 5.6

2.67 gm of MPS was in 20 ml distilled H_2O. The pH was adjusted to 5.6 with 5M KOH and volume was made to 25 ml distilled H_2O and stored at room temperature.

2.1.4.2. Fixation solution; pH-5.6

Components	**Stock concentration**	**working concentration**
Formaldehyde	0.75% (v/v)	0.3%
0.5M MES	0.002% (v/v)	10mM
Mannitol	5.46% (w/v)	0.3M

2.1.4.3. 50 mM pH-7.0 NaH_2PO_4

Stock solutions:

1. 50 mM solution of $NaH_2PO_4.2H_2O$ (0.78 gm in 100 ml)
2. 50 mM solution of $Na_2HPO_4.2H_2O$ (0.71 gm in 100 ml)

For the preparation of 100 mM Na_2PO_4 (Phosphate buffer); pH-7.0, about 39.0 ml of 1. and about 69.0 ml of 2. stock solution were mixed and checked the pH for 7.0.

2.1.4.4. Histochemical reagent

X-gluc (5-bromo-4-chloro-3-indolyl glucuronide or 5-bromo, 3-indol β-D glucuronide): For the preparation of 10 ml of X-gluc, 10 mg of X-gluc was dissolved in 100 µl of diethyl formamide in a reagent bottle. The final volume was done with 50 mM Na_2PO_4 at pH 7.0.This was stored at -20^{0}C in eppendrof tube.

2.1.4.5. 70% ethanol

Degreening was performed with 70% ethanol.

2.2. METHODS

For different types of experiment such as seed germination and seedling development, regeneration initiation and shoot differentiation, shoot elongation, root initiation and for various transformation experiments, different types of media were used.

2.2.1. Preparation of stock solutions for MS medium

Different stock solutions were prepared as the first step for the preparation of medium. The various constituents of the medium were prepared into stock solutions for ready use during the preparation of medium. As different constituents were required in different concentrations, separate stock solutions for macro- and micro-nutrients, vitamins, plant growth regulators etc. were prepared.

2.2.1.1. Stock solution A (Macro-nutrients) for MS medium

This stock solution was made in such-a-way that its strength was 10 times more than the final strength of the medium in 500 ml distilled water. For this purpose, 10 times the weight of different salts required for 1 litre of medium were weighted correctly. Then the salts were sequentially dissolved one after another in a 500 ml volumetric flask with 350 ml of distilled water. The final volume of the solution was made up to 500 ml by further addition of distilled water. The solution was filtered through Whatman No. 1 filter paper to remove all the solid contaminants like dust, cotton etc. and was poured into a clean plastic container. After labeling, the solution was stored in a refrigerator at 4^0C for several weeks.

2.2.1.2. Stock solution B (Micro-nutrients) for MS medium

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

2.2.1.3. Stock solution B1 (all micro-nutrients except iron) for MS medium

This part of the stock solution was made with all the micro nutrients except $FeSO_4.7H_2O$ and Na_2-EDTA. This was made 100 times the final strength of necessary components in 500 ml of distilled water as described for the stock solution A. The solution was filtered and stored at 4^0C for several weeks.

2.2.1.4. Stock solution B2 (Iron chelate solution) for MS medium

The second solution was also made 100 times the final strength of $FeS0_4.7H_2O$ and Na_2-EDTA in 500 ml distilled water in a conical flask and heated slowly at low temperature until the salts were dissolved completely. Finally the solution was filtered and stored in refrigerator at 4^0C for several weeks.

2.2.1.5. Stock solution C (Organic constituents) for MS medium

It was also made 100 times the final strength of the medium in 500 ml of distilled water. This solution was also filtered and stored at 4^0C future use.

2.2.1.6. Stock solutions for growth regulators

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

2.2.1.6.1. Auxins

Indole-3-acetic acid (IAA)

Indole-3-butyric acid (IBA)

α-naphthalene acetic acid (NAA)

2.2.1.6.2. Cytokinins

6-benzylaminopurine(BAP)

6-furfurylaminopurine (Kn)

The growth regulators were dissolved in appropriate solvent as shown against each of them (Sigma Plant Cell Culture Catalogue):

Growth regulator	Solvent
IAA	1N NaOH
IBA	1N NaOH
NAA	1N NaOH
BAP	1N NaOH
Kn	1N NaOH

To prepare any one of the mentioned hormonal stock solution, 20 mg of the hormone was placed on a clean plastic weighting boat and dissolved in 1 to 2 ml of particular solvent. The mixture was then washed of with distilled water and collected in a 200 ml measuring cylinder. It was then made up to 200 ml with the addition of distilled water. The solution was filtered and poured into a clean plastic container and stored in a refrigerator at 4^0C for future use.

2.2.1.7. Preparation of stock solution of antibiotics

Three types of antibiotics were used in different bacterial and plant regeneration medium in transformation experiments. These antibiotics were:

Kanamycin (Duchefa, Netherland)

Cefotaxime (Duchefa, Netherland)

Carbinicilin (Duchefa, Netherland)

For the preparation of kanamycin, cefotaxime and carbinicilin stock solutions, 1 gm of each antibiotic was separately dissolved in 10 ml of deieonized water giving a final concentration. After filter sterilization, these solutions were stored in 2 ml eppendrof tubes at -20^0C in the dark as stock.

2.2.2. Preparation of one litre of MS medium

To prepare one litre of medium, the following steps were carried out successively:

1. For the preparation of desired medium (such as MS) thirty grams of sucrose was dissolved in 500 ml of distilled water in a 1 litre volumetric flask.
2. 50 ml of stock solution A, 5 ml of stock solution B and C were added to this 500 ml distilled water and mixed well.
3. 100 mg of myo-inositol (Sigma, USA) was added to this solution and were dissolved completely.
4. To obtain different required concentrations, various hormonal supplements were added to this solution either individually or in combinations and were mixed thoroughly. Since each of the hormonal stock solution contained 20.0 mg of the chemical in 200 ml of solution, the addition of 10 ml of any hormonal stock solution to make 1 litre of medium resulted 1.0 mg/l concentration of that hormonal supplement. Different concentrations of hormonal supplements were prepared by adding required amount of the stock solution to the medium following the similar procedure described earlier.
5. The whole mixture was then made up to 1 litre with further addition of distilled water.
6. The pH of the medium was adjusted to 5.8 using a digital pH meter (JENWAY- 3010,U.K) with the help of 1N NaOH or 1N HCl, whichever was required.
7. To solidify either 8.0 g (at 0.8%) of bacto- agar (Sigma, USA) or 0.3g (at 3%) of phytagel (Sigma, USA) was added to the desired medium.
8. To dissolve solidifying agent (agar, phytagel etc.) quickly the whole mixture was heated in a microwave oven (Emerson, Korea).

2.2.3. Media sterilization

Fixed volume of hot medium was dispensed into culture vessels, i.e. test tube or conical flasks. The culture vessels were plugged with non-absorbent

cotton and covered with aluminium foil and marked with the help of a glass marker to indicate specific hormonal supplements. The culture vessels were then autoclaved (EYELA AUTOCLAVE MAC_ 501, Japan) at 15 lb/sq. inch pressure at 121^0C temperature for 20 minutes.

2.2.4. Preparation of seed germination and seedling developing medium

To obtain cotyledonery leaves surface sterilized seeds were placed on MS basal medium containing 0.3% (0.3g/100 ml) Sigma band phytagel. The pH was adjusted to 5.8-6.0. The method of preparation and sterilization of this medium similar to the procedure described earlier.

2.2.5. Preparation of *Agrobacterium* culture medium

For the growth of *Agrobacterium tumefaciens* strain LBA4404, YMB medium was prepared with following constituents:

Mannitol	1%
Yeast extract	0.04%
$MgSO_4.7H_2O$	0.02%
NaCl	0.01%
$K_2HPO_4.3H_2O$	0.05%

The pH was adjusted to 7.0-7.2 with 1N HCl before adding the agar at 1.5% w/v. After autoclaving the medium waited to cool down to 50-55^0C and antibiotic kanamycin was added at a rate of 200µl for each 100 ml of medium to obtain final concentration 200mg/l. But for the preparation of maintenance culture medium (solid medium), same components of YMB were used with agar (1.5% w/v).

2.2.6. Preparation of co-culture medium

For co-cultivation (infected tomato cotyledonery explants with the *Agrobacterium*) best regeneration medium without any antibiotic were used.

2.2.7. Preparation of selection medium for selection pressure

Regeneration medium autoclaved and cool down at 50-55^{0}C and added Kanamycin at different concentration to determine the selection concentration. It was done inside laminar flow and separated into Petri dishes and wait till it becomes solidified.

2.2.8. Precautions to ensure aseptic conditions

All inoculation and aseptic manipulation were carried out in a Laminar Flow cabinet (ESCO; class II, Type R/B_3 Biohazard safety cabinet, Singapore). The cabinet was switched on for at least half an hour before use and cleaned with 70% alcohol to overcome the surface contaminations. The instruments like scalpels, forceps, inoculation loop, Petri dishes, micro-pipette tip, eppendrof tubes etc. were sterilized by steam sterilization methods. During the entire period of inoculation the scalpels, forceps and inoculation loop were kept immersed into absolute alcohol contained in a glass jar inside the cabinet. At the time of inoculation these were again sterilized by flaming method inside the cabinet. Both the hands were rained with 70% alcohol and dried. All measures were taken to obtain maximum contamination free condition during the surgical operation of the explants. After autoclaving bacterial media were poured inside the laminar air flow cabinet to avoid contamination. Antibiotics used for various media were filter sterilized and stored in sterile eppendrof tubes. Inside the laminar air flow cabinet antibiotics were added to the medium if it were required. After each transformation experiment, Agrobacterial suspension used and contaminated Petri dishes, cotton, filter papers, instruments; glass cuvettes were autoclaved

to destroy genetically engineered *Agrobacterium* as a part of biosafety procedure. Any contaminants and old bacterial cultures were also autoclaved before discarding them.

2.2.9. Culture techniques

The following culture techniques were employed in the present investigation:

1. Establishment of axenic culture
2. Explant culture and shoot regeneration
3. Subculture
4. Rooting
5. *Agrobacterium* culture
6. Infection and Incubation
7. Co-culture
8. Selection and Shoot regeneration

2.2.9.1. Establishment of axenic culture

Surface sterilization of the seeds was the first step of axenic culture. Seeds were immersed in 70% ethanol for 1 minute followed by 5.25% Clorox and treatment with or without 2 droops of tween 20 and shaken continuously for 5-8 minutes. Seeds were then washed with distilled water for three times respectively for 1 minute, 5 minutes and 10 minutes. Seeds were then kept in a rotator shaker (EDISON; NJ, U.S.A) for 24 -36 hr in 150 rmp at 28^0C while immersed in sterile distilled water.

Surface sterilized seeds were then placed on sterilized filter paper soaked with distilled water and kept in dark or in light at 25 ± 2^0C to test seed viability and the effect of light in germination. Some of those seeds were placed on the flasks containing solid MS basal medium, for seedling development.

2.2.9.2. Explant culture and Shoot regeneration

Explants were collected from seedlings for *iv- vitro* shoot regeneration. Each cotyledonary leaf was transversely cut into two to three segments and used as explants for multiple shoot regeneration. The explants were placed in different regeneration medium both in dorsal (underside of a leaf) and ventral (upper side of a leaf) position of cotyledonary leaf.

2.2.9.3. Subculture

Regenerated cultures were subcultured to fresh media containing the same hormonal supplement for further proliferation and development. Subculture was performed regularly at an interval of three to four weeks for maintenance. Cultures were routinely examined for different morphogenic development.

2.2.9.4. Rooting

Well developed shoots around 3-4 cm long, were placed individually in the rooting medium to obtain sufficient root formation.

2.2.9.5. *Agrobacterium* Culture

Two kinds of culture media were needed for genetically engineered *Agrobacterium tumefaciens* strain LBA4404. One media was used for maintaining *Agrobacterium* stock and the other for the infection of explants.

