IN VITRO SELECTION OF CALLI FOR SALT TOLERANCE IN TOMATO (*Solanum lycopersicum* L.)

BY

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CERTIFICATE

This is to certify that thesis entitled, "IN VITRO SELECTION OF CALLI FOR SALT TOLERANCE IN TOMATO (Solanum lycopersicum L.)" submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in GENETICS AND PLANT BREEDING, embodies the result of a piece of bona fide research work carried out by ANJU BISWAS, Registration No: 08-03071 under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

I further certify that such help or source of information, as has been availed of during the course of this investigation has been duly been acknowledged.

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DEDICATED TO

MY

BELOVED PARENTS

LIST OF ABBREVIATION

Abbreviations	Full word
%	Percentage
°C	Degree Celsius
1N	1 Normal
BAP	6-Bezylaminopurine
BARI	Bangladesh Agricultural Research Institute
BBS	Bangladesh Bureau of Statistics
Cm.	Centimeter
CRD	Completely Randomized Design
et al.	And others
etc.	Etcetera
DAT	Days After Treatment
DW	Distilled Water
FAO	Food and Agricultural Organization
g	Gram
g/l	Gram per liter
HgCl	Mercuric Chloride
i.e	That is
IAA	Indole-3-Acetic Acid
Intl.	International
J.	Journal
mg	Milligram(s)
mg/l	Milligram per liter
ml	Milliliter
mM	mili mole
MS	Murashige and Skoog
NAA	Napthalene Acetic acid
NaCl	Sodium chloride
NaOCl	Sodium Hypoclorite
NaOH	Sodium Hydroxide
No.	Number
pH	Negative logarithm of hydrogen ion concentration (-log[H+])
SAU	Sher-e-Bangla Agricultural University
Sci.	Science
Univ.	University

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ABSTRACT

Soil salinity is one of the most important abiotic stresses that limit crop production. Responses of six breeding lines (BD-7755, BD-7757, BD-9008, BD-9011, BD-10122, BD-10123 which were named as G1, G2, G3, G4, G5 and G6 respectively) of tomato (*Solanum lycopersicum* L) to NaCl stress were studied in callus induction. Hypocotyl and cotyledon segments were chosen as explants for callus induction *in vitro*. The six investigated tomato genotypes differed in their callus growth. Tomato seeds were cultured for callus formation and that callus were treated with 0 mM (control), 50 mM, 100 mM, 150 mM and 200 mM NaCl in nutrient solutions. The effect of the stress applied on the callus was evaluated in 10 DAT (Days After Treatment) and 17 DAT. Different concentrations of NaCl in the medium significantly affected the biomass callus size and callus weight of tomato. G2 and G6 showed excellent performance of tolerance up to 50 mM of NaCl. G6 showed better performance under high salt concentrations i.e., at 100 mM and 200 mM but not at low salt stress. It indicates the expression of functional gene occurs at high salt stress. However it is possible to select callus line tolerant to elevated levels of NaCl stress by sudden exposure to high of NaCl, accordingly a NaCl tolerant cell line was selected from hypocotyls and cotyledon derived callus of tomato which proved to be a true cell line variant. The interaction effect of variety and treatment revealed that genotype G6 and G1 were the highest and lowest performer respectively. These findings indicated some salt tolerant tomato genotypes which will be promising for regeneration and for future breeding program. It is quiet necessary to asses accumulation of proline and the anti-oxidant enzymes like Super Oxide Dismutase, Ascorbate Peroxidase and Catalase from the control and stressed callus as they are the indicator of salt tolerance. It is evident that tissue culture technique was able to evaluate several genotypes for salt tolerance into cell level under controlled environment with relatively little space and less time required comparing with such process studies at the whole plant level.

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INTRODUCTION

CHAPTER I

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most important solanaceous vegetable crops in the world in terms of both production and harvested area. Though it is a self crossing annual crop, nowadays, tomatoes are grown round the year. Due to increasing consumption of tomato products, the crop is becoming promising. In Bangladesh, the yield of tomato is not enough satisfactory in comparison to the other tomato growing countries of the World (Aditya *et al.*, 1999). The low yield of tomato in Bangladesh however is not an indication of low yielding potentially of this crop but of the fact that the low yield may be attributed to a number of reasons, viz. unavailability of quality seeds of high yielding varieties, land for production based on light availability, fertilizer management, pest infestation as well as production in abiotic stress conditions especially high salinity.

Salinity is one of the major stress factors among the abiotic stresses. In the world, about 400 million hectares of land are affected by high salinity. In Bangladesh about 1 million hectares of land are affected by high salinity in the coastal regions and it is increasing day by day. Salinity affects almost every aspect of the morphology, physiology and biochemistry of plants and significantly reduces yield (Aazami *et al.*, 2010; Amini and Ehsanpour, 2006; Zhang *et al.*, 2004). As saline soils and saline waters are common around the world, great effort has been devoted to understanding physiological aspects of tolerance to salinity in plants, as a basis for plant breeders to develop salinity-tolerant genotypes. In spite of this great effort, only a small number of cultivars, partially tolerant to salinity, have been developed. Further effort is necessary if the exploitation of saline soils and saline waters that are not currently usable is to be achieved. Salinity affects yield and quality, so that yield characters must be taken into account when breeding for salinity tolerance. But not only yield-related characters are important. As salinity affects almost every aspect of the physiology and biochemistry of the plant, the enhancement of crop salt tolerance will require the combination of several too many physiological traits (Cuartero *et al.*, 2006; Cuartero and Ferna´ndez-Mun˜oz, 1999; Flowers and Yeo, 1995), not simply those directly influencing yield. As salinity in soils is variable and plant tolerance depends

on the stage of plant development, plants should be phenotyped at several salinity concentrations and at the most sensitive plant stage(s).

