Effect of growth regulators on shoot induction and plant regeneration in tomato (*Lycopersicon esculentum* Mill.)

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

Department of Mathematics
and Natural Sciences,
Biotechnology Programme,
BRAC University,
Dhaka, Bangladesh.

Submitted by
Amitav Das
Student ID: 08276001
Session: 2008-2009
November, 2011.

Acknowledgement

All praise goes to the almighty God who has created us with infinite love and affection. My honor and thankfulness is expressed to my deepest respectable and most honorable supervisor Dr. Zeba Islam Seraj, Professor, Department of Biochemistry and Molecular Biology, Dhaka University and Dr. Aparna Islam, Assistant Professor, Biotechnology programme, BRAC University, for constructive guidance, valuable advice, constructive criticism, affectionate feelings and constant encouragement throughout the period of research work and also in the preparation and completion of this manuscript have given the thesis a better finishing.

I wish to express sincere thanks and gratitude to Professor Naiyyum Chaudhury, Coordinator, Biotechnology programme, BRAC University and Professor A. A. Ziauddin Ahmad, Chairperson, Department of Mathematics and Natural Sciences, BRAC University, for their valuable suggestion, cooperation and encouragement in this study.

I am thankful to my companions, seniors and relatively juniors and also Lab. Assistants for their hard work and helpful kind cooperation from the beginning to the end of the project work. I am also grateful to my family members for their encouragement and moral support that inspired me to move forward.

Finally, all my praises go to the Almighty, whose benevolence and mercy has made this dissertation successful.

November, 2011 ***(Amitav Das)***

Certificate

This is to certify that the research work embodying the results reported in this thesis entitled "Effect of growth regulators on shoot induction and plant regeneration in tomato (Lycopersicon esculentum Mill.)" Submitted by Amitav Das, has been carried out under my supervision in the Plant Biotechnology Laboratory, Department of Biochemistry and Molecular Biology, Dhaka University. It is further certified that the research work presented here is original and suitable for submission for the partial fulfilment of the degree of Master of Science in Biotechnology, BRAC University, Dhaka.

(Dr. Zeba Islam Seraj)

Professor

Department of Biochemistry and Molecular Biology,
University of Dhaka.

(Dr. Aparna Islam)

Assistant Professor

Department of Mathematics and Natural Sciences,
BRAC Univeristy, Dhaka.

ABBREVIATIONS

The following abbreviations have been used throughout the text.

B-3	BINA tomato-3
BAP	6-Benzylaminopurine
BINA	Bangladesh Institute of Nuclear Agriculture
BR	Bahar
IAA	Indole-3 Acetic Acid
IBA	Indole-3 Butyric Acid
MS	Murashige and Skoog (1962) medium
Na-FeEDTA	Sodium salt of Ferric Ethylene Diamine Tetra Acetate
NaOH	Sodium Hydroxide
PR	Pussa Rubby

Abstract

Tomato (*Lycopersicum esculentum* Miller) is one of the most important winter vegetable crops in Bangladesh. To meet the increasing demand of tomato in Bangladesh, it is necessary to develop new varieties adapted to various abotic and botic stresses. To achieve high yield potential and wide adaptability, tissue culture may play a role. For this, it is necessary to investigate the effect of growth regulators on shoot induction and plant regeneration in tomato. In this study four locally grown tomato (*Lycopersicon esculentum* Mill.) varieties, namely, BINA-3, BARI-3, Bahar, Maple and one Indian commercial variety, Pussa Rubby (PR) were selected to observe the effects of growth regulators on plant regeneration in tomato. Cotyledonary leaf explants of tomato were collected from 8-10 days old *in vitro* germinated seedlings. Different concentrations and combinations of growth regulators were added to MS media to observe shoot initiation and root induction. Among all the combinations, MS media containing 1.5mg/l BAP and 0.2mg/l IAA showed best result with lowest callus formation and increased number of shoot in all the varieties. In this medium the best response was found for Maple (93.33 %) followed by Pussa Ruby (86.67 %) and for the other varieties such as BARI-3, BINA-3 and Bahar gave almost similar response to that combination of the medium. The survival response of all varieties was in between 80 % to 90 %. Among the varieties, Maple showed the best survival response which was almost 90 % while BINA-3 showed the survival response of almost 80 %. Among all the varieties, BINA-3 showed highest number of fruits per plant while BINA-3 and BARI-3 showed highest average number of seeds per fruit. Viability response of all the five varieties was in between 75 % to 82.5 %. Among the varieties, BINA-3 showed the best viability response which was almost 82.5 % while Maple showed the viability response of almost 75 %. Finally this study has shown that 1.5 mg/l BAP with 0.2mg/l IAA containing MS media is better for shoots induction and 0.2mg/l IAA containing ½ strength MS media showed best response to root formation for all the five varieties. Each experiment was conducted three times (n=3). Regeneration percentages were analyzed by using SPSS version 16 and significant differences among means were assessed by the Duncan test ($P < 0.05$).

Contents

List of Tables

List of Figures

Chapter-1
INTRODUCTION

Introduction

Tomato is one of the most important winter vegetable crop in Bangladesh. The scientific name of this plant is *Lycopersicum esculentum* Miller belongs to the family of Solanaceae. It was originated in South America and spread around the world following the Spanish colonization of the Americas. The cultivated tomato is a short-lived diploid (2n =2x =24) dicotyledonous annual plant, typically grown to 1–3 meters (3–10 ft) in height. The leaves are 10-12 cm long with 5-9 leaflets, each leaflet up to 8 cm long. The flower is 1-2 cm across, yellow with five pointed lobes on the corolla. 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).

Tomato has achieved tremendous popularity over the last century and it is grown in almost every country of the world. It is the second most important vegetable crop in the world after potato (Bhatia *et al.*, 2004; Foolad, 2004). Tomato is consumed in many forms, such as, raw vegetable, added to other food items or as processed products such as paste, whole peeled, diced, juice, sauces and soups (Khoudi *et al.*, 2009). About 130 million tons of tomatoes were produced in the world in 2008 (http://en.wikipedia.org/wiki/Tomato#Modern_uses_of_tomatoes; dated:1/4/2011). According to report published by Food and Agriculture Organization (FAO) of the United Nations (2008), the top producers of tomatoes were China (33811702 ton), USA (12575900 ton), Turkey (10985400 ton), India (10260600 ton) and Italy (5976912 ton).

Tomato is also good for weight loss, obesity, eye disorders, night blindness, urinary tract infection, liver disorders, indigestion, constipation, diarrhea and diabetes (www.pyroenergen.com/.../tomatoes-medicinal-properties.htm, dated 1/4/2011). Tomato has detoxification effect in the body. According to World Health Organization (WHO) report (2009), 51 mg of chlorine and 11 mg of sulfur in 100 grams size of tomato have a vital role in detoxification process. Tomato is a good source of calcium and iron. It also contains some amounts of phosphorus, sulphur and potassium. Tomato is rich in vitamin C (19.1mg), vitamin B (0.059 mg), vitamin A (623 I.U) and is also cholesterol free (USDA Standard Reference Release 13 Nov, 1999) (Block *et al.*, 1992; Gerster, 1997; Rao and Agarwal, 2000).

In Bangladesh, the demand of tomato is increasing day-by-day in the agro. and food industries. It is cultivated all over the country due to its adaptability to wide range of soil and climate (Ahamed *et al.*, 1995). The cultivated area under tomato in Bangladesh was 17, 813.8 hectares, total production was 1,20,000 metric tons having an average yield of 6.7 metric tons per hectare (BBS, 2010).

Tomato has achieved tremendous popularity. 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 producing new varieties, generation of tomato is severely hampered 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. 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 disease reduces plant vigor and yield potential of fruit to a considerable extent (Jones *et al.*, 1991). The diseases that are caused by bacteria include bacterial canker by *Corynbacterium michiganense*, bacterial speck caused by *Pseudomonas syringe* and bacterial wilt caused by *Ralstoni solanacearu.* The main fungal diseases that affect the production of tomato are Verticillium (*Verticillium dahlia*) wilts and powdery mildew caused by *Leveillula taurica,* early and late blights caused by *Alternaria solani* and *Phytopthora infestants,* respectively, anthracnose caused by *Collitotrichum phomoides.* Tomato productions are also damaged by many pests from the first emergence till harvest. Among them Aphids, Flea beetles, Leaf miners, Spider mites etc create problems to plant bed tomatoes while 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).

Diseases 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. Conventional breeding methods were tried to develop the characters of agronomic importance in tomato, but it was not so successful because of high degree of self pollination and non-availability of suitable

wild germplasm. Besides this, conventional breeding method takes long time, extending over seven to eight years involving crossing and selection of desirable traits.

Except sexual breeding techniques, there are also some processes of creating variability including induction of somaclonal variation through tissue culture, somatic hybridization and genetic engineering. 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).

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. The regeneration of plants by tissue culture method is an important and essential component of biotechnological research and also required for genetic manipulation. A breeding program associated to biotechnological tools depends upon the development of an efficient *in vitro* plant regeneration system (Hosseini *et al.*, 2006).

Tomato is considered to be a model species for introduction of agronomically important genes into dicotyledonous crop plants (Wing *et al.*, 1994). The most frequently used way of regeneration in tomato is via shoot organogenesis from callus developed from leaf or cotyledon explants or directly from thin cell layers of the inflorescence (Compton and Veilleux, 1991).

In vitro plant regeneration of tomatoes using protocols for adventitious shoot regeneration from cotyledon segments has been reported (vanRoekel *et al.*, 1993). The system is as follows: a bud induction phase, culturing the explants in medium supplemented with cytokinin (Compton and Gray, 1993); an elongation phase, transferring the shoot buds to medium with a lower concentration of cytokinin (Dong and Jia, 1991) ; and a rooting phase, using a culture medium supplemented with auxin (Compton and Gray, 1994; Abu El-Heba, 2004) .

Tomato regeneration has been previously reported *via* organogenesis in several articles using different explants, such as, leaf (McCormic *et al.*, 1986; Gaffer, 1997; Oktem *et al.*, 1999;

Kartha *et al.*, 1976, and Padmanabhan *et al.*, 1974), meristems (Mirghis *et al.*, 1995), stems and anthers (Zamir *et al.*, 1980), hypocotyls (Ohki *et al.*, 1978). In addition, (Pozueta-Romero, *et al.*, 2001) regenerated shoots of three tomato cultivars after 14 days from the hypocotyl after removing the primary and axillary meristems (Ghada A *et al.*, 2008).