For maintenance, one single colony from previously maintained *Agrobacterium* stocks was streaked into freshly prepared Petri dish containing YMB medium having appropriate antibiotics. The Petri dish was sealed with parafilm and in culture room for at least 48 hours in the dark. These plates were then stored at 4^0C to check over growth and these strains were thus ready to use for liquid culture. The cultures were subcultured regularly at each week in freshly prepared media to maintain the stocks (Fig. 2.).

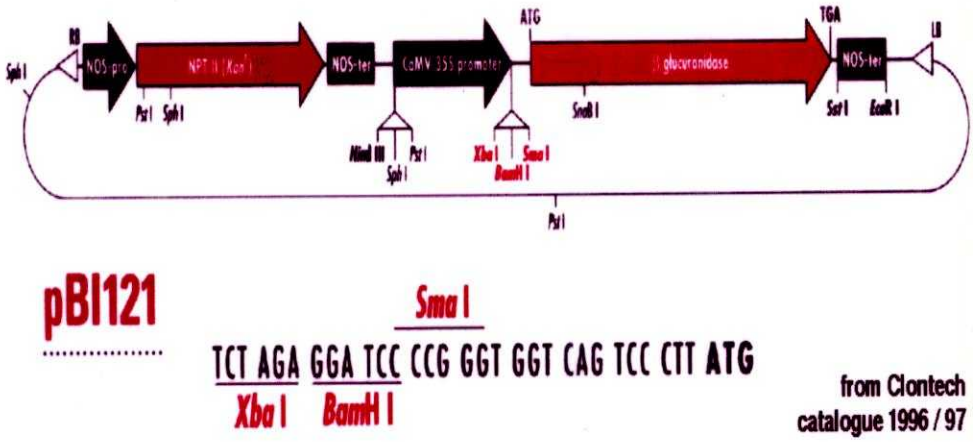


Fig. 1. Region between left (LB) and right (RB) border of the plasmid pBI121. This has been made by Clontech Laboratories Inc.USA.

Fig. 2. 48 hours old culture of *Agrobacterium tumefaciens* strain LBA4404 on YMB maintenance medium.

For infection, a single streak was taken from these *Agrobacterium* stocks with an inoculation loop and was inoculated in a conical flask containing liquid YMB medium with required antibiotics. This culture was kept on the shaker at 120 rmp. for one day and allowed to grow at 28^0C to get optimum population of *Agrobacterium* for infection and co-cultivation of explants.

2.2.9.6. Infection and Incubation

Overnight grown liquid *Agrobacterium* culture was used for infection and incubation. Prior to this Optical Density of the bacterial suspension was determined at 600 nm with the help of a spectrophotometer (Analytikjena Specord 50, Germany). Different OD were used in different experiment to determine optimum density of *Agrobacterium* culture. Following the determination of density, to get suitable and sufficient infection of the explants, cut explants were dipped in bacterial suspension for different incubation periods before transferring them to co-cultivation medium. During infection liquid MS medium were used as bacterial resuspension media.

2.2.9.7. Co-culture

Following infection and incubation, the explants were co-cultured in regeneration medium. Before transferring all the explants to co-culture media they were soaked in filter papers for a short period of time to remove excess bacterial suspension. All this explants were maintained in co-culture medium for 2-3 days. The Petri dishes were placed in the dark at 25 ± 2^0C temperature. The plates were checked daily to discard the contaminated plates and to note the behavior of the explants.

2.2.9.8. Selection and Shoot regeneration

During co-cultivation *Agrobacterium* used to grew on the culture medium. It is important to check such growth during the regeneration of infected explants. Appropriate concentration of antibiotics such as cefotaxime and

carbinicilin were added to the regeneration medium along with effective concentration of kanamycin to allow the transformed cells to regenerate.

2.2.9.9. Selection and rooting

The healthy shoots grown under selection pressure were transferred to the rooting media containing ½ or ¼ MS media with or without IAA or IBA.

2.2.10. Control experiment during transformation

As controls, non-infected explants were cultured in normal regeneration medium and after 2 weeks of shoot initiation it was subcultured in selection medium to detect the effect of selective agents on this control shoots. These controls were maintained with each set of transformation experiments to perform various comparative studies.

2.2.11. Transplantation

The plantlets with sufficient root system were taken out from the culture vessels and the roots were washed under running tap water. The plantlets were then transplanted to small pots containing grown soil, sand and cowdung in the ratio 1:2:1. Pots were then covered with transparent perforated polythene bags. Inner sides of these bags were moistened with water to prevent desiccation. To reduce sudden shock, the pots were kept in growth room for two weeks, of which polythene covers were maintained for the first week and without cover for the second week. These plantlets were exposed to natural environment for 2-8 hours daily and again placed in growth room for another week. Three weeks after transplantation, when the regenerated plants were fully established in the small pots, they were then transferred to large pots for further growth and to get fruits from those regenerated plants.

2.2.12. GUS (β-glucuronidase) histochemical assay

From each batch of explants following each transformation experiment, randomly selected co-cultured tissues (explants) were examined for GUS histochemical assay. For this experiment co-cultured explants were fixed in fixation solution by the help of vacuum infiltration for 3-5 minutes. The explants were then washed three times in 50 mM phosphate buffer and immersed in X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide) solution and incubated at 37^0C overnight. A characteristic blue color would be the expression of GUS (β-glucuronidase) gene in the plant tissue. Proper control for GUS histochemical assay was done with the explants having no *Agrobacterium* infection.

After X-gluc treatment explants were transferred to 70% alcohol for degreening. Following degreening explants were observed under stereomicroscope (Olympus, Japan). For confirmation slides were prepared with transformed and control tissues and were observed under microscope.

CHAPTER 3:
RESULT

The responses of four varieties of tomato (*Lycopersicon esculentum* Mill.), namely, Bahar (BR), Bina tomato 5 (B-5), Bina tomato 3 (B-3) and Pusa Ruby (PR) growing in Bangladesh, were investigated to develop *in vitro* regeneration and transformation protocol. The present investigation has been carried out in three phases. In the first phase, genotype independent *in vitro* regeneration protocol was tried to develop, and in the second phase, various factors affecting *Agrobacterium*-mediated transformation efficiency were evaluated to develop a transformation protocol. In the third and last phase, analysis of putative transgenic plants was carried out.

3.1. *In vitro* regeneration

The cotyledonary leaf explant, used for direct regeneration, was collected from aseptically grown seedlings following germination. Following successful shoot development, they were cultured for root formation. Regenerated plantlets were acclimatized in soil and were allowed to grow under field conditions to obtain flowers and fruits. Finally viability of seeds, collected from mature fruits of these regenerated plants was tested.

3.1.1. Surface sterilization and *in vitro* germination of seeds

These experiments were preformed to find out a proper procedure for surface sterilization of seeds to obtain maximum germination under *in vitro* condition. When seeds were tested for 5 mins in sodium hypochlorite solution, contamination was recorded to be 13%, 12%, 10% and 12% in BR, B-5, B-3 and PR varieties, respectively. Increase of Clorox ™ treatment though decreases the contamination rate to 0 %, but this was achieved in compensation of lower rate of germination in all the varieties (Fig. 3). However, when surface sterilization was conducted with two drops of Tween-20 in Clorox ™ and few hours shaking in sterile distilled water after

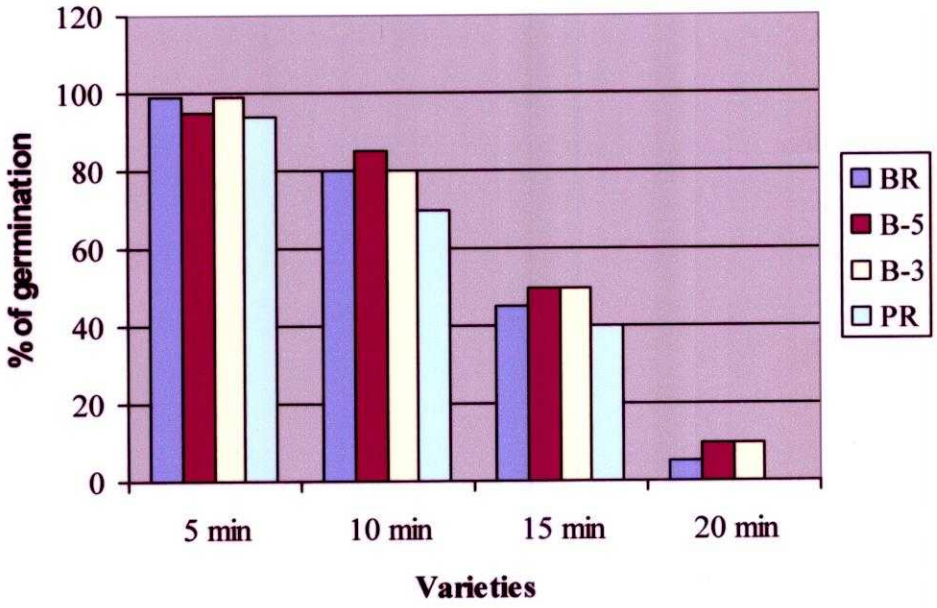


Fig. 3. Effect of various time periods of Clorox treatment on germination percentage of four varieties of tomato viz. BR, B-5, B-3 and PR.

sterilization, contamination was reduced to 0% while attaining almost cent percentage germination in all four varieties (Table 3.1.).

Table 3.1. Effect of Tween-20 and continuous shaking for decontamination of four varieties of tomato seeds.

Varieties	Tween-20 addition	Shaking with DDH_2O	% of contamination
BR	-	-	13
	+	-	1
	-	+	6
	+	+	0
B-5	-	-	12
	+	-	0
	-	+	6
	+	+	0
B-3	-	-	10
	+	-	2
	-	+	6
	+	+	0
PR	-	-	12
	+	-	1
	-	+	6
	+	+	0

Note: + = present; – = absence. Total number of seeds per experiments 100 in all cases. Each experiment was conducted 15 times.

3.1.2. Seedling development

In the earlier study it was observed that germination percentage increase when sterilized seeds were agitated in distilled water for few hours. To minimize time requirement effects of light and agitation (shaking) period on germination was further tested in the present study.

It was observed that both darkness and agitation reduced the time required for germination in all the varieties. The percentage of germination for the variety B-3 was found to be highest (96%) compared to other varieties and

the time required for germination was also lower for B-3 than the others. Lowest germination percentage (70%) and the highest time requirement for germination were found in PR variety (Table 3.2.).

After germination seedlings were allowed to develop under light. It was observed that seeds of BR, B-5, B-3 and PR varieties took 3-4, 3-4, 2-4 and 4-6 days, respectively, to germinate and all the four varieties took another 5-8 days to attain the maturity to collect cotyledonary leaf explants.

Table 3.2. Effects of light and agitation on time requirement for germination in all four tomato varieties (BR, B-5, B-3 and PR).

Varieties	Agitation duration following sterilization (hrs.)	% of seed germination	Days required for germination	
			Dark	Light
BR	24-36	65	5-6	6-7
	36-48	66	2-3	4-5
	48-60	71	2-3	3-4
B-5	24-36	87	5-6	6-7
	36-48	90	4-5	5-6
	48-60	92	3-2	5-4
B-3	24-36	91	4-5	5-6
	36-48	95	3-4	4-5
	48-60	96	2-3	3-4
PR	24-36	60	5-6	7-8
	36-48	64	4-5	6-7
	48-60	70	3-5	4-5

Note: A total of 100 seeds were treated per experiment.

3.1.3. Determination of suitable medium for *in vitro* regeneration of shoots

These experiments were performed to determine suitable MS medium supplemented with different concentrations of auxins and cytokinins for *in*

vitro shoot regeneration. In the present study, effect of different concentrations of hormonal combinations on regeneration initiation and numbers of shoots formation per explants was observed.