As salinity is a major factor in limiting crop productivity in semi-arid areas of the world, the selection of salt tolerance lines continues to challenge plant scientists, especially those working in physiology and genetics. If it is possible to use cell and tissue culture techniques, together with conventional breeding and genetic engineering (Mohamed *et al.*, 2011; Shatnawi, 2006; Cano *et al.*, 1998), for the development of plants with increased tolerance to salt stress, the major problem in selecting salt tolerant lines could be the difficulty of screening thousands of plants without a reliable selection for criterion. *In vitro* culture, besides its use as a tool for obtaining salt tolerant plants, may offer potential for quick evaluation of germplasm against salt stress. *In vitro* techniques make it possible to screen the required number of genotypes rapidly since *in vitro* plant exhibit their capacity to withstand the stress (Tewary *et al.*, 2000). In many species like tobacco, grape, rice citrus and carrot salt tolerant lines have been isolated using *in vitro* techniques (Vijayan *et al.*, 2003; Tewary *et al.*, 2000; Ben-Hayyim, 1987).

Tomato is sensitive to moderate levels of salt in the soil. Tomato genotypes' response to salinity is genetic and species dependant and there is too much interest in screening and breeding for higher salt tolerance (Mohamed *et al.*, 2007; Amini and Ehsanpour, 2006). Tomato is also a favorable food crop for *in vitro* and genetic studies due to its low chromosome no i.e., 2n=2x=24 and due to comprehensive knowledge of tomato genetics. A considerable improvement has already been made by exploiting the natural variation through conventional breeding in tomato. Even though the success made in the last century, traditional breeding efforts alone cannot meet the increasing demand of tomato consumers in the 21st century. Therefore, plant cell and tissue culture techniques are being used for the genetic improvement and developing salt tolerant lines of tomato plant throughout the world. Several *in vitro* investigations have been conducted on tomato in different applications. In tomato, a positive correlation between growth of calluses and whole plants has been observed under saline condition (Tal *et al.*, 1978; Perez-Alfocea *et al.*, 1994). The genetics of physiological characters together with other

tolerance components related to metabolic defenses against salinity have to be studied in order to advance the breeding of tomato genotypes tolerant to salinity.

This study was conducted to explore the bioassay so as to establish a reproducible protocol for selecting of different salt tolerant callus lines in tomato in different concentrations of NaCl. With conceiving the above scheme in mind, the present research work has been undertaken in order to fulfill the following objectives:

1. To study the *in vitro* biomass changing of callus under different concentration of NaCl.
2. To investigate the callus induction ability of six genotypes of tomato under different NaCl concentrations.
3. To identify and select the salt tolerant callus lines.
4. To find out the effect of NaCl at varied levels on biomass character of six tomato genotypes by using hypocotyls and cotyledons explants.
5. To establish an effective callus induction protocol for six genotypes of tomato under control and salt stress condition.
6. To develop of somaclonal variant from the salt tolerant calli.
7. To assay the magnitude of genetic divergence in tomato genotypes regarding callus induction.

CHAPTER-II

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

Tomato is one of the popular and most important vegetable crops of Bangladesh and as well as many countries of the world. Tomato is used as a fresh vegetable and can be processed as paste, juice, ketch-up, sauce, powder or as a whole. Nutritionally, it is a significant dietary source of minerals, vitamin A and C, organic acid and essential amino acids. Its centre of origin is presumed to be in the present state of Mexico. It is believed that the tomato was introduced in subcontinent during the British regime. It is popular for its taste, nutritional status and various uses. The crop is adapted to a wide variety of climates ranging from the tropics to a few degree of the Arctic Circle. The present leading tomato producing countries of the world are China, United States of America, Turkey, India, Egypt, Italy, Iran, Spain, Brazil Mexico, and Russia (FAO, 2010).

The crop has received much attention by the researchers on various aspects of its production under different adverse condition especially salinity. Many studies on the genetic variability have been carried out in many countries of the world. The work so far done in Bangladesh is not adequate and conclusive. Nevertheless, some of the important and informative works and research findings so far been done at home and abroad on this aspect have been reviewed in this chapter under the following:

2.1 Tomato

The cultivated tomato, *Solanum lycopersicum* L. is grown worldwide for its fruits. Tomatoes are native to South America, but were brought to Europe sometime in the 1500s, where they soon became popular and were exported around the world. For long time tomatoes were known by the name *Lycopersicon esculentum* Mill. but recent work by scientists has shown that they are really part of the genus *Solanum* – as Linnaeus recognized when he first described the species. Today scientists and plant breeders all use the name *Solanum lycopersicum* for the cultivated tomato. Tomato is a favorable food crop for genetic and *in vitro* study for its low chromosome number and easy *in vitro* handling. Tomato is also amenable to physiological and cytogenetic investigation due to its

genetic uniformity resulting from autogamy (Rick, 1980). The wild species of tomato bear a wealth of genetic variability. Less than 10% of the total genetic diversity in the *Lycopersicon* gene pool is found in *L. esculentum* (Miller and Tanksley, 1990). The center of diversity for tomato is located in western South America, and the cherry tomato *L. esculentum var. cerasiforme* is considered as the most likely ancestor of cultivated tomatoes. Karyotypes of the *Lycopersicon* species are very similar with little or no structural difference among species (Barton, 1950). As a crop plant, tomato is one of the best-characterized plant systems. It has a relatively small genome of 0.95pg or 950 Mb per haploid nucleus, (Arumuganathan and Earle, 1991) and features such as diploidy, self pollination, and a relatively short generation time make it amenable to genetic analysis. Tomato is moderately tolerant to salinity and is typically cultivated in regions that are exposed to soil salinization (Cuartero *et al.*, 1999).

2.1.1 Tomato status in Bangladesh compared to world aspects

Many developing countries like Bangladesh benefited from the green revolution in cereal production in the past but were not able to substantially reduce poverty and malnutrition. Vegetable production can help farmers to generate income which eventually alleviate poverty. Among the vegetables tomato is one of the most important vegetables in terms of acreage, production, yield, commercial use and consumption. At present 6.10% (BBS, 2005) area is under tomato cultivation both in winter and summer. It is the most consumable vegetable crop after potato and sweet potato occupying the top of the list of canned vegetable (Chowdhury, 1979). It is cultivated all over the country due to its adaptability to wide range of soil and climate (Ahmed, 1976). However, the yield of the crop is very low compared to those obtained in some advanced country (Sharfuddin and Siddique, 1985). In Bangladesh congenial atmosphere remains for tomato production during low temperature winter season that is early November is the best time for tomato planting in our country (Hossain *et al.*, 1986). It is a good source of vitamin C (31 mg per 100g), vitamin A, calcium, iron etc. (Matin *et al.*, 1996). Although tomato plants can grow under a wide range of climatic conditions, they are extremely sensitive to hot and wet growing conditions, the weather which prevails in the summer to rainy season in Bangladesh. But limited efforts have been given so far to overcome the high temperature

barrier preventing fruit set in summer-rainy (hot-humid) season. Its demand for both domestic and foreign markets has increased manifold due to its excellent nutritional and processing qualities (Hossain *et. al*, 1999). Considering the growing demand and importance of tomato, Bangladesh Agricultural Research Institute (BARI) has taken initiative to develop off-season summer and rainy season tomatoes. So far BARI has developed and released 2 hybrid tomato varieties i.e. BARI hybrid tomato-3 and 4 which can be grown during summer and rainy season under polytunnel. Bangladesh is producing a good amount of tomatoes. In Bangladesh tomato has great demand throughout the year but it is available and cheaper during the winter season. In Bangladesh it is cultivated as winter vegetable, which occupies an area of 58854 acres in 2009-10 (BBS, 2010). The total production of tomato was 339 lac tons in China, 137 lac tons in USA, 109 lac tons in Turkey, 103 lac tons in India and 92 lac tons in Egypt in 2008 (FAO, 2010). The total production of tomato was 190 thousand metric tons in Bangladesh in the year of 2009-2010 (BBS, 2010). The average tomato production in Bangladesh is 50-90 tons/ha (BARI, 2010). The best tomato growing areas in Bangladesh are Dinajpur, Rajshahi, Dhaka, Comilla and Chittagong.

2.1.2 *In vitro* investigations in tomato

Several *in vitro* investigations have been conducted on tomato in different applications i.e., production of virus free plants (Moghaieb *et al.*, 2004), genetic transformation (Park *et al.*, 2003) and studies about the effect of variety and plant growth regulators on callus proliferation and regeneration (Chaudhry *et al.*, 2007). Most of the reports about adventitious regeneration in tomato deal with induction of regeneration in hypocotyls or cotyledon explants (Moghaieb *et al.*, 2004, Raiziuddin *et al.*, 2004; Brichkova *et al.*, 2002). Shoot formation from different explants as apical meristem, cotyledons, stems internodes, leaves, anthers and inflorescences has been reported in tomato (Afroz *et al.*, 2010; Jatoi *et al.*, 1999; Compton and Veilleux 1991; Branca *et al.*, 1990; Young *et al.*, 1987). *In vitro* anther culture stands out and is an increasingly powerful tool when integrated into breeding programs (Jose, 2007; Hu and Zeng, 1984). This technique allows the acceleration of plant breeding by providing homozygous doubled haploids within a comparatively short time (Nurhidayah *et al.*, 1996). In addition, obtaining haploid plants