The hormones, auxins and cytokinins control plant development through a multitude of complex interactions. The balance between auxins and cytokinins controls the formation of roots, shoots, and callus tissue *in vitro* (DeRopp, 1954; Skoog and Miller, 1957). The mode of interaction between auxins and cytokinins can therefore be synergistic, antagonistic, or additive and is dependent on the type of tissue and on the plant species in which the interaction occurs. Although the molecular mechanisms underlying most of these auxin-cytokinin interactions are unknown, they are thought to include mutual control of auxin and cytokinin metabolism, interactions in the control of gene expression, and post-transcriptional interactions (Coenen and Lomax, 1997). Since the pioneering work, zeatin has been widely accepted as the only cytokinin capable of inducing satisfactory growth in tomato explants (Jabeen *et al.*, 2009; Aileen O'Connor-Sánchez *et al.*, 2010). Selective shoot regeneration medium (SRM) containing Myo-inositol, Vitamins and Zeatin showed increased shoot regeneration from the cotyledons (Ahsan *et al.*, 2007). Coconut water (CW) and BAP successfully replaced zeatin in olive micropropagation (Peixe *et al.*, 2007). CW had been reported in other important crop micro propagation, such as, monopodial orchid hybrid *Aranda deborah* (Lakshmanan *et al.*, 1995), legume crop and *Arachnis labrosa* (Temjensangba and Deb, 2005) and also in monocots like maize (Baskaran *et al.*, 2006) and sugarcane (Desai *et al.*, 2004; Amber *et al.*, 2010). While working with Bangladeshi tomato varieties, good callus was obtained by Begum and Miah (1993) 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. But using Indian variety (*Lycopersicon esculentum* cv.PKM.1), Jawahar *et al.*, (1997) induced callus from hypocotyls of tomato on MS medium supplemented with 2 mg/l IAA and 1 mg/l BAP and after subculture in the same medium they got good shoot proliferation.

Many differentiated shoots and shoot primordia were visible on primary leaf explants whereas, it was observed only a high rate of callus proliferation and formation of roots in the case of cotyledon explants. Primary leaves were three times more efficient than cotyledons

in terms of percentages of regeneration productivities (P) when calculated for both types of explants and were found: 0.64 for primary leaves and 0.15 for cotyledons. Although many shoot primordial were initiated on cotyledon explants, only few normal plants were recovered (4 out of 170 cotyledon explants), hence primary leaves were used as explant. Zeatin at 1 mg/l for shoot regeneration and 0.2 mg/l for shoot elongation combined with 0.1 mg/l IAA gave the best results in terms of regeneration percentages, productivity and morphological quality of the regenerated plants compared to the medium containing BAP and IAA (Khoudi *et al.*, 2009).

This similarity of hormonal supplements indicates that the overall hormonal demand is same for the tomato plants. *In vitro* regeneration through organogenesis and somatic embryogenesis can be used for multiplication of genetically identical clones and it is an integral part of genetic transformation procedures. The *in vitro* morphogenetic responses of cultured plants are affected by different components of the culture media and it is important to evaluate their effect on plant regeneration. Although advances are being made toward better understanding of metabolic processes correlated with regeneration, determining of the conditions for *in vitro* plant regeneration is still largely an empirical process. Thus, *in vitro* regeneration can be difficult to achieve for some plant species or particular genotypes within a species. Somatic embryogenesis in tomato is still at its infancy, and efficient procedures for large-scale production *via* somatic embryogenesis are yet to be developed (Bhatia *et al.*, 2004).

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

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 was-

▲ Establishment of *in vitro* regeneration methodology following evaluation the effectiveness of various plant growth regulators.

▲ Analysis the varietal response toward different hormonal supplements to observe effect of genotype.

▲ Observe the reproducibility of the regenerants to establish a germline, which will specifically be useful during transgenic development.

Chapter-2
MATERIALS

Materials

2.1 Plant materials

Five verities of tomato seeds were used for this research work. They are: BINA-3, BARI-3, Bahar, Pussa Rubby and Maple. The collection areas of these varieties are as follows:

1) **BINA-3 and Bahar**: Collected from Bangladesh Agricultural University (BAU), Mymensingh.
2) **BARI-3**: Collected from Bangladesh Agricultural Research Institute (BARI), Gazipur.
3) **Pussa Rubby**: Collected from Lal Teer Seed Company, Dhaka, Bangladersh.
4) **Maple**: Collected from Maple Agrobusiness Company, Dhaka, Bangladersh.

The important characteristics of these varieties are as follows:

2.1.1 BINA-3

It is a summer variety that was formed by the cross between Bahar and S1 mutant by radiating gamma ray (Hamid, 2004). This summer variety was released in 1997 by the National Seed Board. The plant height is 80-85 cm and leaf is curl shaped and light green, fruit number/plant is 12-14. The average weight of fruit is 82 gm and yield rate is 38-42 ton/ha and vitamin-C content is 19.5 mg/100 gm. Its fruit size is small and taste is sour. It requires 60-65 days maturing after transplantation. Maximum fruit yield is 48 ton/ha (av. 40 ton/ha) (Dutta, 2004).

2.1.2 BARI-3

This variety was released in 1996 by the Bangladesh Agricultural Research Institute (BARI) Gazipur, Bangladesh. Fruits are fleshy, semi-globe and red in color. Number of fruits/plant is about 28-30 and average fruit weight is about 85-90 gm. Yield is 2-2.5 kg/plant and 85-90 ton/ha.

2.1.3 Bahar

This variety was developed by Bangladesh Institute of Nuclear Agriculture (BINA) through the cross between "Anobic" and "Ocshert". The variety was released by the National Seed Board in 1992. It is medium height with 80-85 cm and determinate. Fruits are big and average weight is 110 gm and yield rate is 60-70 ton/ha, fruits are tasty with red colored, its vitamin-C content is 21.19 mg/100 gm. Maximum fruit yield is 75 ton/ha (av.65 ton/ha) (Dutta, 2004).

2.1.4 Pussa Rubby

This variety was released by International Agricultural Research Institute (IARI), New Delhi, India. Plants are erect in nature. Fruits are medium sized and red in color. This variety is suitable for spring-summer and autumn-summer seasons. Yield capacity is 32.5 ton/ha and fruiting time is 65-70 days after planting.

2.1.5 Maple

It is a high yielding tomato variety developed by Maple Agrobusiness Company, Dhaka, Bangladesh. Plants are medium sized and sowing time is autumn-winter. Fruits are round shaped with red colored and contain high defense quality against pathogens. Fruits are collected within 90-95 days after plantation with average weight of 90-100 gm and fruiting capacity is 20-25 ton/acre.

2.2 Different culture media

Different culture media were used for this present experiment which is as follows:

2.2.1 Seed germination and seedling development medium

MS basal (Murashige and Skoog, 1962) media solidified with Phytagel were used for seed germination and seedling development.

2.2.2 Regeneration initiation and shoot differentiation media

Cotyledonary explants collected from seedlings were cultured on modified MS media with different concentrations and combinations of various growth regulators (Auxins and Cytokinins) for regeneration of shoot. After shoot initiation same media was used as subculture media for shoot elongation and multiplication.

2.2.3 Root induction media

Half strength of MS basal media supplemented with different concentrations and combinations of Auxins, namely, NAA, IAA and IBA were used for root induction and development.

Chapter-3
METHODS

Methods

For seed germination and seedling development, regeneration initiation and shoot differentiation, elongation and for root initiation different types of media were used.

3.1 Preparation of stock solutions

As the first step, different stock solutions were prepared for the preparation of MS media. The various constituents, namely, macronutrients and micronutrients, vitamins, plant growth regulators etc. of the medium were prepared into stock solutions for ready use during the preparation of medium.

3.2 Stock solution A (Macro nutrients) for the MS medium

This stock solution was prepared in such a way that its strength was 20 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 upto 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°C for several weeks.

3.3 Stock solutions B (Micro nutrients) for MS medium

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

3.3.1 Stock solutions 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 (Macro nutrient). The solution was filtered and stored at 4°C for several weeks.

3.3.2 Stock solutions B2 (Iron chelate solution) for MS medium

The second solution was made 100 times the final strength of $FeSO_4.7H_2O$ 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°C for several weeks.

3.4 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°C for future use.

3.5 Stock solution for growth regulators

The following growth regulators were used in this experiment:

3.5.1 Auxin

Indole-3-acetic acid (IAA)

Indole-3-butyric acid (IBA)

Alpha-napthalene acetic acid (NAA)

3.5.2 Cytokinin

6-Benzylaminopurine (BAP)

The growth regulators were dissolved in appropriate solvent as shown against each of them.

Growth Regulator	Solvent
IAA	1N NAOH
IBA	1N NAOH
NAA	1N NAOH
BAP	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 respective solvent. The mixture was then washed off 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°C for future use.

3.6 Preparation of MS medium

To prepare one liter of MS medium, the following steps are carried out:

1) For the preparation of MS medium, 30 gm of sucrose was dissolved in 500 ml of distilled water in a volumetric flask.

2) 50 ml of stock solution of A, 5 ml of stock solution of B and C were added into 500 ml distilled water and were mixed well.

3) 100 mg of myo-inositol (Sigma, USA) was added to this solution and were dissolved completely.

4) For obtaining 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 mg of the chemical in 200 ml of the solution, the addition of 10 ml of any hormonal stock solution to make 1 litre of medium resulted 1mg/l concentration of that hormonal supplements.

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

6) The pH of the medium was adjusted to 5.8 using a digital pH meter (JENWAY-3010, UK) with the help of 1N NaOH or 1N HCl, whichever was required.

7) To solidify 0.3 g (at 3%) of phytagel (Sigma, USA) was added to the desired medium.

8) To dissolve phytagel (solidifying agent), the whole mixture was quickly heated in a microwave oven (Emerson, Korea).

3.7 Media sterilization

The culture vessels like test tube or conical flasks etc. were filled with fixed volume of media and plugged with non-absorbent cotton and covered with aluminum foil and then marked with a glass marker to indicate necessary supplements. The culture vessels were then autoclaved (Eyela Autoclave MAC-501, Japan) at 15 lb/sq. inch. pressure at 121°C temperature for 20 minutes.