3.1.3.1. Response of cotyledonary leaf explant towards multiple shoot regeneration using different concentrations of BAP in MS medium for BR, B-5, B-3 and PR tomato varieties

MS medium supplemented with different concentrations of BAP (1.0-7.0 mg/l) were used for induction of multiple shoots using cotyledonary leaf explant. Results of this experiment are presented in Table 3.3. It was found that BAP had a positive effect towards multiple shoot regeneration. When explants were inoculated on MS medium without BAP supplementation, either no response or development of roots directly from the explants was observed (Fig. 4). In all varieties presence of low concentration of BAP (0.5 mg/l) shoot regeneration occurred following callus formation (Fig. 5). However, with the increase of BAP, shoots found to regenerate directly from the explants. Highest numbers of shoots per explant were obtained on MS medium supplemented with 2.0 mg/l BAP in all the varieties (Fig. 6). It was observed that in B-5 and B-3 number of shoots per explant increased with the increase of BAP concentrations. But after subculture in the same media (BAP 5.0-7.0 mg/l), most of the shoots showed abnormal morphology such as abnormal leaf formation, branching and vetrifications (Fig. 7).

Table 3.3. Response of BR, B-5, B-3 and PR explants towards multiple shoot regeneration using different concentrations of BAP in MS medium.

Varieties	Concentration of BAP (mg/l)	Total no. of explants inoculated	% of responsive explants	Time required for initiation of regeneration (Days)	No. of shoots/explants after 40-50 days of culture
BR	1.0	15	73	15-16	4-5
	2.0	11	91	12-16	5-6
	3.0	25	72	12-16	3-4
	5.0	16	69	12-16	3-4
	7.0	18	50	15-20	1-2
B-5	1.0	10	60	10-12	7-8
	2.0	7	86	10-15	6-8
	3.0	16	75	10-12	3-4
	5.0	9	89	15-17	2-3
	7.0	9	33	15-17	1-2
B-3	1.0	12	62	10-15	4-6
	2.0	12	83	10-12	6-10
	3.0	12	91	15-18	4-5
	5.0	17	77	15-18	5-4
	7.0	13	48	12-15	3-4
PR	1.0	12	83	15-20	3-4
	2.0	14	93	12-15	4-5
	3.0	15	67	16-20	4-5
	5.0	12	75	15-20	2-3
	7.0	15	53	15-20	1-2

3.1.3.2. Effects of various combinations and concentrations of BAP and auxins on regeneration and development of multiple shoots in BR, B-5, B-3 and PR varieties

In the present experiment, effect of IAA was also tested in the shoot regeneration medium. It was found that MS medium supplemented with only IAA had no positive effect towards shoot regeneration. In MS medium supplemented with 1.0 mg/l IAA, explants of all four varieties formed roots from every cut ends. Roots were very well developed and long (Fig. 8).

When MS medium was supplemented with different concentrations of BAP in presence of 0.1 mg/l IAA, almost similar type shoot initiation responses were observed in all four varieties (Table 3.4.). Maximum number of multiple shoots were obtained in MS media supplemented with 2.0 mg/l BAP and 0.1 mg/l IAA. Though 2.0 mg/l BAP with 0.1 mg/l IAA gave better response, subculture in the same media lead to slower growth and elongation. So, 1.0 mg/l BAP and 0.1 mg/l IAA combination in MS media found to be the best among all the media tried. Multiple shoots regenerated on this medium is presented in Fig. 9. On the other hand, increase of IAA concentration (1.0 mg/l) in presence of BAP (1.0 mg/l) on MS media showed negative impact on shoot regeneration (Table 3.4.).

When only 0.1 mg/l NAA was used callusing was observed on the cut surfaces of explants for all the varieties (Fig.10). Addition of 0.1 mg/l BAP with 0.1 mg/l NAA resulted formation of non-regenerative callus (Fig. 11).

3.1.4. Position effect of explants on regeneration efficiency

Cotyledonary leaf explants were placed in the medium both in dorsal and ventral position. Dorsal position showed maximum response in shoots initiations (Figs 12-13). It was also observed that regeneration could occur from one or both the cut ends of cotyledonary leaf segments (Fig. 14-15).

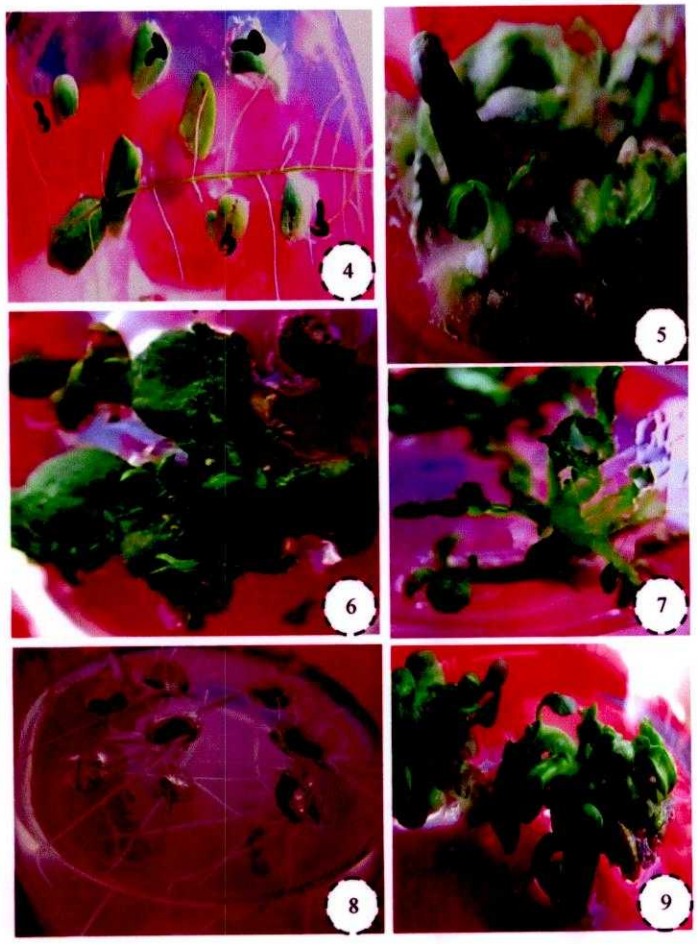


Fig. 4. Root formation from the cotyledonary leaf explants of B-3 in MS medium without hormonal supplementation.
Fig. 5. Shoot initiation from callus following culture in MS medium containing 0.5 mg/l BAP in B-3.
Fig. 6. Direct shoot initiation from cotyledonary leaf explants of B-3 in MS medium supplemented with 2 mg/l BAP after 15 days.
Fig. 7. Abnormal morphology of regenerated shoots in MS medium having 5.0 mg/l BAP in B-3 after 21 days.
Fig. 8. Root formation at every cut ends of cotyledonary leaf explants of B-5 cultured in MS medium containing 1.0 mg/l IAA after 28 days.
Fig. 9. Multiple shoot regeneration in B-5 on 1.0 mg/l BAP and 1.0 mg/l IAA supplemented MS medium. Photograph had taken after 28 days.

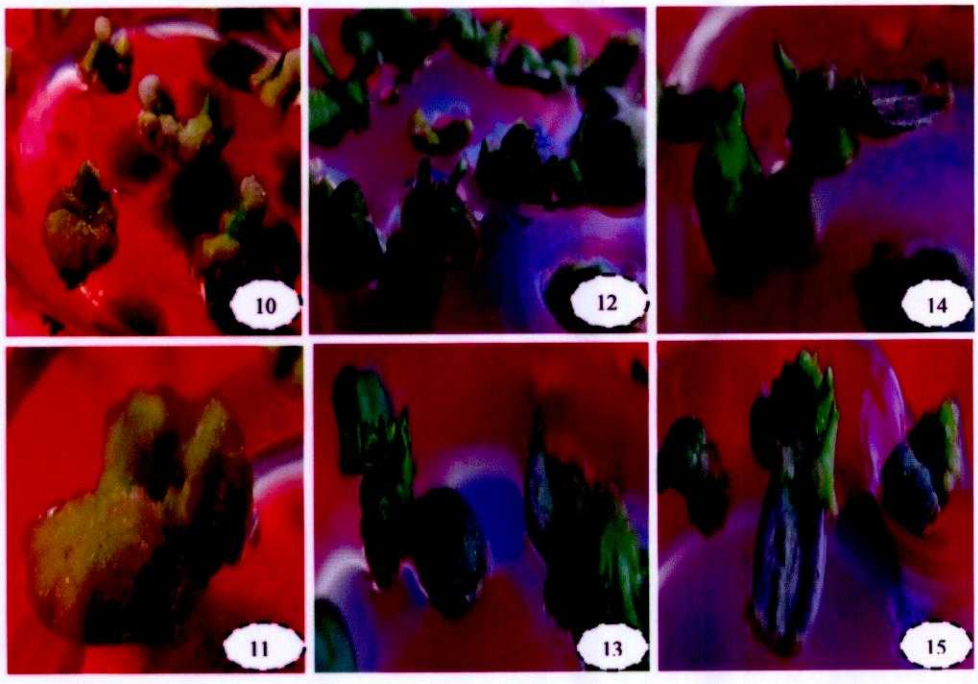


Fig. 10. Callusing on cut ends of the explants cultured on MS medium supplemented with only 1.0 mg/l NAA in B-5.
Fig. 11. Non-regenerative callus formation in B-5 variety when cultured on 0.1 mg/l BAP and 0.1 mg/l NAA supplemented MS medium.
Fig. 12. Cotyledonary leaf explants of BR on regeneration medium at ventral position.
Fig. 13. Same as Fig. 12. in case of PR.
Fig. 14. Cotyledonary leaf explants of BR on regeneration medium at dorsal position showing regeneration initiation from the both cut ends.
Fig. 15. Same as Fig. 14 in case of PR.

Table 3.4. Response of explants of BR, B-5, B-3 and PR towards multiple shoot regeneration using different concentrations of BAP with IAA in MS media.

Varieties	Hormonal Concentrations (mg/l)		No. of explants inoculated	% of responsive explants	Days to regeneration initiation	No. of shoots/ explants
	BAP	IAA				
BR	1.0	0.1	12	67	18-20	5-6
	2.0	0.1	25	80	18-20	6-10
	5.0	0.1	22	77	15-17	3-4
	1.0	1.0	10	60	20-22	3-4
B-5	1.0	0.1	12	75	15-18	7-8
	2.0	0.1	12	83	15-18	7-9
	5.0	0.1	12	83	18-20	2-3
	1.0	1.0	10	40	15-17	2-3
B-3	1.0	0.1	9	66	15-18	6-8
	2.0	0.1	11	91	12-15	6-9
	5.0	0.1	13	92	18-20	2-3
	1.0	1.0	9	49	18-20	4-5
PR	1.0	0.1	12	67	18-20	5-6
	2.0	0.1	8	75	10-12	7-6
	5.0	0.1	12	58	15-18	3-4
	1.0	1.0	10	30	18-20	4-5

3.1.5. Sub-culture of regenerated shoots

In the present study, for elongation of regenerated shoots subculture was performed in every 3-4 weeks and was made on the same hormonal condition that was used for regeneration initiation. (Figs 16-19).

3.1.6. Induction of roots from regenerated shoots

Root formation is an essential step to produce plantlets. *In vitro* regenerated shoots did not produce root spontaneously. So, the elongated shoots (3-4 cm) were excised and cultured to produce roots.

In the present experiment, half strength of MS medium with or without different concentrations and combinations of auxins such as IAA, IBA and NAA were used to select suitable medium for *in vitro* root formation for all

Fig. 16. Multiple shoot regeneration at the cut end of B-3 explants after 3 weeks, culturing on MS medium containing 2 mg/l BAP.
Fig. 17. Same as Fig. 16. in case of BR.
Fig. 18. Elongated shoots in B-3 after sub-cultured in the same regeneration medium.
Fig. 19. Same as Fig. 16. in case of BR.

four varieties of tomato. Results of these observations are presented in Table 3.5. All three auxins showed positive response towards healthy, well-developed roots. In case of IAA thin long roots were found to initiate from the cut ends at the base of shoots. Among the three concentrations of IAA tested, 0.2 mg/l found to be the best. In case of IBA, similar types of root were obtained in all concentrations. The root number was less than what was observed in presence of IAA. The root growth in IBA supplemented media was slow and sometime found to develop callus before root initiation at the shoot base. In presence of NAA two types of roots were found: thin elongated long (tap root system) and short (bushy) type (fibrous root system). There was no variation among the type of response in four tomato varieties. Effects of different auxins in the development of roots from regenerated shoots are presented in Figs 20-29.