from segregant generations facilitates genetic analysis, eliminating the complexity of the heterozygous state (Moraes-F, 1990). Improving the quality of *in vitro* cultured shoots of tomato by using activated charcoal and ascorbic acid is evaluated by Bhatia and Ashwath (2008). *In vitro* culture of immature seed for rapid generation advancement in tomato studied by Bhattarai *et al.* (2009). This offers an opportunity for rapid generation advancement aimed towards population development when coupled with marker assisted selection in tomato breeding for biotic and abiotic stress tolerance. Intra and interspecific variability of *in vitro* culture response in tomatoes were performed by Pratt *et al.* (1997). Embryogenesis induction, callogenesis, and plant regeneration by *in vitro culture* of tomato isolated microspores and whole anthers discussed their application to the production of doubled-haploid plants in tomato (Simarro and Nuez, 2007). Micropropagation in *Lycopersicon* could be a useful option when a large number of rare genotypes (such as interspecific hybrids) is required. As in other crops (Jarret *et al.*, 1980; Bayliss and Dunn, 1979; Pence *et al.*, 1979; Baroncelli *et al.*, 1973), different responses (callus production, regeneration of whole plants, roots and pseudo-fruit differentiation have been reported in tomato, depending on the genotypes, explants, culture media and incubation conditions (Zorzoli *et al.*, 1993a, b; Kurtz and Lieneberger, 1983; Locky, 1983; Kut and Evans, 1982; Tal *et al.*, 1977; Kartha *et al.*, 1976). Engelmann *et. al.* (2010) established and screened multiple *in vitro* tomato cell lines for carotenoid production, test the best producers, and to use the greatest carotenoid accumulator for *in vitro*. *In vitro* selection and screening of abiotic stress tolerant cell lines for genetic variability analysis have been reported in tomato (Martinez *et al.*, 1996; Mercado *et al.*, 2000; Cano *et al.*, 1998; Yusuf *et al.*, 1994; Buiatti *et al.*, 1984).

2.2 *In vitro* culture

"*In vitro*" is a Latin word means in glass (American Psychological Association, 2010; Cheryl, 2007) and *in vitro* culture is the technique or process of maintaining or cultivating cells or tissues derived from a living organism in a culture medium. Studies that are *in vitro* are performed with cells or biological molecules studied outside their normal biological context. For example proteins are examined in solution, or cells in artificial culture medium. Colloquially called "test tube experiments", these studies

in biology and its sub-disciplines are traditionally done in test-tubes, flasks, petri dishes etc. They now involve the full range of techniques used in molecular biology such as the so-called omics. Studies that are conducted using components of an organism that have been isolated from their usual biological surroundings permit a more detailed or more convenient analysis than can be done with whole organisms. *In vitro* studies include cells derived from multicellular organisms (cell culture or tissue culture).

2.2.1 Callus induction and proliferation

Callus Induction and Proliferation Callus production is an essential step in the use of tissue culture studies for various physiological phenomena including resistance against various abiotic stresses. Callus is an unorganized, proliferative mass of predominantly parenchyma cells. Studies have revealed that better response of callus was obtained when callus cultures were kept in dark. Chen *et al.*, (1988) reported that morphogenic callus could form most readily from the leaf explants with most proliferating callus when kept in dark. Aftab *et al.*, (1996) have also reported that embryogenic callus could be obtained from young leaves on modified MS medium under dark conditions. Studies have suggested that amongst all the media tested for callus induction and proliferation by different workers, the best medium was modified MS (Murashige and Skoog, 1962) medium (Liu and Chen, 1974; Guiderdoni, 1986; Aftab *et al.*, 1996, Baksha *et al.*, 2002). Role of auxins have also been studied for callus induction and proliferation. Nadar *et al.*, (1978) found that embryogenic callus forms when auxin is added to the medium. On the other hand, no embryogenesis was observed in callus cultures on auxin-free media. Callus proliferation in modified MS medium with various levels of auxins and cytokinins was also reported by Bhansali and Singh (1982). Studies have shown that amongst different auxins tested for callus induction, addition of 2, 4-D in the medium always produced better callus growth than any other growth regulator. Kulkarni (1989) reported that callus induction and proliferation from immature leaves triggers on medium containing 2,4-D. Karim *et al.*, (2002) also observed that highest percentage of callus induction was obtained on MS basal medium supplemented with 3.0 mg l-1 2,4-D and 10 % coconut milk. Similarly, Mamun *et al.*, (2004) also found that among all the tested auxins (IAA, 2,4-D, IBA, and NAA), the best performance for callus induction was obtained on 3.0 mg l-1 2,4-D. Studies have