3.8 Sterilization of seeds

Seeds were immersed in 70% ethanol for 2 min followed by 5.25% colorx. After that seeds were treated with Tween-20 (2 Drops) and shaken continuously for 20 min. Seeds were washed with distilled water for 5 times respectively (1, 2, 4, 8 and 10 min). Seeds were then kept in Shaker (Eyela, Singapore) at 28°C with 180 rpm for 48 hr.

After 48 hr, seeds were taken out from shaker and kept in solidified Germination Media (MS basal Media) under light and dark condition of (6+8) hr at 25°C ± 2°C for germination.

3.9 Regeneration

From 7-8 days old seedlings cotyledonery leaf explants were collected and placed on MS media supplemented with various hormonal supplements. Cultures were kept under fluorescent light with 25 - 27°C. Each experiment was conducted for three times (n=3). Regeneration percentages were analyzed by using SPSS version 16 and significant differences between means were assessed by the Duncan test (P <0.05).

3.10 Precautions to ensure aseptic conditions

All inoculation and aseptic manipulation were carried out in a Laminar Air Flow Cabinet (ESCO; class 2, Type R/B_3 Biohazard Safety Cabinet, Singapore). The cabinet was switched on for at least 50 minutes before use and cleaned with 70% alcohol to overcome the surface contaminations.

The instruments like scalpels, forceps, inoculation loop, Petri dishes, Micro-pipette tip etc. were sterilized by stem sterilization method. At the time of inoculation, these instruments were again sterilized by flaming method inside the cabinet. After autoclaving media were

poured inside the cabinet. All measures were taken to obtain maximum contamination free condition during the surgical operation of the explants.

3.11 Subculture

Regenerated cultures were subcultured to fresh media containing the same hormonal supplements for further proliferation and development. Subculture was performed regularly at an interval of 25 days for maintenance. Cultures were routinely examined for different morphogenic development.

3.12 Rooting

Well developed shoots about 2-4 cm long were placed individually in test-tube containing rooting media to obtain sufficient root formation.

3.13 Transplantation

Regenerated plantlets with sufficient roots (7-10 cm in length) were considered to transfer in the soil. Before transfer in the soil rooted plantlets were kept into room temperature (30 ± 2^{0}C) for 5-7 days for acclimatization. After proper hardening, plantlets were taken out from the test tubes, washed carefully under running tap water to remove any traces of agar. Each plantlet was transferred into small plastic pot containing garden soil, sand and compost at the ratio of 1:1:1 and kept inside a moist plastic bag for 2 weeks. Finally bags were removed and plants were placed in net house for flower and fruit formation.

3.14 Seed viability test

Seeds were collected from ripen fruit and sun dried. Seeds were counted and then sterilized according to 3.8 and placed in culture room to observe viability through germination response.

Chapter-4
RESULTS

Results

In the present study, cotyledonary leaf explants of tomato varieties viz. BINA-3, BARI-3, Bahar, Maple and Pussa Rubby were collected from 7-8 days old *in vitro* germination seedlings. The effects of hormonal treatments, viz. cytokinins and auxins were observed on *in vitro* shoot proliferation and root induction. The results obtained from each of the experiments are described in details under the following heads:

4.1 Effect of sterilization on *in vitro* germination of seeds

Seeds were surface sterilized and were shaken for few hours before placing on germination medium and incubated in dark. After germination seedlings were allowed to develop under light. It was observed that all the five varieties took 7 to 8 days to attain optimum seedling stage to collect cotyledonary leaf to use as explants. Among the five varieties, BINA-3 showed the highest rate of germination with 96.25 % followed by BARI-3 (93.75%) (Figs.1-5). However, other varieties also showed very good germination response. This response was similar throughout the year indicating prolonged viability of the varieties.

Table-1. Germination response of seeds of five varieties towards seedling development using germination medium.

Varieties	No. of seeds germinated	Germination rate (%)
BARI-3	75	93.75
BINA-3	77	96.25
Pussa Rubby	74	92.5
Bahar	74	92.5
Maple	72	90

Note: MS Media was used for germination and a total of 80 seeds were used for each variety.

4.2 Multiple shoot regeneration response of BARI-3, BINA-3, Pussa Rubby, Bahar and Maple varieties towards different concentrations of BAP and IAA in MS medium.

MS medium supplemented with different concentrations of BAP (1.0–3.0 mg/l) along with various concentrations of IAA (0.1-0.5 mg/l) were used for induction of multiple shoots using cotyledonary leaf explants. The average time for shoot bud initiation in all the varieties was 28 to 30 days. Results of this experiment are presented in Tables 2-6. It was found that BAP with IAA had a positive effect towards shoot regeneration. The eight hormonal combinations were used for regeneration during this experiment.

4.2.1 Effect of different concentrations and combinations of BAP and IAA for BARI-3 variety

For shoot regeneration, the best regeneration response (83.33%) was found in media composition T3 (1.5 mg/l BAP with 0.2 mg/l IAA) (Fig. 6) followed by 80% in media composition T2 (1.5 mg/l BAP with 0.1 mg/l IAA) in BARI-3 (Table 2). Callus development was observed in the medium having 3 mg/l BAP and 0.1 mg/l IAA (T8). Further increase of IAA showed deleterious effect. BAP supplements also showed similar negative effect.

4.2.2 Effect of different concentrations and combinations of BAP and IAA for BINA-3 variety

In case of BINA-3, for shoot regeneration, the best regeneration response (83.99%) was found in T3 media (1.5 mg/l BAP with 0.2 mg/l IAA) (Fig. 7) followed by T4 media composition (1.5 mg/l BAP with 0.5 mg/l IAA) (Table 3). Growth media T6 (2 mg/l BAP with 0.1 mg/l IAA) showed the lowest shoot regeneration and growth media T8 (3 mg/l BAP and 0.1 mg/l IAA) showed callus formation in the medium. Increase of IAA with BAP-2 mg/l showed positive effect.

4.2.3 Effect of different concentrations and combinations of BAP and IAA for Bahar variety

The variety Bahar also showed the best regeneration response (83.33%) in media composition T3 (1.5 mg/l BAP with 0.2 mg/l IAA) followed by 80% in T2 media (1.5 mg/l BAP with 0.1 mg/l IAA) (Fig. 8) and T4 (1.5 mg/l BAP with 0.5 mg/l IAA) (Table 4). Growth media T8 (3 mg/l BAP with 0.1 mg/l IAA) showed the lowest shoot regeneration with callus formation. With increase of IAA along with BAP (1.5 mg/l) showed negative effect. In case of 2 mg/l BAP, increase of IAA showed similar deleterious effect. Increase of BAP supplements showed same negative effect.

4.2.4 Effect of different concentrations and combinations of BAP and IAA for Pussa Rubby variety

In case of Pussa Rubby, the best regeneration response (86.67%) was observed in T3 (1.5 mg/l BAP with 0.2 mg/l IAA) (Fig. 9) medium followed by 80% in T1 media (1 mg/l BAP with 0.1 mg/l IAA) (Table 5). Growth media T8 (3 mg/l BAP with 0.1 mg/l IAA) showed the lowest shoot regeneration. Callus formation was also found in that medium. Increase of IAA along with BAP (1.5 and 2 mg/l) showed deleterious effect. Increase of BAP supplements also showed deleterious effect on shoot regeneration.

4.2.5 Effect of different concentrations and combinations of BAP and IAA for Maple variety

Regeneration response of the Maple variety was found to be the best for shoot regeneration (93.33%) in T3 (1.5 mg/l BAP with 0.2 mg/l IAA) (Fig. 10) medium followed by 80% in medium T1 (1 mg/l BAP with 0.1 mg/l IAA) (Table 6). With the increase of IAA along with BAP (1.5 and 2 mg/l) showed deleterious effect on *in vitro* response was observed. 3 mg/l BAP with 0.1 mg/l IAA (T8) and 2 mg/l BAP with 0.5 mg/l IAA (T7) showed the lowest shoot regeneration while BAP at 3 mg/l in MS medium supported callus development. Also increase of BAP supplements showed similar deleterious effect on shoot regeneration.

Table 2. Shoot regeneration response of BARI-3 variety towards various concentrations and combinations of BAP and IAA in MS media.

Treatments	BAP (mg/l)	IAA (mg/l)	Regeneration rate (%)	No. of Shoot bud developed (after 28 days)	Days requirement for shoot elongation (days)	Total number of shoots developed (after 80 days)	Well developed shoots/ explant (after 80 days)
T1	1	0.1	66.67±2.3a	9	64	20	5
T2	1.5	0.1	80±1.58c	7	66	24	5
T3	1.5	0.2	83.33±5.1b	10	62	25	6
T4	1.5	0.5	76.67±2.45d	8	65	23	4
T5	1.5	1	70±1.19e	7	63	21	3
T6	2	0.1	60±2.38f	6	65	18	4
T7	2	0.5	60±1.57f	8	69	18	2
T8	3	0.1	66.67±3.27a	6	64	20	3

Note: Total number of explants was 30 in all treatments. Values are expressed as means ± SD (n=3); values within the column with the same superscript are not significantly different (P <0.05).

Table 3. Shoot regeneration response of BINA-3 variety towards various concentrations and combinations of BAP and IAA in MS media.

Treatments	BAP (mg/l)	IAA (mg/l)	Regeneration rate (%)	No. of Shoot bud developed (after 28 days)	Days requirement for shoot elongation (days)	Total number of shoots developed (after 80 days)	Well developed shoots/ explant (after 80 days)
T1	1	0.1	67.82±2.82a	8	65	22	4
T2	1.5	0.1	79.76±2.25c	7	66	23	3
T3	1.5	0.2	83.99±3.21b	11	63	26	6
T4	1.5	0.5	76.17±2.67d	8	65	25	2
T5	1.5	1	69.93±4.98ae	8	68	22	2
T6	2	0.1	59.62±1.57f	7	65	18	3
T7	2	0.5	60.28±3.21g	6	70	19	2
T8	3	0.1	67.57±1.09a	6	72	19	2

Note: Total number of explants was 30 in all cases. Values are expressed as means ± SD (n=3); values within the column with the same superscript are not significantly different (P <0.05).