Table3.5. Effects of different concentration of auxins (IAA, IBA and NAA) in half strength of MS (½ MS) medium on rooting in BR, B-5, B-3, and PR.

Varieties	Concentrations of (mg/l)			No. of shoots inoculated	Days required for root initiation	% of root producing shoots	Type of root	Roots/ shoot
	IAA	IBA	NAA					
BR	0.1	-	-	10	5-6	90	L +	15-20
	0.2	-	-	10	5-6	100	L ++	15-18
	0.3	-	-	9	6-7	78	L +	15-18
	-	0.1	-	12	7-10	92	L +	8-10
	-	0.2	-	12	6-8	100	L +	10-12
	-	0.3	-	15	8-10	93	L +	10-12
	-	-	0.1	10	7-8	90	L TRS +	10-12
	-	-	0.2	10	6-7	100	S FRS	12-15
	-	-	0.3	10	7-8	100	S FRS	12-15

Varieties	Concentrations of (mg/l) IAA	IBA	NAA	No. of shoots inoculated	Days required for root initiation	% of root producing shoots	Type of root	Roots/ shoot
B-5	0.1	-	-	10	6-7	80	L +	7-8
	0.2	-	-	9	6-7	89	L + +	8-10
	0.3	-	-	10	5-6	90	L +	6-8
	-	0.1	-	12	5-7	92	L +	6-8
	-	0.2	-	10	5-7	80	L +	5-8
	-	0.3	-	8	6-8	88	L +	7-8
	-	-	0.1	10	5-7	90	L TRS +	8-10
	-	-	0.2	10	6-7	90	S FRS	12-15
	-	-	0.3	12	5-7	83	S FRS	12-15
B-3	0.1	-	-	8	5-6	88	L+	6-10
	0.2	-	-	11	6-7	100	L ++	9-12
	0.3	-	-	10	6-7	80	L +	8-10
	-	0.1	-	10	5-8	90	L +	6-9
	-	0.2	-	15	6-8	87	L +	5-8
	-	0.3	-	10	5-6	90	L+	6-8
	-	-	0.1	10	6-8	80	L TRS +	7-9
	-	-	0.2	15	6-9	87	S FRS	10-15
	-	-	0.3	10	6-9	90	S FRS	10-15
PR	0.1	-	-	8	8-10	88	L +	8-10
	0.2	-	-	9	6-8	89	L+ +	8-15
	0.3	-	-	10	6-7	80	L+	8-10
	-	0.1	-	10	7-8	90	L +	8-10
	-	0.2	-	10	6-8	90	L +	6-8
	-	0.3	-	10	6-7	80	L +	6-8
	-	-	0.1	12	7-8	75	LTRS +	6-8
	-	-	0.2	10	8-10	80	S FRS	8-12
	-	-	0.3	10	6-8	90	S FRS	12-15

Note: Long = L, Short = S, Fibrous root system = FRS, Tap root system = TRS, Good = +, Very good = ++

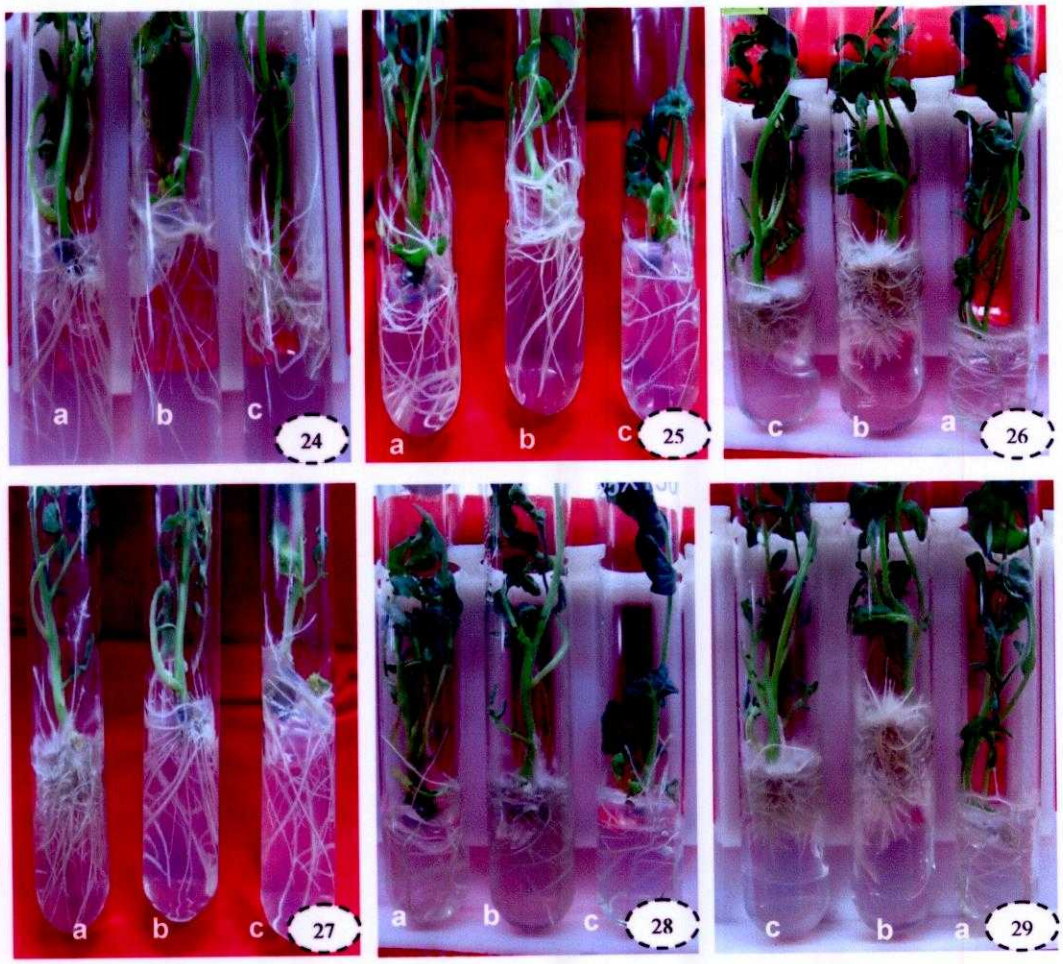
Different stages of root formation in B-3

Fig. 20. Root formation initiation (a), elongation (b) and matured root (c) at the shoot base on ½ MS media supplemented with 0.1 mg/l IAA.

Fig. 21. Same as Fig. 20. in case of ½ MS media supplemented with 0.2 mg/l IAA .

Fig. 22. Same as Fig. 20. in case of ½ MS media supplemented with 0.3 mg/l IAA.

Fig. 23. Rooting at the shoot base when cultured on ½ MS media supplemented with 0.1 (i), 0.2 (ii) and 0.3 (iii) mg/l IBA. Photograph taken 16 days after inoculation.



Matured roots formed at the shoot base of B-5
Fig. 24. Roots formed on ½ MS media supplemented with 0.1 (a), 0.2 (b) and 0.3 (c) mg/l IBA.
Fig. 25. Roots formed on ½ MS media supplemented with 0.1 (a), 0.2 (b) and 0.3 (c) mg/l IAA
Fig. 26. Roots formed on ½ MS media supplemented with 0.1 (a), 0.2 (b) and 0.3 (c) mg/l NAA.
Matured roots formed at the shoot base of PR
Fig. 27. Roots formed on ½ MS media supplemented with 0.1 (a), 0.2 (b) and 0.3 (c) mg/l IAA.
Fig. 28. Same as Fig. 27. in case of IBA.
Fig. 29. Same as Fig. 26. in case of NAA.
Photographs taken of 28 days old cultured plantlets.

3.1.7. Establishment and performance of plantlets in natural environment

Healthy, well-developed rooted plantlets of all four tomato varieties were successfully transplanted into small plastic bags containing soil. Roots that were formed in IAA survived well in the soil than of IBA and NAA where callus or fibrous roots were regenerated. Following transplantation, survival rate of the regenerated plantlets was found to be highest (92%) in B-3 while lowest (66%) in PR. Plantlets of BR, B-5, B-3 and PR transplanted in the small plastic pots containing soil are shown in Figs 30-31, respectively. Following proper acclimatization, the plantlets were transferred to field or larger pots for their further growth. The survival rates of plantlets in the larger pots or in the field were found to be cent percent. The survival performances of plantlets following their transplantation are presented in Table 3.6.

These plants flowered within 5-7 weeks after transferring to the field or large pots(Figs 32-33). It took another 4-6 weeks to obtain mature fruits (Figs 34-40). Time required for *in vitro* regeneration, plantlet development, flowering and fruit setting is presented in Table 3.7.

Table 3.6. Performance of plantlets of four varieties of tomato following transplantation.

Varieties	No. of rooted shoot transplanted	No. of plantlets survived*	% of plantlets survived	No. of plants transferred to field	% of plants survived
BR	40	30	75	30	100
B-5	30	24	83	24	100
B-3	50	46	92	46	100
PR	30	20	66	20	100

Note: * Survibility found to be influenced by the nature of the regenerated roots.

Fig. 30. Regenerated plantlets of BR, B-5 and B-3 transplanted into soil in small poly bags.
Fig. 31. Transplanted plantlets of PR.
Fig. 32. Flower buds (arrow) initiation (after 30 days) on regenerated plantlets of PR variety.
Fig. 33. Flower blossom (arrow) in PR plantlets in the natural environment.
Fig. 34. Fruit sets on *in vitro* regenerated plantlets of BR variety.
Fig. 35. Same in case of B-5 variety.

Fig. 36. Fruit sets on *in vitro* regenerated plantlets of B-3 variety.
Fig. 37. Same in case of PR variety.
Fig. 38. Matured fruits on regenerated BR plantlets.
Fig. 39. Same in case of B-5 plants.
Fig. 40. Same in case of B-3 plants.
Fig. 41. Germination test to check the viability of seeds collected from fruits of regenerated plantlets of B-3. Note that all the seeds germinated under test condition.

Table 3.7. Time required for plantlet development, flowering and fruit setting for all four (BR, B-5, B-3 and PR) tomato varieties.

Varieties	Days required							
	Seedling development	Regeneration Initiation	Matured developed shoots (weeks)	Initiation of roots (days)	Fully developed roots (weeks)	aft wering ransplantation eks)	Fruit setting (days)	Fruits maturation (weeks)
BR	10-12	12-16	5-7	5-6	3-4	4-6	12-15	4-8
B-5	10-12	10-15	5-6	6-7	3-5	3-5	10-15	4-8
B-3	8-10	10-12	5-6	6-7	13-4	3-5	10-15	4-8
PR	10-15	12-15	5-7	6-8	3-5	4-6	10-15	4-8

3.1.8. Viability of seeds collected from regenerated plantlets

The seeds of all the varieties collected from fruits of regenerated plantlets were found viable in the viability test. Here BR, B-5, B-3 and PR showed 90-99%, 95-100%, 98-100% and 90-95% viability in the germination test. The response of these *in vitro* regenerated plant seeds was found almost identical to the natural seeds that were used in the present study (Fig. 41).

3.2. *Agrobacterium*-mediated genetic transformation

In this phase, a suitable protocol for *Agrobacterium*-mediated genetic transformation of tomato was tried to establish. For this purpose, a genetically- engineered *Agrobacterium* strain was used to transform cotyledonary leaf explants of BR, B-5, B-3 and PR varieties of tomato. In this experiment different parameters of transformation were optimized to obtained transgenic plants.