further indicated that use of 2,4-D in MS medium not only results in an earlier (7-10 days) callus induction but it also improves the callus proliferation (Nagai *et al.*, 1991; Islam *et al.*, 1996). In a study by Snyman *et al.*, (2000), it was found that a good mass callus could be obtained on MS medium containing 3.0 mg l-1 2,4-D after 3-4 weeks of inoculation. Ramanand *et al.*, (2006) also found that when the young meristematic leaf sheath explants were aseptically inoculated on agar (7.5 g l-1) gelled Murashige and Skoog (MS) medium containing 20 g l-1 sucrose and different concentrations of NAA, IBA and 2,4-D for callus formation, maximum (67.3 %) explants showed callus initiation within 10-14 days at 4.0 mg l-1 2,4-D. The addition of kinetin and coconut water to the callus initiation medium was found to be inhibitory to embryogenesis (Fitch and Moore 1993). On the contrary, the role of 2,4-D in the medium has also been reported to be crucial in obtaining embryogenic calluses in sugarcane. Studies have suggested that the addition of 2,4-D in the medium favors the formation of embryogenic callus (Himanshu *et al.*, 2000; Snyman *et al.*, 2001). Foranzier *et al.*, (2002) have also supported the observation that mostly embryogenic callus forms in the medium supplemented with 2,4-D. In another study by Marcano *et al.*, (2002), efficient embryogenic callus formation was achieved using young leaf explants cultivated on modified MS medium containing 13 µM 2, 4-D. Niaz and Quraishi (2002), however, found that the use of NAA in addition to 2,4-D improves the callus and embryogenic response. They found that 1 mg NAA and 3 mg l-1 2,4-D was optimal for embryogenesis. Like many earlier findings, Gandonou *et al.*, (2005) also reported that embryogenic callus could be obtained in sugarcane on MS medium supplemented with 3 mg l-1 2,4-D. Although 2,4-D has proven efficiency for good in vitro response, thidiazuron (TDZ) has also been used for this purpose. Gallo-Meagher *et al.*, (2000) established embryogenic callus on MS basal medium having 2,4-D, kinetin and NAA. Various concentrations of thidiazuron were also tested for embryogenic callus induction. It was observed that TDZ has a positive effect on embryogenic callus induction. Use of TDZ for tomato callus induction is however scanty.

2.2.2 *In vitro* selection of callus line

In vitro selection on NaCl-containing media seems a promising approach for selecting cell lines which tolerate salt in their nutritional environment. Despite the complex nature of salt

tolerance expressed by plant cells, there are reports on the *in vitro* isolation of salt tolerant cell lines and the plants regenerated from such cell lines displaying acquired traits of tolerance at the whole plant level (Croughan *et al.* 1981, Winicov 1996). Cell lines with enhanced resistance to salt have been isolated from many plant species (Ochatt *et al.*, 1999; Tal, 1996; Gulati and Jaiwal, 1994). In most studies, NaCl was used as the selection agent. NaCl selection is likely to produce genotypes with resistance to Na^+ and Cl^- ions, but not necessarily to other toxic ions contributing to salinity in certain agricultural situations (Rains *et al.*, 1986). The results obtained with single different salts might differ from those obtained when tissues are grown on salt mixtures to which plants may be exposed in nature. In recent years *in vitro* culture techniques are being used as a useful tool to elucidate the mechanism involved in salt tolerance by using *in vitro* selected salt tolerant cell lines (Gu, *et al.* 2004; Venkataiah. *et al.*, 2004; Davenport, *et al.*, 2003; Lutts *et al.*, 1999; Naik and Harinath, 1987). Besides, these lines have been used to regenerate salt tolerant plants (Chen *et al.*, 2001; Jaiswal and Singh, 2001; Miki *et al.* 2001; Ochatt *et al.*, 1999). The selection of crop varieties for greater tolerance to saline environment will allow greater productivity from large saline lands.

2.3 Salinity

Salinity is a measure of dissolved salts in sea water. It is calculated as the amount of salt (in grams) dissolved in 1,000 grams (1 kilogram) of seawater. Soil salinity is the salt content in the soil, the process of increasing the salt content is known as salinization ("Soil salinity" in Water Wiki, the on-line Knowledge and Collaboration Tool). Soil salinity causes due to the excess accumulation of salts, typically most pronounced at the soil surface, can result in salt-affected soils. Salts may rise to the soil surface by capillary transport from a salt-laden water table and then accumulate due to evaporation. They can also become concentrated in soils due to human activity, for example the use of potassium as fertilizer, which can form sylvite, a naturally occurring salt. As soil salinity increases, salt effects can result in degradation of soils and vegetation. Salinization as a process can result from, high levels of salt in water, landscape features that allow salts to become mobile (movement of water table), climatic trends that favor accumulation, human activities such as land clearing, irrigation, salt runoff from streets (in

winter if the streets are salted for snow). Salinity is detrimental effects on plant growth and yield and soil erosion ultimately, when crops are too strongly affected by the amounts of salts.

2.3.1 Effect of salt on developmental stages of plant and crop production

Salt stress is a polymorphous stress that affects plant growth and reduces yield through four direct ways: First, the presence of salt reduces the ability of the plant to take up water which leads to reductions in the growth rate. This is referred to as the osmotic effect of salt stress, which starts immediately after the salt concentration around the roots increases over a threshold level. There is a second and slower response due to the accumulation of ions in leaves. This ion-specific phase of plant response to salinity starts when accumulated salt reaches toxic concentrations in the leaves. The third one is the oxidative stress linked to the production of reactive oxygen species (ROS), which cause damage to lipids, proteins and nucleic and the last one is nutritional stress (Rao and patil, 2012 ; Vidal *et al.,* 2009; Nublat *et al.*, 2001; Hernandez *et al.*, 2000; Gomez--Cadenas *et al.*1998). Within many species, documented genetic variation exists in the rate of accumul ation of Na^+ and Cl^- in leaves, as well as in the degree to which these ions can be tolerated (Munns and Tester 2008). For most species, Na^+ appears to reach a toxic concentration before Cl^- does. However for some Cl^- considered to be the more toxic ion (Lopez-Climent *et al.*, 2008; Moya *et al.*, 2003).