Table 4. Shoot regeneration response of Bahar variety towards various concentrations and combinations of BAP and IAA in MS media.

Treatments	BAP (mg/l)	IAA (mg/l)	Regeneration rate (%)	No. of Shoot bud developed (after 28 days)	Days requirement for shoot elongation (days)	Total number of shoots developed (after 80 days)	Well developed shoots/ explant (after 80 days)
T1	1	0.1	76.67±6.23a	9	69	23	2
T2	1.5	0.1	80±1.25b	10	74	24	2
T3	1.5	0.2	83.33±2.34b	11	73	25	4
T4	1.5	0.5	80±1.11b	10	73	24	3
T5	1.5	1	73.33±0.29c	8	71	22	3
T6	2	0.1	66.67±0.57d	8	66	20	3
T7	2	0.5	60±0.67e	7	77	18	2
T8	3	0.1	50±1.87f	8	75	15	3

Note: Total number of explants was 30 in all cases. Values are expressed as means ± SD (n=3); values within the column with the same superscript are not significantly different ($P < 0.05$).

Table 5. Shoot regeneration response of Pussa Rubby variety towards various concentrations and combinations of BAP and IAA in MS media.

Treatments	BAP (mg/l)	IAA (mg/l)	Regeneration rate (%)	No. of Shoot bud developed (after 28 days)	Days requirement for shoot elongation (days)	Total number of shoots developed (after 80 days)	Well developed shoots/ explant (after 80 days)
T1	1	0.1	80±1.78a	7	66	24	3
T2	1.5	0.1	73.33±2.57c	8	61	22	5
T3	1.5	0.2	86.67±4.89b	8	65	26	6
T4	1.5	0.5	76.67±2.22d	7	65	23	4
T5	1.5	1	76.67±3.57d	7	70	23	4
T6	2	0.1	70±4.97c	6	70	21	3
T7	2	0.5	63.33±3.33e	5	69	19	3
T8	3	0.1	63.33±2.51e	5	69	19	3

Note: Total number of explants was 30 in all cases. Values are expressed as means ± SD (n=3); values within the column with the same superscript are not significantly different ($P < 0.05$).

Table 6. Shoot regeneration response of Maple variety towards various concentrations and combinations of BAP and IAA in MS media.

Treatments	BAP (mg/l)	IAA (mg/l)	Regeneration rate (%)	No. of Shoot bud developed (after 28 days)	Days requirement for shoot elongation (days)	Total number of shoots developed (after 80 days)	Well developed shoots/ explant (after 80 days)
T1	1	0.1	80±2.27a	8	62	24	4
T2	1.5	0.1	73.33±6.57c	9	65	22	4
T3	1.5	0.2	93.33±3.47b	12	65	28	5
T4	1.5	0.5	76.67±1.45d	10	66	23	4
T5	1.5	1	76.67±1.22d	9	60	23	3
T6	2	0.1	70±3.47e	8	63	21	3
T7	2	0.5	63.33±2.59f	7	66	19	3
T8	3	0.1	63.33±3.45f	7	65	19	2

Note: Total number of explants was 30 in all cases. Values are expressed as means ± SD (n=3); values within the column with the same superscript are not significantly different (P <0.05).

4.3 ANOVA results of the experiment

With regard to overall varietal response towards all the tested hormonal supplements, Mapple showed the best response. Highest response achieved is 93.33 % while lowest is 63.33 % (Fig. 11). Among the treatments T3 (1.5 mg/l BAP + 0.2 mg/l IAA) showed the best hormonal supplement because all the varieties showed the best response in this medium. Therefore, this hormonal combination can be considered as optimum to give genotype independent good response. Increase of either BAP or IAA supplementation found to have negative impact of regeneration. In general treatment T6, T7 and T8 found to give less responsive result which is most of the time less than treatment T1 and T2, which have very less amount of hormonal supplements.

Table 7. Duncan test results on shoot regeneration response of all the five varieties.

Treatments	BAP (mg/l)	IAA (mg/l)	No. of shoot regenerated				
			BARI-3	BINA-3	Bahar	Pussa Rubby	Maple
T1	1	0.1	66.67b±1.2	67.82b±1.6	76.67b±1.2	80c±.74	80c±1.55
T2	1.5	0.1	80c±1.3	79.76c±1.9	80a±1.63	73.33d±.65	73.33d±1.65
T3	1.5	0.2	83.33d±1.9	83.99d±1.1	83.33c±1.61	86.67e±1.48	93.33e±1.26
T4	1.5	0.5	76.67e±1.6	76.17e±1.2	80a±1.72	76.67b±1.47	76.67b±1.15
T5	1.5	1	70f±.85	69.93f±1.74	73.33d±1.91	76.67b±1.91	76.67b±0.42
T6	2	0.1	60a±.95	59.62a±1.77	66.67e±1.15	70f±1.18	70f±1.86
T7	2	0.5	60a±1.92	60.28a±1.87	60f±1.41	63.33a±1.21	63.33a±1.28
T8	3	0.1	66.67b±1.24	67.57b±1.23	50g±1.3	63.33a±1.36	63.33a±1.33

Note: Values are expressed as means ± SD (n=3). Values within the column with the same superscript are not significantly different (P <0.05).

From Table 7 it is found that BARI-3 variety, growth media T6 (2 mg/l BAP with 0.1 mg/l IAA) and T7 (2 mg/l BAP with 0.5 mg/l IAA); BINA-3 variety, growth media T6 (2 mg/l BAP with 0.1 mg/l IAA); Bahar variety, growth media T8 (3 mg/l BAP with 0.1 mg/l IAA); Pussa Rubby variety, growth media T7 (2 mg/l BAP with 0.5 mg/l IAA) and T8 (3 mg/l BAP with 0.1 mg/l IAA) and for Maple variety growth media T7 (2 mg/l BAP with 0.5 mg/l IAA) and T8 (3 mg/l BAP with 0.1 mg/l IAA) showed the lowest shoot regeneration rate and also lowest number of shoot development (Figs.11 and 12).

4.4 Effect of different concentrations of IAA and IBA for root induction

Root formation is one of the essential steps to produce plantlets. Regenerated shoots did not form root spontaneously. For this reason, elongated shoots (3-4 cm) were excised and cultured on root induction medium to produce roots. In the present experiment, half strength of MS medium with different concentrations of auxins, such as, IAA and IBA, were used for *in vitro* root formation in all five varieties of tomato. Irrespective of hormonal supplements, all cultured shoot showed rooting response and showed positive response towards healthy root development. Results of these observations are presented in the Table 8. In case of IAA thin long roots were initiated from the cut ends at the base of shoots. While in case of IBA, though similar type of roots formed in all concentrations but the root growth in IBA supplemented media was slow. Among the four combinations tested, 0.2 mg/l IAA showed best result with increased number of average root for all varieties that were used for this

experiment. All the varieties showed good number of roots with 0.2 mg/l IAA and BARI-3 showed the highest average root number among the varieties. Effects of different auxins in the development of roots from regenerated shoots are presented in the (Figs.13-16).

Days required for root initiation varied to the different types of media. The minimum days (6-7) required for root initiation when the explant treated with half MS medium supplemented with 0.2 mg/l IAA. It was also found that, well develop roots (8-10 cm in length) were observed in 16-20 days.

Table 8. Effects of different concentrations of auxins (IAA, IBA) in half strength of MS (1/2 MS) medium on rooting in BARI-3, BINA-3, Bahar, Pussa Rubby and Maple.

Varieties	Concentrations (mg/l)		Days needed for root initiation	Days required for root development	Average root number	Average root length (cm)	Root type
	IAA	IBA					
BARI-3	0.2	-	7-9	16	11	9.4	L^{++}
	0.5	-	7-8	18	6	9.2	FRS
	-	0.2	8-9	19	8	10.2	TRS
	-	0.5	7-8	16	8	9.5	TRS
BINA-3	0.2	-	6-7	17	9	10.85	FRS
	0.5	-	6-7	16	6	10.8	L^{++}
	-	0.2	7-8	20	8	9.5	LFRS
	-	0.5	6-7	18	5	10	L^{+}
Bahar	0.2	-	5-8	19	8	10.2	FRS
	0.5	-	8-9	17	6	9.5	TRS
	-	0.2	9-10	16	7	9.8	L
	-	0.5	7-8	15	6	9.5	L
PR	0.2	-	7-8	17	10	8	L^{++}
	0.5	-	7-9	18	7	7.6	L^{+}
	-	0.2	9-10	16	7	8.6	LTAP
	-	0.5	7-8	15	5	9.6	FRS
Maple	0.2	-	8-9	18	9	10.5	TRS
	0.5	-	9-10	18	7	9.8	L^{++}
	-	0.2	10-11	19	8	9.2	L^{+}
	-	0.5	8-9	17	8	10.6	LFRS

Note: Good=+, Very Good=++, L=Long, TRS=Tap Root System, FRS=Fibrous Root System. Total number of shoots was 30 in all cases.

4.5 Establishment and performance of plantlets in natural environment

Healthy and fully developed rooted plantlets of all five varieties were successfully transplanted into plastic pots containing soil. Roots formed in IAA showed better survivability in the soil than IBA where callus or fibrous roots were regenerated.

Following transplantation, survival rate of the regenerated plantlets were found to be the highest (90%) in Maple variety while similar result was observed in other varieties. Transplanted plantlets in plastic pots are shown in Figs.17-18. The survived plantlets were transferred to the field and were found to be established cent percent. The survival performances of plantlets following their transplantation are presented in the Table 9.

Table 9. Performances of plantlets of five varieties of tomato during transplantation.

Varieties	No. of plantlets transplanted in plastic pot	No. of plantlet survived in pot	% of plantlets survived in pot	No. of plants transferred to the field/larger pots	% of survival rate of plants in the field/larger pots in the environment
BARI-3	30	26	87	26	100
BINA-3	30	24	80	24	100
PR	30	26	87	26	100
Bahar	30	26	87	26	100
Maple	30	27	90	27	100

4.6 Flowering on the regenerated plants of five tomato varieties in the field

The transplanted plants flowered within five to seven weeks after transferring to natural environment (Figs.19-21). All the plants showed 100% flowering response. Flowers appeared on the apical meristem. All these characteristics are same at the naturally grown tomato flower. The data is presented in the Table 10. The minimum time requirement (33 days) required for flowering on BINA -3 and Bahar varieties followed by PR (34 days), Maple (36 days) and BARI-3 (38 days). The maximum number of flower (9 per plant) observed in BARI-3 and BINA -3 followed by PR (8 per plant) and Maple (8 per plant) varieties after 6 weeks old of transplantated plant.