3.2.1. Determination of specific *Agrobacterium*-tomato cultivars (host) compatibility and efficiency of *Agrobacterium* strain LBA4404 towards transformation

In this investigation, genetically-engineered *Agrobacterium* strain LBA4404 was used for transformation experiments to test its compatibility with BR, B-5, B-3 and PR varieties of tomato. Following transformation experiments transgene transfer was monitored by histochemical assay of the GUS reporter gene in randomly selected 10-15% of the infected explants.

All the four tomato varieties were found to be compatible with the *Agrobacterium* strain LBA4404. GUS positive (blue colored) regions were detected at the cut ends of the cotyledonary leaf explants (Figs 42-43). In most of the cases the entire cut surface of the explants was found to be blue following GUS transient assay (Figs 44-45). Control plants (non-infected explants) were not found blue in this experiment (Fig. 46). Moreover, GUS positive regions were found on the epidermal layer as well as within the tissue underneath the epidermis (Fig. 47). Histochemical staining of the cotyledonary leaf explants indicated the presence of numerous blue cells near vascular tissue (Fig. 48). Characteristic blue colors were also detected in the internal tissues as well as on the leaf hairs of the infected explants (Figs 49-50). Following GUS histochemical assay, it was revealed that all the four varieties showed positive response towards transformation. Among all four varieties, B-3 showed highest transformation ability (96.7%), whereas BR, B-5 and PR showed 83.3%, 93.3% and 93.3%, respectively, GUS positive explants. Thus, all varieties were found to be compatible towards the *Agrobacterium* strain LBA4404 (Fig. 51).

3.2.2. Influence of optical density of *Agrobacterium* suspension on transformation

The relationship between optical densities of *Agrobacterium* suspension (OD_{600}) and transformation efficiency of cotyledonary leaf explants was investigated. Bacterial suspensions with optical densities of 0.79, 0.64 and

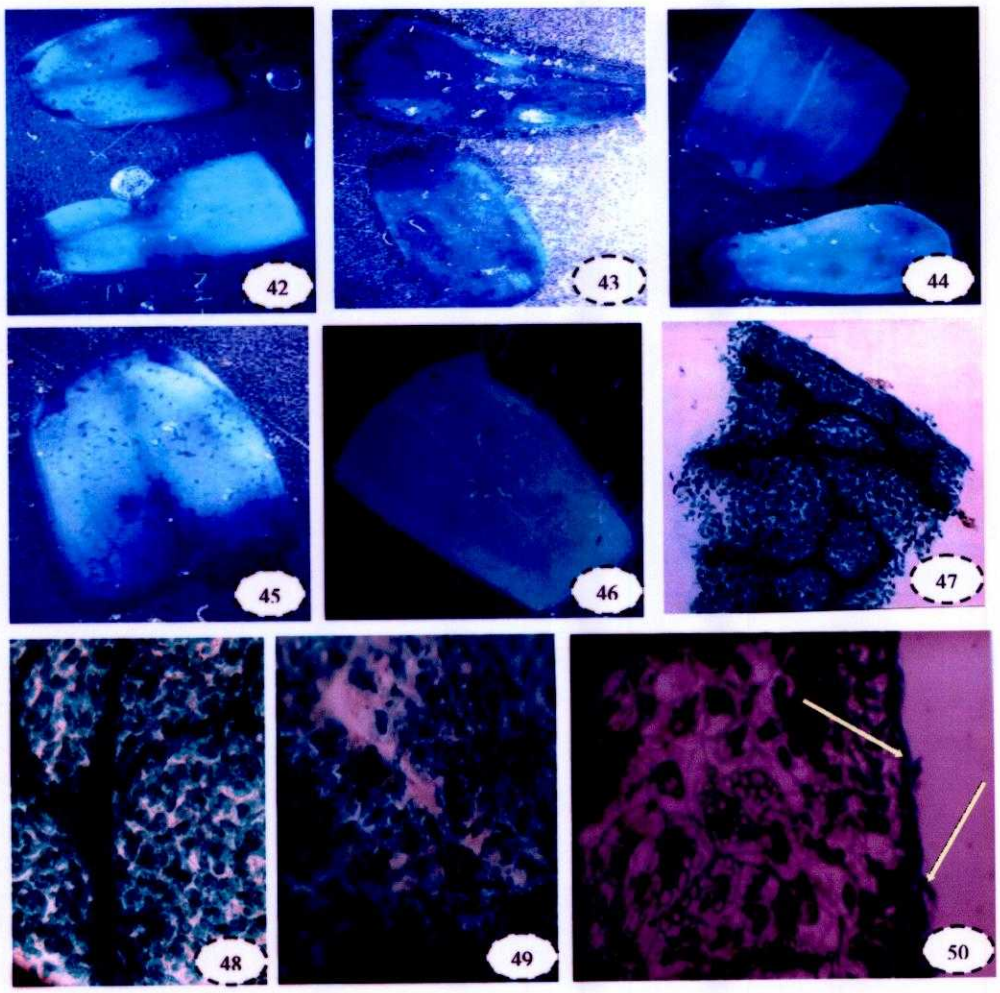


Fig. 42. Histochemical localization of GUS activity (blue zone) at the entire cut surface of cotyledonary leaf explants of BR (× 6).
Fig. 43. Same in case of B-5 (× 6).
Fig. 44. Same in case of B-3 (× 8).
Fig. 45. Same in case of PR (× 8).
Fig. 46. Same in case of control PR (× 8).
Fig. 47.Characteristic GUS positive blue color found within the tissue underneath the epidermis of the cotyledonary leaf explant of B-3 (× 45).
Fig. 48. Cotyledonary leaf explants of B-3 indicated the presence of numerous blue cells near vascular tissue (× 170).
Fig. 49. Magnified view of Fig. 48 (× 245).
Fig. 50. Characteristic blue color was detected in the internal tissues as well as leaf hairs (arrows) (× 45).

0.42 were used in these experiments. For the cotyledonary leaf explants of all the four varieties, transformation efficiency was found to be increased with the increase of optical density of the *Agrobacterium* suspension. Through histochemical experiment it was deducted that maximum percentage of transformation was observed at OD_{600} of 0.79 while minimum percentage was at OD_{600} of 0.42 in all varieties (Fig. 52). For B-3, at OD_{600} of 0.79 maximum transformation percentage was found 96% GUS positive. Similar transformation efficiency was recorded (90-94%) in B-5, BR and PR varieties at the same condition. For BR and PR varieties minimum 40% transformation efficiency (GUS positive) were found at 0.42 OD_{600} whereas 50% GUS positive were found for B-3 and B-5 varieties.

3.2.3. Influence of incubation period on transformation of cotyledonary leaf explants of four tomato varieties (BR, B-5, B-3 and PR)

In this experiment, effect of different incubation periods of cotyledonary leaf explants within the *Agrobacterium* suspension were investigated. The incubation period was found to influence *Agrobacterium*-mediated transformation efficiency in all four varieties. Different incubation periods ranging from 10 to 20 minutes were applied using OD_{600} nm of 0.8. In transient assay 100% of transformation (GUS +ve) was observed when the explants were incubated for 15 minutes in case of BR and B-3 (Table 3.8.). In the same condition, B-5 and PR also showed highest 92% and 90% GUS positive explants, respectively. Minimum (63%) GUS positive response was found for 20 mins incubation period in B-5 when the other varieties showed minimum of 71-77% GUS positive response in the infected explants. It was interesting to see that the percentage of transformation was increased with the increase of incubation period up to a certain time. However, increase of incubation period further led to decrease in transformation efficiency.

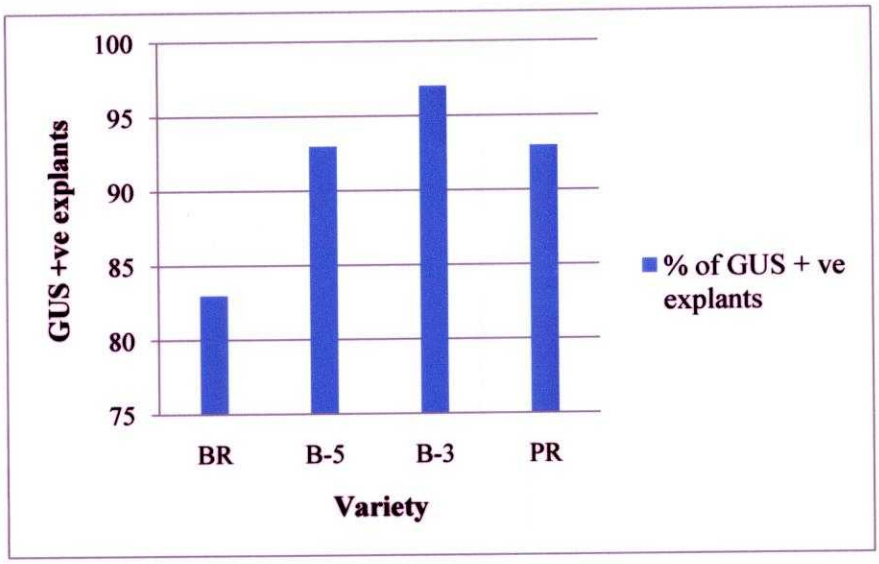


Fig. 51. Results of transient GUS assay following infection of cotyledonary leaf explants of the all four varieties (BR, B-5, B-3 and PR) of tomato using LBA4404 strain of *Agrobacterium tumefaciens.*

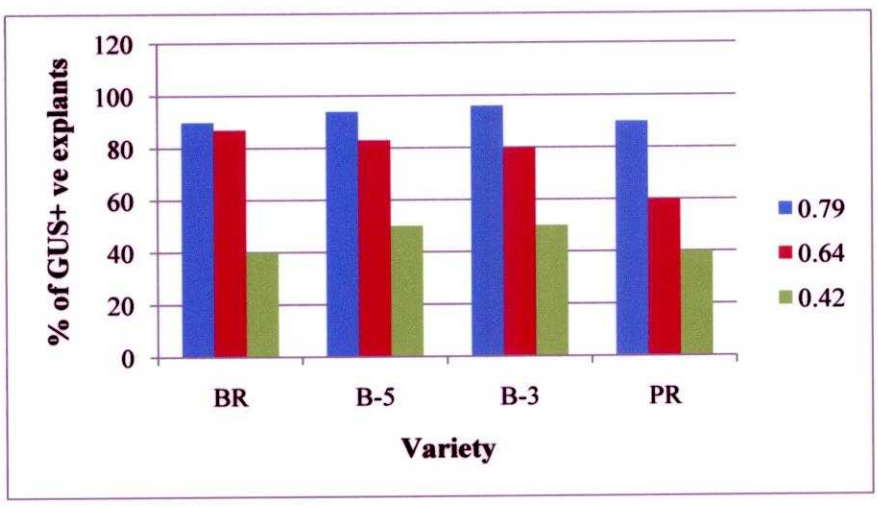


Fig. 52. Influence of optical density (OD_{600}) of *Agrobacterium* suspension on transformation efficiency analyzed by transient GUS histochemical assay for all the four tomato varieties (BR, B-5, B-3 and PR).

Table 3.8. Effect of different incubation periods on *Agrobacterium*-mediated genetic transformation of cotyledonary leaf explants of tomato varieties (BR, B-5, B-3 and PR) analyzed through transient GUS histochemical assay.

Varieties	Incubation period (min)	No. of explants assayed for GUS expression	% of explants +ve to GUS expression
BR	10	10	90
	15	9	100
	20	7	71
B-5	10	8	88
	15	12	91
	20	8	63
B-3	10	9	89
	15	10	100
	20	9	78
PR	10	9	78
	15	10	90
	20	8	75

Note: Total of 20 explants was used in each treatment.

3.2.4. Influence of pre-culture on transformation efficiency of cotyledonary leaf explants of four tomato varieties (BR, B-5, B-3 and PR)

Pre-culture is another factor which might influence the transformation efficiency and regeneration following infection in tomato. In this experiment, cotyledonary leaf explants of all varieties were pre-cultured for 4-6 days in regeneration medium before infection with *Agrobacterium* suspension. Pre-cultured and freshly collected explants were incubated for 10-15 mins in bacterial suspension having an OD_{600} 0.8 to find out the significance of pre-culture on transformation efficiency. It was found that there was no influence of per-culture in transformation efficiency in all four varieties (Figs 53-56). But after infection, pre-cultured explants regenerated faster and performed better than the non-pre-cultured explants (Table 3.9.).