2.3.2 Mechanism of salt tolerance

The salt tolerance of a plant is the degree to which the plant can withstand, without significant adverse effects, moderate or high concentrations of salt in water on its leaves or in the soil within reach of its roots. In practice, salt tolerance is a relative term. Researchers who assess and describe such phenomena often rely on definitions of degrees of tolerance that are specific to their particular studies. Despite physiological evidence that control of Na^+ invasion of the tissues is a key determinant of salt tolerance (Niu *et al.*, 1995; Yeo and Flowers, 1986), the mechanisms involved in this control are poorly understood. There is an ongoing debate regarding whether Na^+ enters the cells by K^+ transport systems and, if so, what kind of K^+ transport systems could be involved (Amtmann and Sanders, 1999; Walker *et al.*, 1996; Rubio *et al.*, 1995). Based on our present knowledge, two kinds of

transport systems are likely to play a major role in Na^+ transport: transporters of the HKT1 family, with the Arabidopsis member suspected of transporting Na^+ more efficiently than K^+ (Uozumi *et al.*, 2000), and the Na^+/H^+ tonoplastic antiporter, which is suspected to play a role in sequestering Na^+ in the vacuole (Garbarino and DuPont, 1988; Blumwald and Poole, 1985). A report presenting the effects of the overexpression of a Na^+/H^+ tonoplastic antiporter in Arabidopsis has provided the first experimental evidence that control of Na^+ transport within tissues has a great effect on salt tolerance (Apse *et al.*, 1999). Thus, it is important to identify variants altered in functions involved in the control of Na^+ transport and Na^+ accumulation to evaluate the impact of these alterations on salt tolerance.

2.4 Salt tolerance in tomato *in vitro*

In Bangladesh, tomato is important vegetable crop ranks second to potato among vegetable crops based on cultivated area. It is grown throughout the country where irrigation water and arable land are available. Soil salinity is one of the most important abiotic stress that limit crop production (Osman *et al.*, 2011; Debez *et al.*, 2006). Up to 20% of the irrigated arable land in arid and semiarid regions is already salt affected and is still expanding (Muhling and Lauchli, 2003). Although tomato is moderately salt tolerant, it affect adversely in different regions of Bangladesh by salt stress. Hence, there is a need to select salt tolerance cultivar of tomato using modern biotechnological approaches especially *in vitro* studies as it is a very advantageous method for screening of different cultivars for salt stress. In tomato, a positive correlation between growth of calluses and whole plants has been observed under saline condition (Tal *et al.*, 1978; Perez-Alfocea *et al.*, 1994).

2.4.1 Effect of NaCl on callus

The primary characteristics associated with salt tolerance to date are: (i) NaCl transport, (ii) Na+ distribution and (iii) components at the cellular level. Among these three characteristics, cellular components appear to be the most amenable to study and manipulation at the present time. NaCl decrease the growth of calli as the salinity is high (Vaziri *et al.*, 2004). This effect is more in calli grown in presence of light compared to those grown in the dark. Some parts of calli grown in the light show necrosis. The adverse effect of salt is more pronounced on total protein content of callus (Priya *et al.*, 2011).

Previous results also suggest that the proline accumulation in callus is an index of salinity tolerance. NaCl also effects callus color in the medium. Gupta *et al.* (2014) studied the effect of NaCl on callus and obtained yellow-green and compact calli from *in vitro* raised Stevia leaves sub-cultured on MS medium supplemented with 2.0 mg l^{-1} NAA and different concentrations of NaCl (0.05-0.20%).

2.4.2 Genotypic differences of tomato for salt tolerance *in vitro*

Salt tolerance in plants depends primarily on the genotype which determines the alteration on processes such as, exclusion of the salt, uptake and transport of salt by roots, together with metabolic and physiological events occurring at cellular level (Silva, *et al.*, 2003). The selection of salt tolerant lines continues to challenge plant scientists, especially those working in physiology and genetics. Most crop plants, including the cultivated tomato, are sensitive to salinity, although differences between tomato cultivars have been reported (Cano *et al.*, 1998; Rus, *et al.*, 2001). One strategy to reduce the deleterious effects of soil salinity on tomato production is by development of Salt -tolerant cultivars (Nabors *et al.*, 1980). The screening of a large number of genotypes for salinity tolerance under *ex vitro* conditions is rather difficult since it requires a large amount of resources and space and complex interactions between the plant and different soil components. *In vitro* culture, on the other hand, is an ideal system for screening salt-tolerance in plants, since it can be carried out under controlled conditions with limited space and time (Ghoshal and Bajaj, 1984). Therefore, many attempts have been made to screen genotypes *in vitro* using shoot apices. Chandler *et al.,* (1988) screened genotypes of sugar beet, tobacco, Chinese cabbage and canola on media with different salt concentrations. *In vitro* culture of tomato has been successfully exploited for selection of tolerant cell lines for various abiotic stresses under laboratory conditions, by exploiting the genetic variability arising during *in vitro* culture conditions (Buiatti *et al.*, 1984). It requires comparatively less effort and fewer resources than selection under field conditions. Selection for salinity tolerance can be carried out *in vitro*, by culturing explants, callus, cell suspension, protoplasts, embryos or microspores in the presence of screening agent, e.g. NaCl (Cano *et al.*, 1998). *In vitro* selection and screening for salinity tolerance have been reported in tomato by (Yusuf *et al.*, 1994; Cano

et al., 1998; Mercado *et al.*, 2000) An *in vitro* shoot apex culture could be a better system for testing and selecting for salt tolerance (Martinez *et al.*, 1996).