Table 10. Flowering response of regenerated plantlets of the five tomato varieties.

Varieties	No. of plant transferred to natural environment	Days required for flowering	No. of flowers/plant
BARI-3	26	38	9
BINA-3	24	33	9
PR	26	34	8
Bahar	26	33	6
Maple	27	36	8

4.7 Fruiting of regenerated plants of five tomato varieties

After flowering it took almost 2 weeks to set fruit and another 2-5 weeks to obtain mature fruits (Table 11) (Figs. 22-26). All the varieties showed 100% fruiting. Among all the varieties, BINA-3 and Maple showed lowest average time for fruit setting. While Pussa Rubby and Bahar showed highest average time for fruit maturation.

Table 11. Fruiting response of the five tomato varieties in the natural environment.

Varieties	Total number of plant	Average time required for fruit setting (Days)	Average time required for fruit maturation (Weeks)
BARI-3	26	13-15	4-8
BINA-3	24	10-15	4-8
PR	26	13-15	5-8
Bahar	26	11-15	5-8
Maple	27	10-15	4-8

Fruits vary in diameter from 1.5 to 7.5 cm or more and are usually red, scarlet, or yellow; they vary in shape from almost spherical through oval and elongate to pear-shaped according to the respective varietal characteristics. The fruits are soft, succulent berry, containing two to many cells of small seeds surrounded by jellylike pulp. Among all the varieties, BINA-3 showed highest number of fruits (8 per plant) followed by BARI-3 (7 fruits per plant) and Bahar (7 fruits per plant) (Table 12).The maximum average weight of ripened fruit was observed 75.27 gm in BARI-3 variety followed by BINA-3 (65.52 gm). The highest average

number of seeds per fruit was recorded in BINA-3 (80 seeds per fruit) followed by BARI-3 (70 seeds per fruit) and the lowest number of seeds (46 seeds per fruit) was recorded in the variety Bahar, where seed number was half of that of the BINA-3 variety.

Table 12. Characteristics of ripened fruits of five tomato varieties

Varieties	Fruits/Plant	Average weight (gm)	Average seed number/Fruit	Fruit size
BARI-3	7	75.27	70	Semi Round
BINA-3	8	65.52	80	Semi round
PR	6	11.86	63	Round
Bahar	7	82.8	46	Round
Maple	6	77.7	62	Heart Shaped

Note: Average number of fruits is 10 for all varieties.

4.8 Viability of seeds collected from regenerated plants

After fruit ripening, and seeds were collected from fruits for viability checking. All the five varieties showed good germination efficiency during viability checking and BINA-3 showed highest germination efficiency having 82.5% rate among the varieties (Fig. 27). The data is presented in Table13.

Table-13. Viability response of seeds collected from ripened fruits of five tomato varieties

Varieties	Total number of seed taken for germination	No. of seeds germinated	% of seed germination
BARI-3	40	32	80
BINA-3	40	33	82.5
PR	40	32	80
Bahar	40	31	77.5
Maple	40	30	75

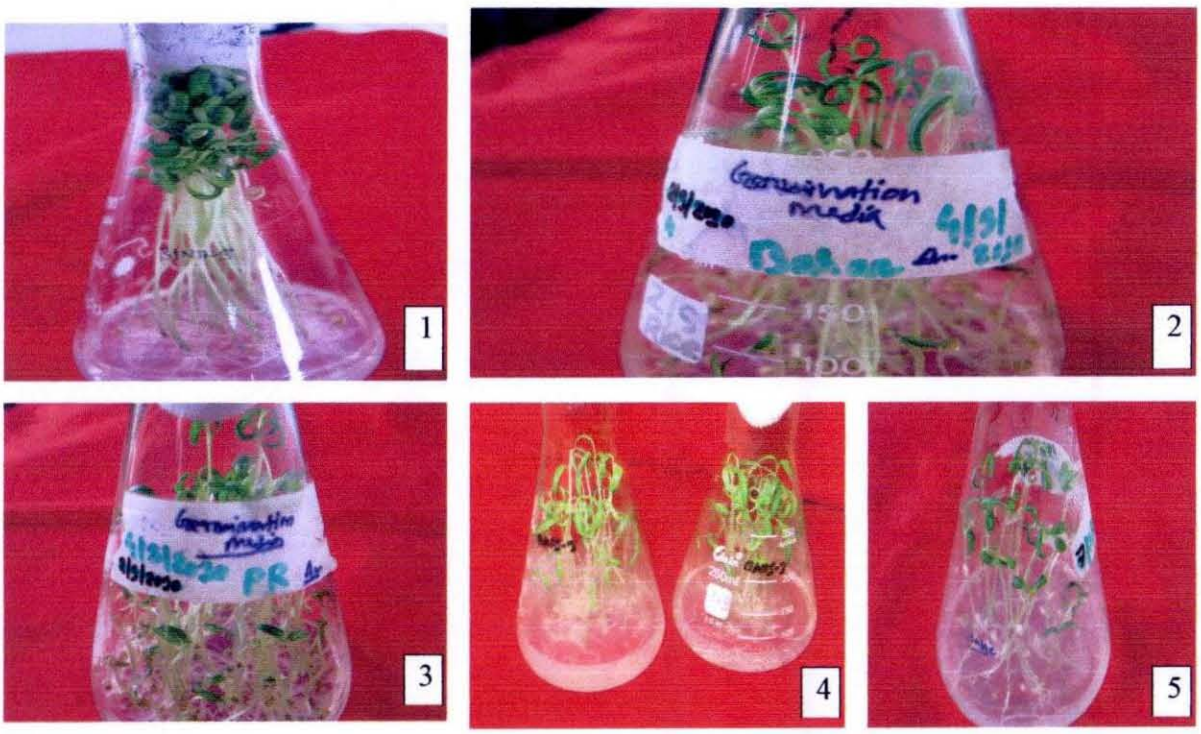


Fig-1. 7days old seedlings of BINA-3 on germination media.

Fig-2. 7days old seedlings of Bahar on germination media.

Fig-3. 7days old seedlings of Pussa Rubby on germination media.

Fig-4. 7days old seedlings of BARI-3 on germination media.

Fig-5. 7days old seedlings of Maple on germination media.

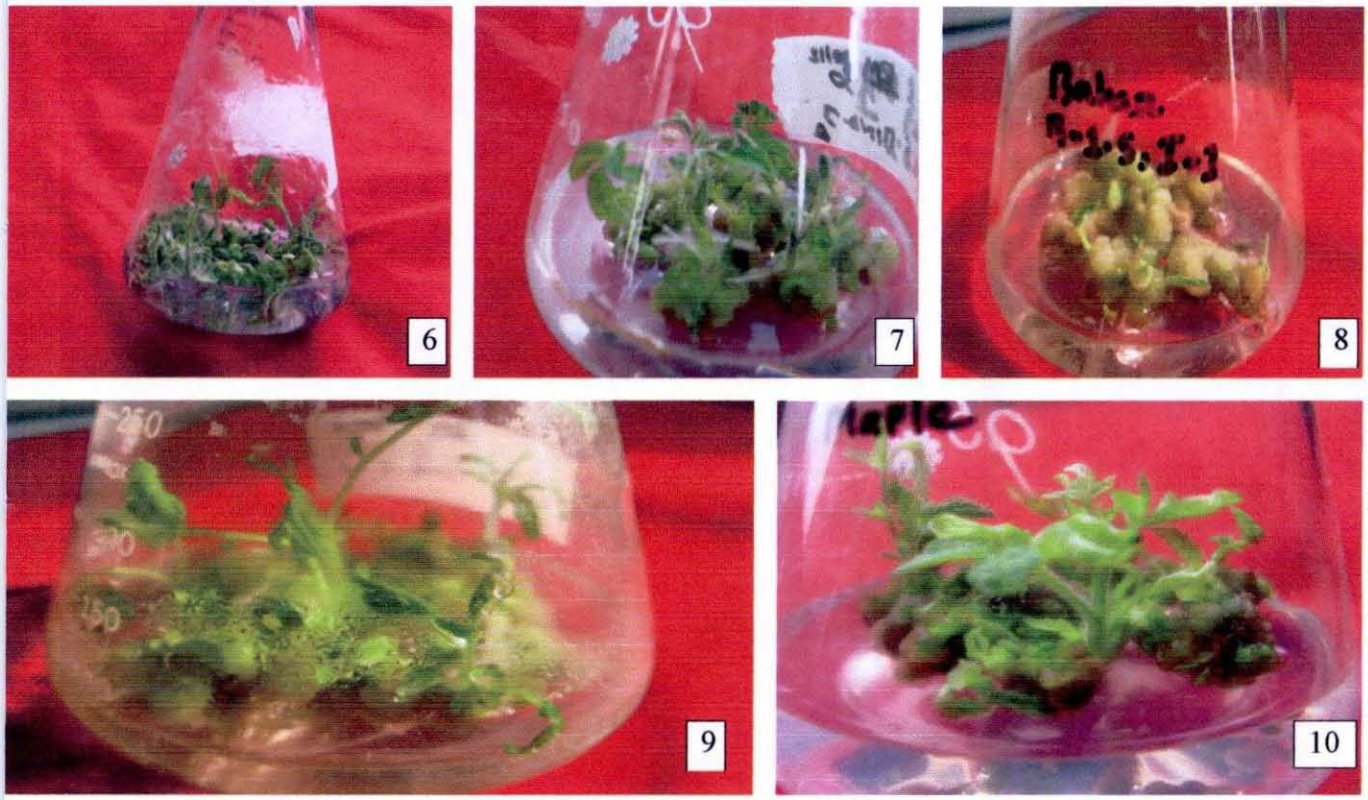


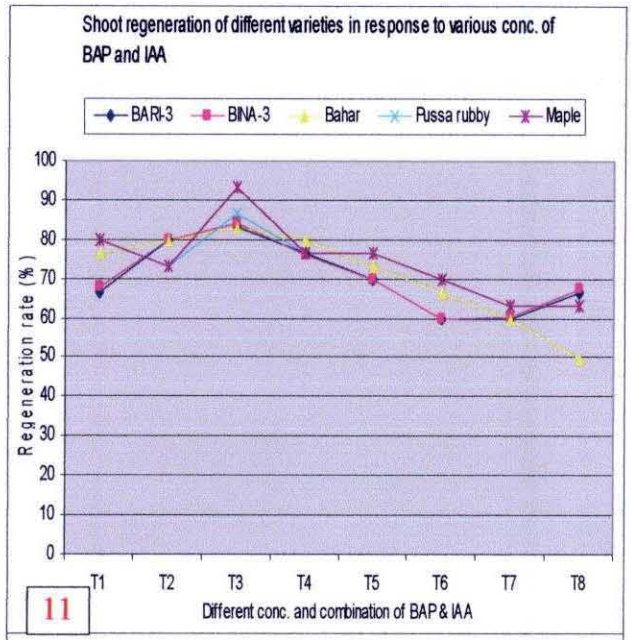
Fig.6 Multiple shoot regeneration of BARI-3 in MS medium containing BAP-1.5 mg/l and IAA-0.2 mg/l. Photograph had been taken after 75 days.