Fig. 53. Cotyledonary leaf explants of B-3 placed on selection media following transformation. The explants were subjected to pre-culture on regeneration media before infection with *Agrobacterium tumefaciens.*
Fig. 54. Non- pre-cultured cotyledonary leaf explants of B-3 on selection media after infection. Note that explants are healthier in Fig. 53. than that in this photograph which may be due to pre-culture.
Fig. 55. Regeneration initiation from infected explants of B-3 which were subjected to pre-culture on selection media.
Fig. 56. Same as Fig. 5, but the explants were not give any pre-culture. Note that following transformation the no. of shoots and regeneration is slower than pre-cultured explants (Fig. 55).

Table 3.9. Influence of pre-culture on transformation efficiency of cotyledonary leaf explants of four tomato varieties (BR, B-5, B-3 and PR).

Varieties	% of GUS +ve explants		Days required for regeneration initiation	
	Pre-cultured	Non pre-cultured	Pre-cultured	Non pre-cultured
BR	99	100	15-20	20-30
B-5	90	92	15-20	20-30
B-3	100	100	15-20	20-30
PR	90	90	15-20	20-30

Note: Total of 20 explants used in each case.

3.2.5. Influence of co-cultivation period on transformation of cotyledonary leaf explants of four tomato varieties (BR, B-5, B-3 and PR)

Duration of co-cultivation was found to influence the transformation efficiency and subsequent regeneration capacity. It was found that, percentage of transformation could be increased with the increase of co-cultivation period maintaining constant OD_{600} (0.79 or 0.64) of bacterial suspension and a constant incubation period (15 mins). Correspondingly the transformation percentage was found to decrease with the decrease of co-cultivation period (Table 3.10.). In addition to this, more than 3 days of co-cultivation found to promote overgrowth of bacteria on the infected explants of all four varieties. For this reason a good number of explants cultured for more than 3 days in co-culture media found to suffer from poor health showing browning at the cut surfaces. Finally, these explants failed to regenerate. From this experiment it was found that 3 days of co-cultivation was the best condition for obtaining maximum transformation without sacrificing the regeneration ability of the infected tissue.

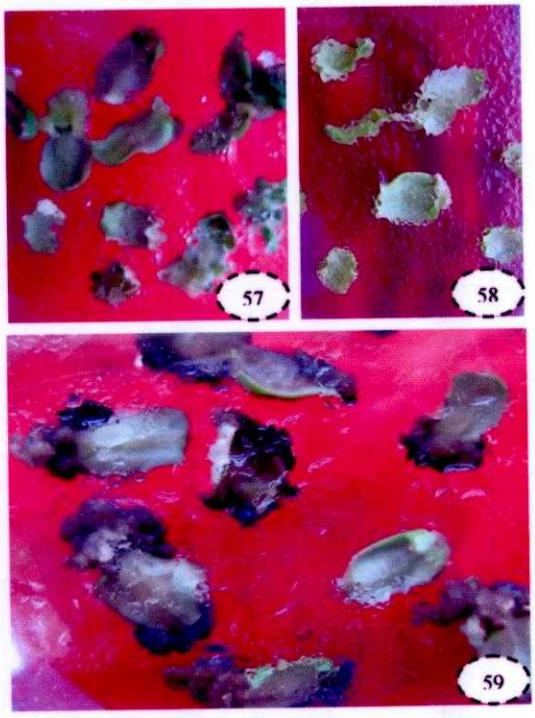
Table 3.10. Influence of co-cultivation periods on transformation of cotyledonary leaf explants of four tomato varieties (BR, B-5, B-3 and PR).

Variety	OD_{600} of bacterial suspension	Co-culture period (hrs)	No. of explants Infected	No. of explants assayed for GUS expression	% of GUS + ve explants
BR	0.76	72	30	10	90
		48	15	5	80
	0.64	72	20	8	88
		48	10	5	40
B-5	0.76	72	40	18	95
		48	15	10	70
	0.64	72	30	12	83
		48	20	6	50
B-3	0.79	72	40	25	96
		48	15	10	70
	0.64	72	35	10	80
		48	30	8	50
PR	0.79	72	20	10	90
		48	15	10	70
	0.64	72	10	5	60
		48	10	5	40

3.2.6. Determination of kanamycin concentration in selection media for cotyledonary leaf explants of tomato

In this investigation the response of non-transformed cotyledonary leaf explants of the variety B-3, towards different concentrations (50,100,150,200 and 300 mg/l) of kanamycin was evaluated. Sensitivity of the explant tissue was compared with control having no kanamycin.

It was found that, in the control set of experiment (without kanamycin), all the incubated explants survived and found to regenerate. It was also found that the survival percentage of the explants decrease gradually with the increase of kanamycin concentration in the regeneration media. In 150 mg/l kanamycin the explants were found to be albino and in 200 mg/l or more kanamycin they were found to turn deep brown and finally died (Figs 57-59).



Effect of different concentrations of kanamycin on cotyledonary leaf explants.
Fig. 57. Control cotyledonary leaf explants remain green and regenerate in the regeneration media having no kanamycin.
Fig. 58. Cotyledonary leaf explants become albino at 150 mg/l kanamycin containing regeneration media.
Fig. 59. In regeneration media containing 200 mg/l kanamycin cotyledonary leaf explants failed to regenerate.
Photographs taken two months after inoculation.

All the non-transformed explants in presence of 200 mg/l kanamycin failed to survive within 20 days.

Therefore, the optimum kanamycin concentration was found to be 200 mg/l for the selection of transformed shoots. Shoots, survived in this selection pressure for more than 15 days, were considered as transformed.

3.3. Transgenic tomato plant regeneration and analysis

In the third and last phage, attempts were made to obtain transgenic tomato plants using the optimized parameters and to confirm transgenic status following GUS histochemical assay. Following transformation the putative transgenic plantlets were subjected to GUS histochemical assay to confirm transgenic status before transferring to soil. These T_0 plants flowered and gave fruits which again checked through GUS assay to analyze the transformation status.

For these experiments, cotyledonary leaf explants of B-3 tomato were infected with *Agrobacterium tumefaciens* strain LBA4404 having an OD_{600} 0.8, the infection period 15 mins and was co-cultivated for 3 days.

3.3.1. Regeneration of putative transformed shoots

To support regeneration of transformed shoots explants were subjected to selection medium (MS +2.0mg/l BAP +200mg/l kanamycin) immediately after transformation. To control Agrobacterial growth 50 mg/l cefotaxime and 100 mg/l carbenicillin was also added to the medium. Shoot regeneration initiated in these explant within 20-30 days. Following subculture in the same medium green shoots were found to elongate. However, some green shoot died after browning (Figs 60-61). Thus, shoots survived in this selection pressure for more than 15 days, were considered as putatively transformed.

3.3.2. Effect of kanamycin on root induction from transformed shoots

In this investigation suitable media for root formation after transformation were determined. IAA or IBA supplemented ½ or ¼ strength of MS media with different concentrations of antibiotics were used for this purpose.

It was found from the experiments that, in compare to ½ strength MS media, ¼ strength MS media with IBA (0.5 mg/l) gave better response and form healthy roots and no callus formation (Fig. 62) (Table 3.11). The use of antibiotics in rooting media was modified. Keeping the kanamycin concentration constant (200 mg/l), the concentrations of cefotaxime and carbenicillin were made half compare to shoot regeneration media. This was done because higher concentrations of cefotaxime and carbenicillin found to promote callus at the shoot base instead of root formation (Fig. 63). It was also found that kanamycin had no significant role in callus formation at this stage (Fig. 64). During these experiments transformation efficiency was found to be 11.11% for B-3 variety.

Table 3.11. Determination of root induction media for putative transformed shoots in B-3 variety.

Medium	Hormonal concentrations (mg/l)		% of root producing shoot
	IAA	IBA	
½ MS	0.2	-	70 #
	0.5	-	50 #
	-	0.5	50***
¼ MS	0.2	-	75***
	0.5	-	60***
	-	0.5	80***

Note: Total of 20 shoots used in each cases. Healthy root = ***, root with callus = #.

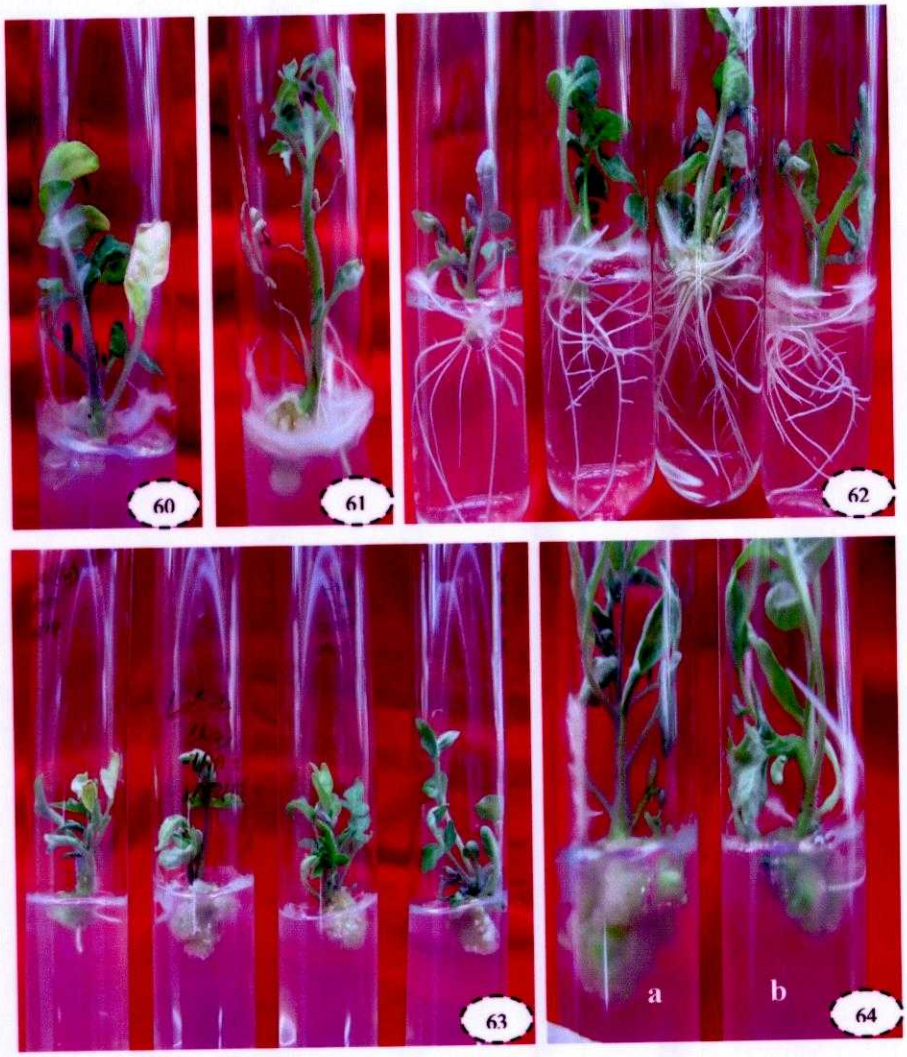


Fig. 60. Browning of shoot regenerated after Agrobacterial transformation, under selection pressure (200 mg/l kanamycin).
Fig. 61. Same as Fig. 60. but new leaves found to regenerate under the selection pressure.
Fig. 62. Healthy roots without callus formation found at the shoot base on ¼ MS media (containing kanamycin 200 mg/l, carbenicillin 50 mg/l and cefotaxime 25 mg/l) supplemented with 0.5 mg/l IBA in B-3.
Fig. 63. Callus formation at the shoots base of the putative transformed shoot of B-3 in ¼ MS media containing kanamycin 200 mg/l, carbenicillin 100 mg/l and cefotaxime 50 mg/l.
Fig. 64. Callus formation at the shoot base of the putative transformed shoots cultured on ¼ MS media containing 0.5 mg/l IBA, 100 mg/l carbenicillin, and 50 mg/l cefotaxime with (a) or without (b) kanamycin selection. Note that there is no effect of kanamycin on callus formation.