Significant differences were also found among genotypes in several other published reports. Genotypic variation was found when seeds of fourteen tomato (*Lycopersicon esculentum* Mill.) cultivars were germinated under 0, 25, 50, 75, 100 mM NaCl (Mohammad *et al.*, 2006). According to their germination response, the cultivars were selected as, salt-tolerant, moderately salt-tolerant and salt sensitive. Differences were also found from callus of 0.2 g in callus relative growth rate (RGR), fresh and dry weights, proline, Na^+ and K^+ contents from 0.2 g callus of hypocotyls in tomato grown under previous salt levels for four weeks. Rooting parameters are the most useful traits for rapid evaluation and screening of tomato species and segregating populations through *in vitro* shoot apex culture (Cano *et al.*, 1998). He studied the possibility of using *in vitro* shoot apex culture to evaluate salt tolerance of cultivated (*Lycopersicon esculentum* Mill.) and wild (*Lycopersicon pennellii* (Correll) D'Arcy) tomato species and related to the response obtained by callus culture. Both apices and calluses were grown on media supplemented with 0, 35, 70, 105, 140, 175 and 210 mM NaCl, and growth and physiological traits were determined. Most apices of *L. esculentum* did not develop roots from low NaCl levels, whereas the apices of *L. pennellii* were able to develop roots at the different salt levels. This different degree of salt tolerance between *L. esculentum* and *L. pennellii* was not, however, clearly shown on the basis of the shoot growth of the plantlets. The callus response was similar to that shown by the rooting parameters, as callus growth in response to increased salinity was much greater in *L. pennellii* than in the tomato cultivar. K^+ decreased more and proline accumulated less with salinity in shoots of *L. esculentum* compared to *L. pennellii*, whereas the opposite response was obtained in calluses. Liza *et al.* (2013) also evaluated salt tolerance activity of callus in different genotypes of tomato where she used the cotyledon as explants and induced in MS medium which was supplemented with different concentrations of hormones for callus induction.

Performance of salt-tolerant selected genotypes of two processing tomato varieties (*Lycopersicon esculentum* Mill.), Riogrande and Chibli F_1 conducted by Messai *et al.*, (2007) , derived from *in vitro* regeneration under salt stress (34 mM), was evaluated under

greenhouse conditions. This study was conducted with salinized solution culture at 10 mM (control) and 44 mM NaCl. The following parameters were recorded, plant height, plant dry weight, number of tomato fruits/plant, fruit weight and size, fruit yield/plant and fruit quality. Results showed that salt-tolerant plants of both varieties produced fruits with a better quality. There was an increase of total soluble solids content (+47% Chibli F1 and +33% Riogrande, regarding control), fruit firmness (+33% Chibli F1 and +25% Riogrande) and a decrease of fruit juice pH. However, this increase in fruit quality was associated with a decrease in fruit size (-15% Chibli F1 and -20% Riogrande) and weight (-16% Chibli F1 and -19% Riogrande). Nevertheless, a significant increase in fruit yield was observed (+30% Chibli F1 and +20% Riogrande). These findings show also a better salt tolerance of Chibli F1 regenerated plants and they may be useful for exploitation of saline water (34 to 50 mM of NaCl) of Sahel regions in Tunisia.

Smolik *et al.,* (2011) carried out an experiment that characterize phenotype response to salt stress under *in vitro* conditions of four tomato genotypes:'Malinowy Ożarowski', 'Pokusa', 'Awizo' F1 and *Lycopersicon*. Tomato seeds were cultured with 0(control), 50, 75, 100 and 125 mmol·dm-3 NaCl in nutrient solutions. The effect of the stress applied on the morphological traits was evaluated in 14 day-old seedlings. Statistical correlations were found in shoot and root length as well as in the number of roots, with the exception of wild form *L. chmielewskii*. The analysis of variance showed that the highest shoots were grown on 50 and 100 mmol·dm-3 NaCl solution. The shortest shoots were observed in the control. Longer roots (8.6 cm) were developed by the plants from the solutions containing 100 mmol·dm-3 NaCl, the shortest (6.6 cm) – 75 mmol·dm-3 NaCl. NaCl concentration in the medium significantly affected the number of tomato roots.