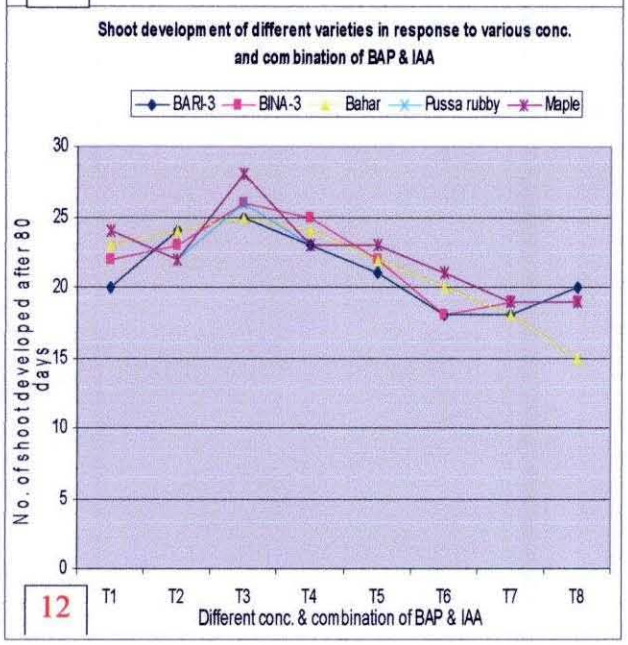
Fig.7. Multiple shoot regeneration of BINA-3 in MS medium containing BAP-1.5 mg/l and IAA-0.2 mg/l. Photograph had been taken after 70 days.

Fig.8.Shoot initiation of Bahar variety in MS medium containing BAP-1.5 mg/l and IAA 0.1 mg/l. Photograph had been taken after 30 days.

Fig.9. Multiple shoot regeneration of PR in MS medium containing BAP-1.5 mg/l and IAA-0.2 mg/l. Photograph had been taken after 74 days.

Fig.10.Multiple shoot regeneration of Maple in MS medium containing BAP-1.5 mg/l and IAA-0.2 mg/l. Photograph had been taken after 72 days.





Treatments	BAP (mg/l)	IAA (mg/l)
T1	1	0.1
T2	1.5	0.1
T3	1.5	0.2
T4	1.5	0.5
T5	1.5	1
T6	2	0.1
T7	2	0.5
T8	3	0.1

Fig.11. Overall shoot regeneration response of all the five varieties toward various concentrations and combinations of BAP and IAA in MS medium.

Fig.12. Total number of shoot developed of all the five varieties in response to various concentrations and combinations of BAP and IAA in MS medium (after 80 days).

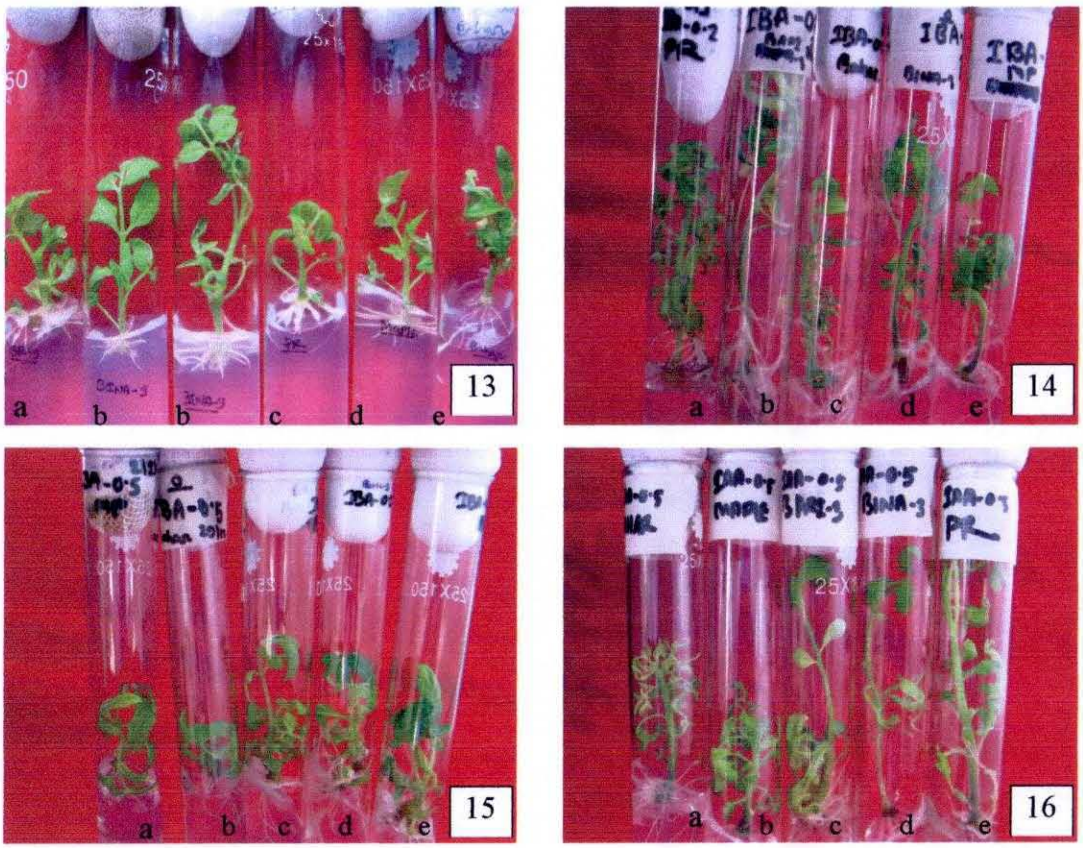


Fig.13. Comparative picture of root development of all five varieties on ½ MS media supplemented with 0.2 mg/l IAA. Photograph had been taken after 7 days (a =BARI-3, b=BINA-3, c=Pussa Rubby, d=Bahar, e=Maple).

Fig.14. Comparative picture of root development of all five varieties on ½ MS media supplemented with 0.2 mg/l IBA. Photograph had been taken after 15 days (a =Pussa Rubby, b=BARI-3, c=Bahar, d=BINA-3, e=Maple).

Fig.15. Comparative picture of root development of all five varieties on ½ MS media supplemented with 0.5 mg/l IBA. Photograph had been taken after 15 days (a =Maple, b=Bahar, c=BARI-3, d=BINA-3, e=Pussa Rubby).

Fig.16. Comparative picture of root development of all five varieties on ½ MS media supplemented with 0.5 mg/l IAA. Photograph had been taken after 15 days (a =Bahar, b=Maple, c=BARI-3, d=BINA-3, e=Pussa Rubby).

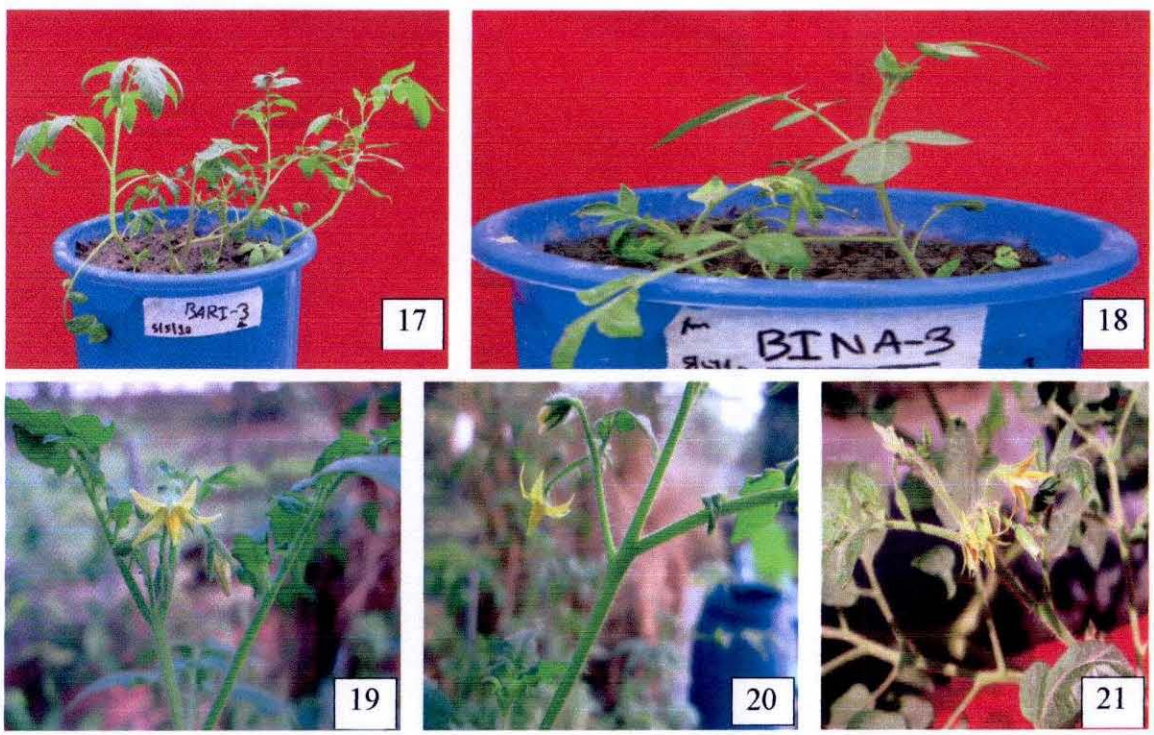


Fig.17. Regenerated plantlets of BARI-3 transplanted into soil in small plastic pots.