3.3.3. Establishment and performance of putative transformed plantlets in natural environment

After sufficient root development the putative transformed plantlets were successfully transplanted into small plastic pots containing soil (Fig. 65). Following proper acclimatization, the plantlets were transferred to larger pots for their further growth (Fig. 66) where they flowered and gave fruits in time as the non-transformed plants (Figs 67-68).

3.3.4. Analysis of putative transgenic plants through GUS histochemical assay

In this study, GUS (β-glucuronidase) gene was used as a screenable marker to analyze transgenic state. Generally transient assay for this marker gene was routinely performed as a preliminary step to monitor DNA transfer during transformation experiments. Assays were also carried out to observe the stable expression of GUS genes in the developing shoots and roots developed under selection pressure. The GUS histochemical assay was further repeated in these regenerated plantlets following successful transplantation and also after flower formation. Fig. 69 is the stereomicroscopic view of leaves from plantlets regenerating from infected explants of B-3 showing stable expression of GUS gene. Fig. 70. shows the magnified view of a leaf from putatively transformed plantlets showing stable expression of GUS gene and Fig. 71. show the uniform stable expression of GUS gene within the leaf tissues of putatively transformed plantlets of B-3. Figs 72 and 73 present the stereomicroscopic view of roots of B-3 showing chimeric expression of GUS gene following selection pressure.

After acclimatized, the plants flowered in due course of time. GUS histochemical assay revealed stable GUS expression in leaf, stem and

Fig. 65. Putative transformed plantlets of B-3 transplanted to soil in small poly bag. Photograph had taken 8 days after transplantation.
Fig. 66. Flowering (arrow) in confined condition of the same plants after attaining maturity.
Fig. 67. Fruit setting on the same plant.
Fig. 68. Mature fruit on B-3 putative T_0 plant.

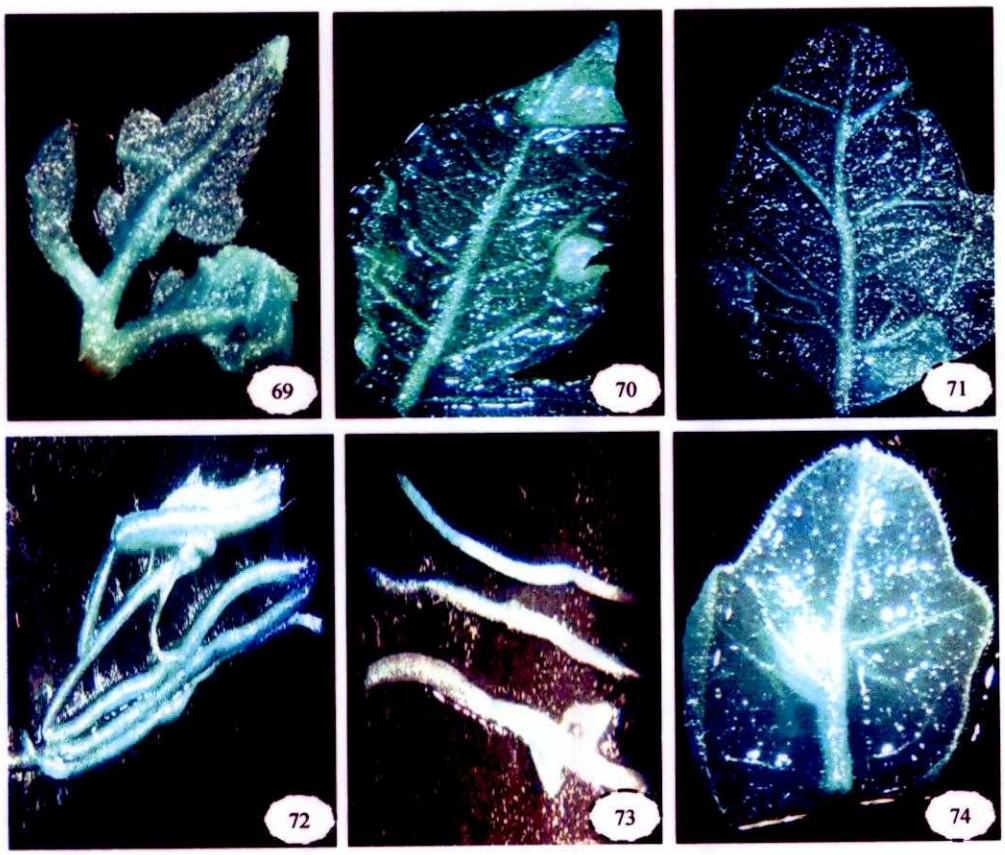


Fig. 69. Stereomicroscopic view of leaf randomly selected from the plantlets regenerated from infected explants of B-3, showing stable expression of GUS gene (× 6).
Fig. 70. Same as Fig. 69 (× 6).
Fig. 71. Same as Fig. 69 showing GUS gene expression on leaf tissue (× 6).
Fig. 72. Stereomicroscopic view of the roots of regenerated putative T_0 plants showing GUS expression (× 6).
Fig. 73. Stereomicroscopic view of the roots of regenerated putative T_0 plants showing GUS expression (× 6).
Photographs taken before the regenerated plantlets transferring in the soil which was 4 weeks old.
Fig. 74. Stereomicroscopic view of a leaf of regenerated putative T_0 plant showing GUS expression (× 6).
Photograph taken two months after transplantation.

flowers of 4 month old B-3 transgenic plants. Figs 74 and 75 showed leaf and stem of such plant respectively. Different parts of flower, like viable pollen (Figs 76-77), corolla (Fig. 78), sepal (Fig. 79), female parts (Fig. 80) and male part (Fig. 81) also showed GUS expression.

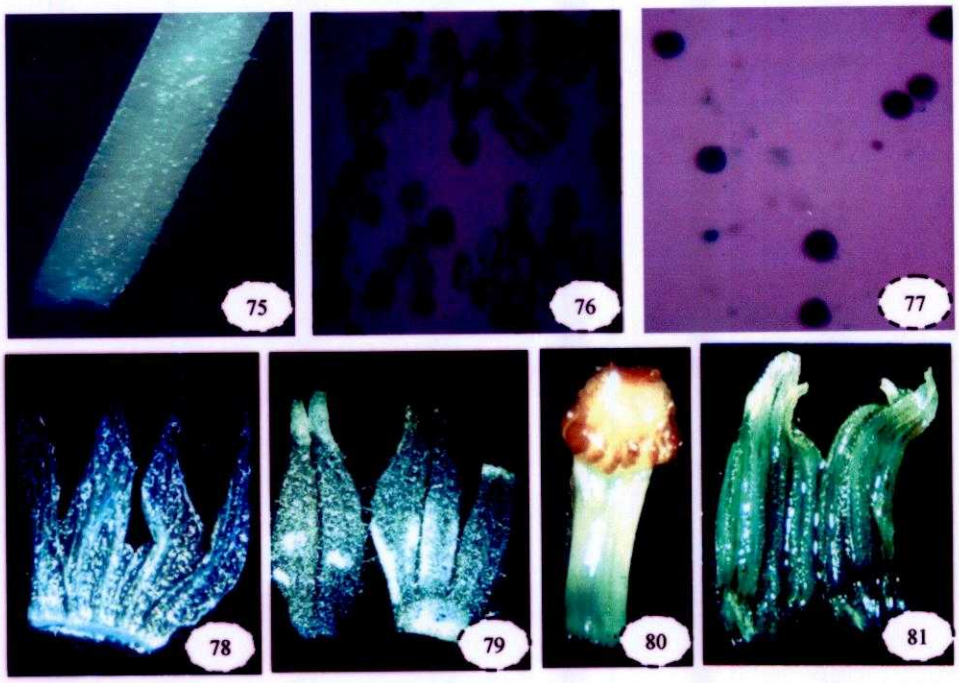


Fig. 75. Stereomicroscopic view of stem showing GUG gene expression (× 6).
Fig. 76. Viable pollen collected from that plant (× 115).
Fig. 77. Viable pollen collected from that plant showing GUS expression (× 115).
Fig. 78. Corolla collected from flower of putative transformed plant showing GUS expression.
Fig. 79. Sepal collected from flower of putative transformed plant flower showing GUS expression (× 6).
Fig. 80. Carpel (female part) of that flower showing GUS expression (× 7).
Fig. 81. Stamen (male part) of that flower showing GUS expression (× 7).

CHAPTER 4:
DISCUSSION

The present investigation aimed at establishing efficient protocols for *in-vitro* regeneration and *Agrobacterium*-mediated transformation for three Bangladeshi tomato (*Lycopersicon esculentum* Mill.) varieties, namely Bahar (BR), Bina tomato 5 (B-5), Bina tomato 3 (B-3), and an Indian commercial variety, Pusa Ruby (PR). The investigation has been carried out in three phases. In the first phase, genotype independent *in vitro* regeneration protocol was established. In the second phase, *Agrobacterium*-mediated transformation protocol was developed. Using these protocols transgenic plants were regenerated and analyzed in the third phase.

Tissue culture protocol which is a prerequisite for genetic transformation begins with selection of suitable explants and determination of appropriate hormonal supplementation for *in vitro* regeneration. Several attempts have been made to establish a suitable regeneration method for tomato. Regeneration in diverse varieties of tomato using various explants, viz. cotyledons, hypocotyls, epicotyls, meristem, leaf, stems, roots, internodes, petiole, anthers and inflorescences has been reported (Padmanabhan et al., 1974; Behki et al., 1976; Kartha et al., 1976; Ohki et al., 1978; Frary and Earl, 1996; Stephanie et al., 2001; Gubis et al., 2003; Reda et al., 2004; Raj et al., 2005; Islam, 2007). Among these explants cotyledonary leaf segments have been reported to be the most responsive explant for tomato regeneration and transformation in various tomato varieties (McCormick, 1991; Frary and Earl, 1996; Gubis et al., 2003; Islam, 2007). For this reason, in the present study, attempts were taken to develop regeneration protocol using cotyledonary leaf explants and to obtain shoots directly from the cut end on the explants without having callus phase.

During the present investigation, cotyledonary leaf explants were collected from aseptically grown seedlings. To obtain aseptic germination, tomato

seeds were treated with 2.5% (v/v) sodium hypochlorite solution and Tween-20 following 2-3 mins wash with 70% ethanol. Same sterilization solution was used by others for Bangladeshi and non-Bangladeshi varieties (Costa, 2000; Gubis et al., 2003; Park et al., 2003; Islam, 2007). However, they reported to use this protocol for a prolonged time, which found to have a negative impact on germination in the present study. On the other hand, Sheeja and Mandal (2003) used freshly made 0.1% $HgCl_2$ as the surface sterilent after using Bavistin (2% w/v) solution with Teepol (5% v/v) for a similar prolonged duration. A simple sterilization protocol was reported by Cortina et al. (2004) where they used only 30% active chlorine for a short time (7 mins) to decontaminate the seeds.

Effects of light and agitation (shaking) on germination following sterilization were observed in the present study. The germination percentage increased with continuous shaking of the surface sterilized seeds within sterile distilled water. It was also observed that both darkness and agitation reduced the time required for germination in all the varieties used. Similar observation was reported by Islam (2007) while working with Bari Tomato-3 (BT-3) and Pusa Ruby.