Fig.18. Regenerated plantlets of BINA-3 transplanted into soil in small plastic pots.

Fig.19. Flowers blossom in Maple plantlets in natural environment. Photograph had been taken after 35 days of plantation.

Fig.20. Flowers blossom in Bahar plantlets in natural environment. Photograph had been taken after 38 days of plantation .

Fig.21. Flowers blossom in Pussa Rubby plantlets in natural environment. Photograph had been taken after 34 days of plantation .

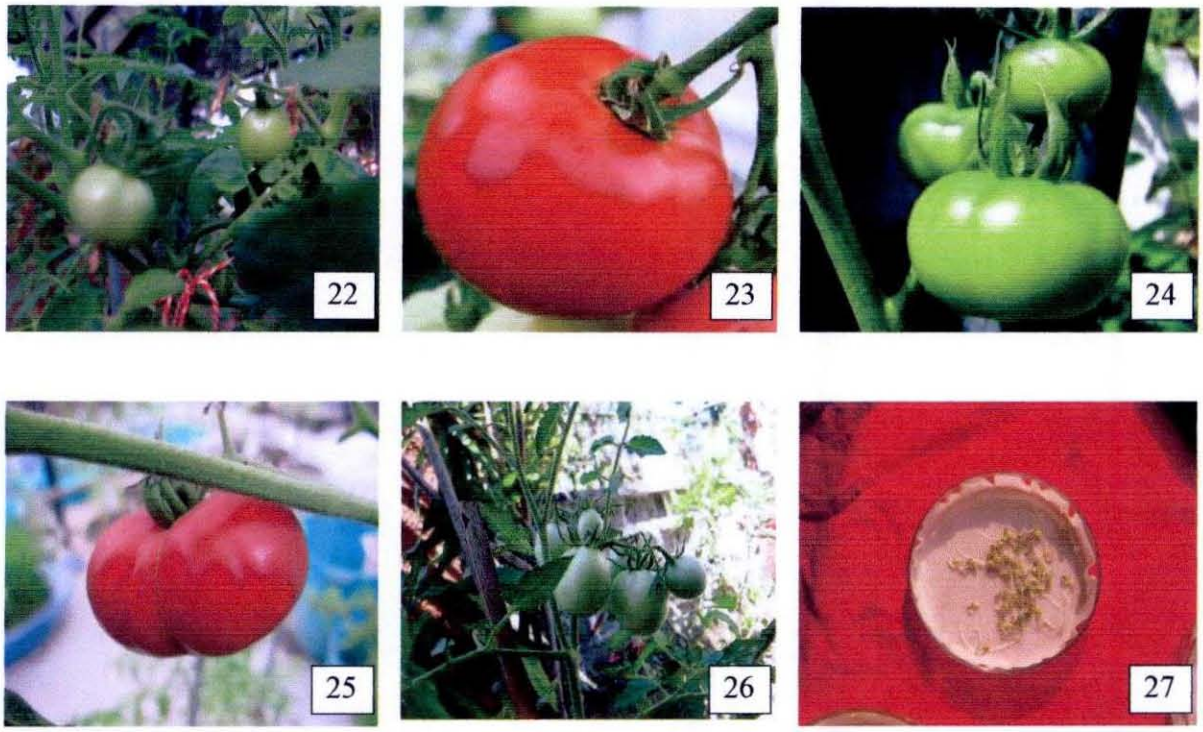


Fig.22. Fruit sets on regenerated plantlets of BINA-3 in natural environment.

Fig.23. Matured fruits on regenerated Bahar plantlets.

Fig.24. Matured fruits on regenerated BARI-3 plantlets.

Fig.25. Matured fruits on regenerated Maple plantlets.

Fig.26. Matured fruits on regenerated Maple plantlets.

Fig.27. Viability test of seeds collected from regenerated Maple plants. Note that most of the seeds germinated under test condition.

Chapter-5
DISCUSSION

Discussion

The purpose of the investigation was to observe the effects of growth regulators on shoot induction and plant regeneration in four Bangladeshi tomato (*Lycopersicon esculentum* Mill.) varieties, namely, BINA-3, BARI-3, Bahar, Maple with an Indian variety, namely, Pussa Rubby.

Tissue culture protocol which is a prerequisite for genetic transformation, starts with selection of suitable explants and determination of appropriate hormonal supplementation for *in vitro* regeneration. 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; Fray and Earl, 1996; Gubis *et al.*, 2003; Raj *et al.*, 2005; Islam, 2007; Chowdhury, 2008). Among these explants cotyledonary leaf segments have reported to be the most responsive explants for tomato regeneration in various tomato varieties (McCormick, 1991; Fray and Earl, 1996; Gubis *et al.*, 2003; Islam, 2007; Chowdhury, 2008).

During the present study, cotyledonary leaf explants were collected from aseptically grown seedlings. MS medium was used as basal media for *in vitro* regeneration, as it is reported to be the most effective media for tomato regeneration (Mirgish *et al.*, 1995; Costa, 2000; Gubis *et al.*, 2003; Islam, 2007; Chowdhury, 2008).

Cotyledonary leaf explants of several verities reported to give best *in vitro* shoot regeneration response when Zeatin was added in addition to IAA in MS media (Costa *et al.*, 2000; Ahasan *et al.*,2007). 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 explants tissue.

After development of seedlings, cotyledonary leaves were excised into several pieces and placed on regeneration media for development of shoots. In this study, when BAP along with IAA were used, shoot initiation was found in all five varieties. Among all the combinations 1.5 mg/l BAP and 0.2 mg/l IAA showed the best result with lowest callus formation and highest number of shoot in all varieties that were used for this experiment. In this medium, the best response was found for Maple (93.33 %) followed by Pussa Ruby (86.67 %) and for

other varieties, such as, BARI-3, BINA-3 and Bahar gave almost similar response to that combination of the medium. In a report, Chowdhury (2008) reported that 2.0 mg/l BAP and 0.1mg/l IAA were found to be the best for shoot formation for varieties BINA-3, BINA-5, Bahar and Pussa Rubby, but the response was similar to only BAP (2.0 mg/l) supplementation. However, a different report came from Islam (2007) where 1mg/l BAP and 0.1mg/l IAA were found to be the best for shoot formation in compare with only BAP (0.5-5mg/l) supplementation in BARI-3 and PR. On the other hand, Jawahar *et al.*, (1997) reported the same hormonal combination to be the best medium for callus induction. In the present study, 1.5 mg/l BAP and 0.2 mg/l IAA was found to be the best medium for shoot regeneration for all five tomato varieties.

Experiments were carried out in half strength of MS medium with different concentrations of auxins to achieve *in vitro* root formation in all five tomato varieties. In the present study, 0.2 mg/l IAA containing ½ strength MS media showed best response for all five tomato varieties, such as, BINA-3, BARI-3, Bahar, Maple and Pussa Rubby which is similar to the findings of Chowdhury (2008). It was also observed that 0.2 mg/l IAA containing ½ strength MS media showed different root types for different varieties, such as, for BARI-3 and PR root type was long and healthy; for BINA-3 and Bahar root type was fibrous root system; and for Maple root type was tap root system which is almost similar to the findings of Chowdhury (2008). Though rooting occurs in IAA, IBA and NAA supplemented media, IAA has been reported to be more preferred rooting hormone for tomato by many (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 while Sheeja *et al.* (2004) used IAA in half strength of MS media which is similar to the present findings. Another report from Zagorska *et al.* (2004) found rooting in half MS media with 0.2mg/l IBA and 0.5mg/ l GA_3.

During the present study, all the five varieties took about four months to develop plantlet from the initiation of the culture which support the report of Oktem *et al.* (1999) and Chowdhury (2008). After completion of rooting stage, plantlets were acclimatized in natural condition where they flowered and got fruits identical to control plants. Seeds were collected from mature fruits (after ripening) which were viable during germination test.

Among all the five varieties, Maple showed best shoot formation and BARI-3 showed best average number of roots while Chowdhury (2008) found that BINA-3 showed highest shoot formation while Bahar showed highest number of roots per shoot. This variation may occur due to the difference in varieties and also the difference in the media composition.

The survival response of all varieties was in between 80 % to 90 %. Among the varieties, Maple showed the best survival response which was almost 90 % while BINA-3 showed the survival response of almost 80 % which almost similar to the findings of Oktem *et al.* (1999). However, a different report came from Chowdhury (2008) where BINA-3 showed the best survival response. This variation may occur due to the difference in the varieties and also difference in the media composition. Among all the varieties, BINA-3 showed highest number of fruits per plant and highest number of seeds per fruit which support the findings of Islam (2007). Viability response of all the five varieties was in between 75 % to 82.5%. Among the varieties, BINA-3 showed the best viability response (82.5 %) which supports the findings of Oktem *et al.* (1999).

Finally, for all the five varieties, 1.5 mg/l BAP with 0.2 mg/l IAA containing MS media is best for shoots induction and 0.2 mg/l IAA containing ½ strength MS media showed the best response for root formation. But Chowdhury (2008) worked on four different varieties, like, BINA-3, BINA-5, Bahar and Pussa Rubby and found that 2 mg/l BAP and 0.1 mg/l IAA were the best for shoot formation and 0.2 mg/l IAA containing ½ strength MS media for best root formation.

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

Chapter-6
REFERENCES

References

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

Ahmed H, Chaudhry Z, Abbas S, Yasmin A, Rashid H (1995) Tissue culture studies in tomato (*lycopersicon esculentum*) var. Moneymaker. Pakistan Journal of Botany 42(1): 155 -163 .

Amber A, Chaudhury Z, Khan MR, Ali GM (2010) Enhanced regeneration in explants of tomato (*Lycopersicon esculentum* L.) with the treatment of coconut water. African Journal of Biotechnology 9(24): 3634-3644.

Baskaran P, Rajeswari BR, Jayabalan N (2006) Development of an *in vitro* regeneration system in Sorghum [*Sorghum bicolor* (L.)] using root transverse thin cell layers (tTCLs). Turkish Journal of Botany 30: 1-9.