During this study, MS medium was used as basal media for *in vitro* regeneration, as it is reported to be the most used medium in case of tomato regeneration (Mirghis et al., 1995; Frary and Earl, 1996; Costa, 2000; Gubis et al., 2003; Islam, 2007). Each cotyledonary leaf segments was placed with the abaxial surface in contact with regeneration media and this dorsal position showed maximum response in shoot initiation in the present study, which supported Costa et al. (1999). To determine appropriate supplementation for regeneration different hormonal concentrations and combinations in MS media were evaluated. Shoot regeneration in tomato has

been majorly reported to be initiated through cytokinin supplementation. In the present study, BAP showed a positive effect on multiple shoot regeneration. Although lower concentration of BAP (0.5 mg/l) resulted indirect shoot regeneration, but higher concentration gave direct shoot formation. Most interestingly, it was observed that though the number of shoots increased with the increase of BAP concentrations, but these shoots showed abnormal morphology when maintained in the same hormonal supplement. Similar response towards increasing BAP concentration was reported by Islam (2007). However, she recorded verification while maintaining cultures at higher BAP concentration. In contrast to these reports, Kartha et al. (1976) and Sheeja et al. (2004) obtained plants from hypocotyle explants using another cytokinin, kinetin. These variations in responses towards hormonal supplementation may be due to variation in explant tissue. Even for the same explant tissue (cotyledonary leaf explant) difference in plant variety also seems to play a role in determining best hormonal supplementation. Cotyledonary leaf explants of several varieties reported to give best *in vitro* shoot regeneration response when Zeatin was added in addition to IAA in MS media (Costa et al., 2000; Gubis et al., 2003; Ahsan et al., 2007).

When IAA (0.1 mg/l) was added with BAP in the present study, shoot initiation was observed in all four varieties. Though 2.0 mg/l BAP with 0.1 mg/l IAA gave best response among the concentrations tried, but the response was similar to only BAP (2.0 mg/l) supplementation. Moreover, subculture in the same media (BAP+IAA supplemented MS media) resulted slower growth and elongation. However, a differing report came from Islam (2007) where BAP (1.0 mg/l) and IAA (0.1 mg/l) were found to be the best for shoot formation in compare with only BAP (0.5-5.0 mg/l) supplementation in Bari tomato-3 and PR. On the other hand, Jawahar et al.,

(1997) reported the same hormonal combination to be the best media for callus induction. While Park et al. (2003) reported shoot regeneration in BAP +NAA containing MS media, the present study showed formation of non-regenerative callus in such hormonal combination. This observation matches that of Islam (2007).

Root formation is an essential step to produce plantlets. As roots were not formed spontaneously, experiments were carried out in half strength of MS medium with or without different concentrations and combinations of auxins to achieve *in vitro* root formation in all four tomato varieties. In the present study, 2.0 mg/l IAA containing ½ strength MS media showed the best rooting response. Though rooting occurs in IAA, IBA and NAA supplemented media, IAA has been report to be more preferred rooting hormone for tomato by others (Jawahar et al., 1997; Oktem et al., 1999; Costa, 2000; Sheeja et al., 2004; Islam, 2007). Oktem et al. (1999) and Costa et al. (2000) used IAA in full strength of MS media or media with modified MS salts for rooting. Whereas, Sheeja et al. (2004) used IAA in half strength of MS media which is similar to the present findings. On the other hand, Zagorska et al. (2004) found rooting in half MS media with 0.2 mg/l IBA and 0.5 mg/l GA_3.

As per the present protocol, all varieties took around four months to develop plantlet from the initiation of the culture. This is similar to the report of Oktem et al. (1999). All the rooted plantlets acclimatized in natural environment where they flowered and formed fruits identical to control plants. Seeds collected from mature fruits were viable during germination test.

The *in vitro* regeneration protocol reported here showed similar response in all four varieties. Thus, the protocol can be considered as genotype

independent, reproducible and also avoid of hormonal complexity that is simple.

In the second phase, a suitable protocol for *Agrobacterium*-mediated genetic transformation of tomato was tried to establish. For this purpose, a genetically engineered *Agrobacterium tumefaciens* strain LBA4404 was used to transformed cotyledonary leaf explants of BR, B-5, B-3 and PR tomato varieties. Transformation of tomato with *Agrobacterium tumefaciens* has been reported since 1986 (McCormick et al., 1986; Chyi and Phillips 1987). However, tomato transformation is still far away from routine. Thus, factors affecting transformation efficiency were evaluated in the present study.

According to Islam (1998) several factors viz. host-bacteria susceptibility, optical density of bacterial suspension, incubation period, and co-cultivation period influence *Agrobacterium*-mediated transformation. During the present study, tissues of all the four varieties of tomato (cotyledonary leaf explant) found susceptible to infection and compatible towards transformation by strain LBA4404. This is supported by a number of reports available (McCormick et al., 1986; 1991; Filatti et al., 1987; Phillips et al., 1987; Davis et al., 1991; Brown et al., 1993; Hamza et al., 1993; Liu et al., 1995; Frary and Earl, 1996; Cortina et al., 2004).

On the other hand, pre-culture of explants suggested by McCormick (1991) did not show any significant improvement regarding transformation frequency in the present study. This is also supported by several other reports (Ahsan et al., 2007; Islam, 2007). However, in the present study, pre-culture found to have positive impact on rapid shoot regeneration after transformation in all tested varieties.

To increase the efficiency of *Agrobacterium*-mediated gene transfer in tomatoes, Ahsan et al. (2007) reported transformation frequency to be highly depends on various parameter like, the wound type of the explants, infection method and co-cultivation temperature. This is in agreement to the present study. In all four tomato varieties, increase in incubation and co-cultivation period as well as optical density ($OD_{600.}$) enhanced the percentage of transformed explants but upto a certain limit. This is in agreement with Islam (2007). Transformation efficiency of explants, in all the four varieties, was found to be maximum with bacterial suspension having optical density of (OD_{600}) 0.8 with 10-15 mins of incubation and 3 days of co-cultivation period. Ling et al. (1998/0; Park et al. (2003); Cortina et al. (2004) and Islam (2007) reported almost similar findings except they used prolonged infection time (30-40 mins).

Yu *et al.* (2001) showed that bacteriostatic antibiotics, namely cefotaxime and carbenicillin used in co-cultivation during the present study are not only antibacterial but also affect regeneration process. Another report from Ling et al. (1998) showed, cefotaxime itself did not inhibit callus growth in culture medium, but it clearly decreased shoot differentiation. Silva and Fukai (2001) reported that the success in transgenic plant regeneration relies on fine balance of four points, such as (1) the plant, (2) the *Agrobacterium,* (3) the selection agent and (4) the agent for eliminating *Agrobacterium sp.* They reported enhanced shoot regeneration in presence of cefotaxime while reduced regeneration in presence of carbenicillin in tobacco under kanamycin selection following infection with *Agrobacteium* strain LBA4404. Using the same plant species and bacterial strain, Islam et al. (2007) observed contradicting report when they tried to regenerate transgenic tobacco under hygromycin selection. They found no regeneration in presence of cefotaxime. Negative effect of cefotaxime was reported in various plants

including tomato (Ling et al., 1998) and chrysanthemum (Silva and Fukai, 2001), while negative impact of both carbenicillin and cefotaxime on regeneration was observed in rose by Li et al. (2002). But in the present study, carbenicillin and cefotaxime showed no significant role in shoot regeneration.

The *Agrobacterium* strain used in this investigation (LBA4404) has *nptII* gene conferring kanamycin resistance to the transformed cells. Therefore, experiments were done to determine the suitable selection concentration of kanamycin. It was found that the survival percentage of the control explants decrease gradually with the increase of kanamycin concentration in the regeneration media. Similar result were found by McCormick (1991); Ling et al. (1998); Tabaeizadeh et al. (1999) and Cortina et al. (2004). Subsequently various selection pressure were used in the above mentioned reports. Ling et al. (1998) used 50 and 100 mg/l kanamycin; while Tabaeizadeh (1999) and Cortina et al. (2004) applied only 100 µg/l kanamycin for the selection of transformed shoots. During the present study, in presence of 150 mg/l kanamycin the explants turned albino and in presence of 200 mg/l or more kanamycin shoots died. Islam (2007) reported similar high concentration for selection of transformed shoots. The suggestion of McCormick (McCormick, 1991) not to decrease kanamycin concentration to obtain large number of transformed shoots was found recommendable in the present study.

During the present study, shoots that survived in the kanamycin selection were induced to form roots in the ½ or ¼ strength of MS media supplemented with IAA or IBA in presence of cefotaxime (25 mg/l), carbenicillin (50 mg/l) and kanamycin (200 mg/l). Kanamycin showed no effect role in callus formation as well as rooting. But the other two antibiotics and half strength of MS media seemed to have a negative impact on rooting in addition to produce callus at

the shoot base. In contrast, Islam (2007) found negative effect of kanamycin in root formation at the base of the shoot.

Expression of GUS gene in the putative transformed plantlets obtained following selection indicates stable integration of GUS genes. Conspicuous blue color was observed in leaves and shoots of the plantlets as well as in the roots and flowers. The blue regions observed in these tissues confirmed the transgenic nature of the plantlets. However, Islam (2007) could not found GUS expression in the regenerated roots even after maintaining selection pressure of kanamycin.

So it is evident from the study that, the selection procedure developed during the study has been found to be effective in recovering transformed plantlets. It is observed that *nptII* gene is efficient selectable marker for tomato as it was reported by Ling et al. (1998), Tabaeizadeh et al. (1999), Park et al. (2003); Cortina et al. (2004) and Islam (2007).

In the present investigation, the transgenic nature of the putatively transformed plants was confirmed through GUS histochemical assay only. However, for confirmation of stable integration of the transgenes into plants, specific molecular techniques like polymerase chain reaction (PCR) analysis and southern hybridization are also necessary. Studies on the inheritance of the transgenic plants are also required to understand the nature of expression of the transgenes. In future transformation experiments, efforts must be made for molecular analysis of transformed plants to study their transgenic nature and to obtain desired transgenics.

CHAPTER 5:
CONCLUSION

Considering all the experiments, the following conclusions can be made:

- A genotype independent regeneration protocol was developed which is simple and efficient. Avoiding complex combinations of different growth regulators only 2.0 mg/l BAP supplemented MS media and 0.2 mg/l IAA supplemented half strength MS media was found to be the best for regeneration and rooting, respectively.

- To analyze and optimize transformation factors an OD_{600} of 0.8 with 10-15 mins of incubation and 3 days of co-cultivation period was found to be the best to achieve maximum transformation ability. B-3 showed highest transformation ability (96.7%).

- As B-3 showed highest transformation ability it was transferred to soil and used to check transformation status.

- Transformed B-3 plantlets were successfully acclimatized in soil and flowered normally. Different parts of the flower were found to be GUS positive and the flowers produced fruit. Seeds from the fruit have been collected for checking inheritance of the transgenes.

- Transgenic status of the plants was confirmed by GUS histochemical assay only. However, for confirmation of stable incorporation of GUS gene or other transgenes into plants, specific molecular techniques like polymerase chain reaction (PCR) analysis and southern hybridization are also necessary.

CHAPTER 6:
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Appendix- 1

Murashige and Skoog (MS) media 1962

Constituents	Concentrations (mg/l)
Macronutrients	
KNO_3	1900.00
NH_4NO_3	1650.00
KH_2PO_4	170.00
$CaCl_2$, $2H_2O$	440.00
$MgSO_4$, $7H_2O$	370.00
Micronutrients	
$FeSO_4$, 7 H_2O	27.80
Na_2-EDTA	37.30
$MnSO_4$, $4H_2O$	22.30
H_3BO_3	6.20
$ZnSO_4$, 4 H_2O	8.60
Na_2MoO_4, 2 H_2O	0.25
KI	0.83
$CuSO_4$, 5 H_2O	0.025
$CoCl_2$, 6 H_2O	0.025
Vitamins	
Glycine	2.00
Nicotinic acid	0.50
Pyridoxine-HCl	0.50
Thiamine-HCl	0.10
Inositol	100.00
Sucrose	30,000.00

Note: p^H adjusted to 5.8 before autoclaving.

Appendix- 2

YM Broth

Constituents	Concentrations (mg/l)
Yeast extract	400.00
Mannitol	10000.00
NaCl	100.00
$MgSO_4$	200.00
$K_2HPO_4.3H_2O$	500.00

Note: p^H adjusted to 7.2 before autoclaving.