Bahman H, Haleh H (2006) Factors influencing regenration and transformation three elite cultivars of tomato (*Lycopersicon esculentum L).* Pakistan Journal Biological Sciences 9(15): 2729-2733.

BBS (2010) Bangladesh Bureau of Statistics, Ministry of Planning, Govt. of Peoples Republic of Bangladesh.

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

Behki RM, Lesley SM (1976) *In vitro* plant regeneration from leaf explants of *Lycopersicon esculentum* (tomato). Canadian Journal of Botany 54: 2409-2414.

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

Block GB, Patterson B, Subar A (1992) Fruit, vegetables and cancer prevention: A review of the epidemiological evidence. Nutrition and cancer 18: 1-29.

Brasileiro ACR, Willadino L, Carvalheira GG, Guerra M (1999) Callus induction and plant regeneration of tomato (*lycopersicon esculentum* cv. ipa 5) via anther culture. Ciencia Rural 29: 619-623.

Butelli EL, Titta M, Gorgio HS, Mock A, Mantos S, Peterek EGW, Schijjlen M, Hall RD, Bovy AG, Luo J, Martin C (2008) Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nature Biotechnology 2(6): 251-254.

Chowdhury J (2008) Establishment of *in vitro* regeneration and transformation protocol in tomato (*Lycopersicon esculentum* Mill). MS Thesis, BRAC University, Dhaka.

Coenen C, Lomax TL (1997) Auxin-cytokinin interactions in higher plants: old problems and new tools. Trends Plant Science 2(9): 351-6.

Compton ME, Gray DJ (1993) Shoot organogenesis and regeneration from diploid, triploid and tetraploid watermelon. Journal of American Society of Horticultural Science 118: 151-175.

Compton ME, Gray DJ (1994) Adventitious shoot organogenesis and plant regeneration from cotyledons of tetraploid watermelon. Horticultural Science 29(3): 211-213.

Compton ME, Veilleiux RE (1991) Shoot, root and flower morphogenesis on tomato inflorescence explants. Plant Cell Tissue Organ Culture 24: 223- 231.

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

Costa GMC, Nogueira FTS, Otoni WC , Brommonschenkel SH , Figeira ML , Cecon PR (1999) Influence of the antibiotic timentin on plant regeneration of tomato (*Lycopersicon esculentum* Mill) cultivars . Plant Cell Tissue Organ Culture 24:115-121.

Daniell H, Khan MS, Allison L (2002) Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. Trends in Plant Science 7: 84-91.

Daniell H, Khan MS (2003) Engineering the chloroplast genome for biotechnology applications. N. Stewart (ed.) Transgenic Plants: Current Innovations and Future Trends, Chapter 5 of 28 pages, Horizon press UK.

DeRopp R (1954) Kinetin and auxin activity. Plant Physiology 31: 253–254.

Desai NS, Suprasanna P, Bapat VA (2004) Simple and reproducible protocol for direct somatic embryogenesis from cultured immature inflorescence segments of sugarcane *Saccharum* sp. Current Science 87: 764-768.

Dong JZ, Jia SR (1991) High efficiency plant regeneration from cotyledons of watermelon (*Citrullus vulgaris Schrad.*). Plant Cell Replication 9: 559-562.

Dutta RK, Sattar MA, Das ML (2004) BINA, A PROFILE. Third edition. Bangladesh Institute of Nuclear Agriculture, Mymensingh.

FAOSTAT (2005) Food and Agricultural Organization of the United Nations, Statistics. Available from http://faostat.fao.org/site/408/default.aspx.

Foolad MR (2004) Recent Advances in Genetics of salt tolerance in Tomato. Plant cell Tissue Organ Culture 76: 101-119.

Frary A, Earl DE (1996) An examination of factors affecting the efficiency of *Agrobacterium*-mediated transformation of tomato. Plant cell Replication 16: 235-240.

Gaffer J, Tiznado ME, Handa AK (1997) Characterization and functional expression of a ubiquitously expressed tomato pectin methylesterase. Plant Physiology 114: 1547-1556.

Gerster H (1997) The potential role of lycopene for human health . Journal of American College of Nutrition 16: 109-126.

Ghada A, El-Heba A, Abdalla NA (2008) A rapid and efficient tomato regeneration and transformation system. Agriculture and Forestry Research 2 (58): 103-110.

Gubi J, Lajchova Z, Farago J, Jurekova Z (2004) Effect of growth regulators on shoot induction and plant regeneration in tomato (*Lycopersicon esculentum* Mill.). Biologia Bratislava 59(3): 405-408.

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

Guerra M , Christina A, Brasileiro R, Willadino L, Carvalheira GG (1999) Callus induction and plant regeneration of tomato (*Lycopersicon esculentum* CV. IPA 5) via anther culture. Cienc Rural 29(4): 15-24.

Hamid MA (2004) BINA udvabito unnoto krishi progukty porichity. Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh .

Heba AE (2004) Evaluation of some promoter's activity of some DNA plant viruses. Cairo University, PhD thesis.

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

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

Jabeen N, Mirza B, Chaudhary Z, Rashid H, Gulfraz (2009) Study of the regeneration of high yielding tomato (*Lycopersicon esculentum* Mill). Pakistan Journal Biological Sciences 13(1): 2221-2375.

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

Jones JB, Jones JP, Stall RE, Zitter TA (1991) Compendium of tomato diseases. The American Photo pathological Society, APS Press St Paul, MN (73).

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

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

Lakshmanan P, Loh CS, Goh CJ (1995) An *in vitro* method for rapid regeneration of a monopodial orchid hybrid *Aranada deborh* using thin section culture. Plant Cell Replication 14: 510-514.

Ling HD, Kriseleit, Ganal M (1998) Effect of ticarcillin/potassium clavulanate on callus growth and shoot regeneration in agrobacterium- mediated transformation of tomato *(Lycopersicon esculentum* Mill.). Plant Cell Replication 17: 843-847.

M E Hossain, Alam M J, Hakim MA, Amanullah ASM, Ahsanullah ASM (2010) An assessment of physicochemical properties of some tomato genotypes and varieties grown at Rangpur. Bangladesh research publication journal 4(3): 235-243.

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

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

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

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

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

Peixe A, Raposo A, Lourenco R, Cardoso H, Macedo E (2007) Effect of growth regulators on tomato. Canadian Journal of Botany 112: 1211-1320.

Potrykus I, Spangenberg G (1995) Gene transfer to plants. Berlin: Springer, p361.

Pozueta-Romero J, Houlne G, Canas L, Schantz R, Chamarro J (2001) Enhanced regeneration of tomato and pepper seedling explants for *Agrobacterium*-mediated transformation. Plant cell tissue organ culture 67(2): 173-180.

Raj SK, Singh R, Pandey SK, Singh BP (2005) *Agrobacterium*-mediated tomato transformation and regeneration of transgenic lines expressing tomato leaf curl virus coat protein gene for resistance against TLCV infection. Current Science 88 (10) : 1674-1679.

Rao A, Agarwal S (2000) Role of antioxidant lycopene in cancer and heart disease. Journal of College Nutrition 19: 563-569.

Rick CM , Yoder JI (1988) Classical and molecular genetics of tomato: highlights and perspectives. Annual Review Genetics 22: 281-300.

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

Sanchez AC, Angel V, May D, Miguel A, Llanes K, Estrada TAG, Ramírez YJP (2010) Efficient plant regeneration from leaf explants of *Solanum americanum.* African Journal of Biotechnology 9(36): 5830-5835.

Schuetze R, Wieezorrek G (1987) Investigations into tomato tissue cultures, 1: shoot regeneration in primary explants of tomato. Archive fuer Zuechtungsforschung 17(1):3-15 .

Scowcroft WR, Brettell RIS, Ryan SA, Davies PA, Pallota MA (1987) Somaclonal variation and genomic flux. Plant tissue and cell culture, p275-286.

Segui-Simarro JM, Nuez F (2007) Embryogenesis induction, callogenesis, and plant regeneration by *in vitro* culture of tomato isolated microspores and whole anthers. Journal of Experimental Botany 58(5): 1119–1132.

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

Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissue cultures *in vitro*. Experimental Biology 11:118–131.

Tanskley SD, Ganal MW, Prince JP, Vicente MC (1992) High density molecular linkage maps of the tomato and potato genomes. Genetics 132: 1141-1160.

Temjensangba Deb CR (2005) Regeneration and mass multiplication of *Arachnis labrosa* : a rare and threatened orchid. Current Science 88: 1966-1969.

Tiwari and Choudhury (1996) Factors affecting the efficiency of tomato cultivation. Plant Tissue Culture and Biotechnology 19(1): 101-111.

Ugraiah A, Karuppusamy S, Pullaiah T (2010) Micropropagation of *Marsdenia brunoniana* - a rare antidiabetic plant. Plant Tissue Culture & Biotechnology 20(1): 7-12.

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

Watterson JC (1986) Disease of tomato crops. Atherton and Rudich (eds), Chapman and Hill Ltd, New York, p 464-462.

Wing RA, Zhang HB, Tanksley SD (1994) Map-based cloning in crop plants: tomato as a model sysytem. Molecular and General Genetics 242: 681-688.

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

Zamir D, Jones R, Kedar N (1980) Anther culture of male-sterile tomato *(Lycopersicon esculentum* Mill.) mutants. Plant Science Letter 17: 352-361.

Appendix

Murashige and Skoog (MS) media (1962)

Constituents	Concentrations(mg/l)
Macronutrients	
KNO_3	1900
NH_4NO_3	1650
KH_2PO_4	170
$CaCl_2.2H_2O$	440
$MgSO_4$	370
Micronutrients	
$FeSO_4, 7H_2O$	27.80
Na_2-EDTA	37.30
$MnSO_4, 4H_2O$	22.30
H_3BO_3	6.20
$ZnSO_4, 4H_2O$	8.60
$Na_2MoO_4.2H_2O$	0.25
KI	0.83
$CuSO_4, 5H_2O$	0.025
$CoCl_2, 6H_2O$	0.025
Vitamins	
Glycine	2
Nicotinic acid	0.50
Pyridoxine-HCl	0.50
Thiamine-HCl	0.10
Inositol	100
Sucrose	30,000