Emilio *et al* (1998) conducted an experiment that the possibility of using *in vitro* shoot apex culture to evaluate salt tolerance of cultivated (*Lycopersicon esculentum* Mill.) and wild (*Lycopersicon pennellii*(Correll) D'Arcy) tomato species was determined and related to the response obtained by callus culture. Both apices and calluses were grown on media supplemented with 0, 35, 70, 105, 140, 175 and 210mM NaCl, and growth and physiological traits were determined. Most apices of *L. esculentum*did not develop roots from low NaCl levels, whereas the apices of *L. pennellii*were able to develop roots at the

different salt levels. This different degree of salt tolerance between *L. esculentum*and *L. pennelliiwas* not, however, clearly shown on the basis of the shoot growth of the plantlets. The callus response was similar to that shown by the rooting parameters, as callus growth in response to increased salinity was much greater in *L. pennelliithan* in the tomato cultivar. KC decreased more and proline accumulated less with salinity in shoots of *L. esculentum* compared to *L. pennellii*, whereas the opposite response was obtained in calluses. The results obtained in this study suggest that rooting parameters are the most useful traits for rapid evaluation and screening of tomato species and segregating populations through *in vitro* shoot apex culture.

Aazami *et al.,* (2010) was conducted an experiment that the response of calli of six tomato cultivars (*Lycopersicon esculentum* Mill.) to salt stress was investigated under *in vitro* conditions. Callus relative growth rate (RGR), dry matter percentage (DM), osmotic potential and proline content were evaluated. Significant differences were found among cultivars regarding above traits. 'PS-10' had the highest RGR, while 'Roma' had the lowest amount of this trait under salt levels. Any increase in salinity levels in the media led to decrease of RGR and in contrast increased DM and osmotic potential in all treatments compared to control. In all cultivars, proline levels increased in response to salinity stress. High callus formation was correlated with low

proline content. 'PS-10' and 'Imperial' had the highest callus formation and the lowest proline content. Significant differences were recorded in regeneration potential of cultivars under salt treatments. 'PS- 10' possessed the highest and 'Roma' had the lowest regeneration rate. It is concluded that the more the salt tolerant genotype the more is the reduction in osmotic potential and proline content.

Mohamed *et al.* (2011) again proved that a plant tissue culture technique is a good method for the evaluation and screening of plant genotypes for salt tolerance. He studied the *in vitro* evaluations of sodium chloride (NaCl) effects on two tomato cultivars (Pearl and Beril) were investigated with four NaCl levels (0, 25, 50 and 75 mM) using hypocotyl and cotyledon explants. The explants were cultured in MS media having 2.0 mg/l BAP along with different concentrations of NaCl. Sodium chloride stress negatively affected the growth traits and chlorophyll content. Significant differences were noticed between the cultivars followed by different NaCl levels, where the Beril responded superior than that of Pearl. The type of explant showed a difference in their response to shoots regeneration under NaCl stress, where the cotyledon explants achieved best results than hypocotyl explants.

CHAPTER-III

MATERIALS & METHODS

CHAPTER III

MATERIALS AND METHODS

The study was conducted at the Genetics and Plant Breeding Laboratory of Sher-e Bangla Agricultural University, Dhaka, Bangladesh during the period from March, 2013 to June, 2014 to study the *in vitro* selection of salt tolerant callus lines in Tomato. The experiment was done in different steps:

1. *In vitro* seed germination of different genotypes.
2. *In vitro* callus initiation and sub-culturing of callus.
3. *In vitro* salt treatment of different concentration (0Mm, 50Mm, 100Mm, 150Mm and 200 Mm) in six genotypes.

The materials and methods of this experiment are presented in this chapter under the following headings –

3.1 Experimental site

The experiment was carried out at the Genetics and Plant Breeding Laboratory, Sher-e-Bangla Agricultural University, Dhaka during the period of March, 2013 to September, 2014. The place is geographically located at about $24^0$75' North latitude and $90^0$50' East longitude.

3.2 Experimental material

3.2.1 Plant materials

A total of six genotypes of tomato originated from different places of Bangladesh were used in this experiment. The materials were collected from Plant Genetic Resource Centre (PGRC) at Bangladesh Agricultural Research Institute (BARI), Gazipur. The name and origin of these genotypes are presented in Table 1.

Table 1: List of the tomato genotypes used in the experiment

SL. No.	Genotypes No.	Name/Acc No. (BD)	Origin
01	G1	BD-7755	PGRC, BARI
02	G2	BD-7757	PGRC, BARI
03	G3	BD-9008	PGRC, BARI
04	G4	BD-9011	PGRC, BARI
05	G5	BD-10122	PGRC, BARI
06	G6	BD-10123	PGRC, BARI

PGRC=Plant Genetic Research Centre, BARI=Bangladesh Agricultural Research Institute

3.2.2 Laboratory materials

Laboratory preparation was started in early March 2013 by collecting chemical and instruments.

3.2.2.2 Chemicals

MS medium, Sterilizing chemicals (Sodium hypo chlorite NaOCl, 70% ethanol), Sucrose, Agar, NaOH (10 N, 1N), HCl, KCl (3M), NaCl (laboratory grade), Absolute Ethanol, Ethanol (70%), Methilated spirit, NAA (1-Naphthaleneacetic acid), BAP (6-Benzylaminopurine).

3.2.2.3 Instruments

Autoclave, Hotplate with magnetic stirrer, Automatic drying oven, Freezers, Furnaces, Incubators, Laminar Air Flow Chamber, Microwave oven, Pipettes, Plant Growth Chamber, Safety Cabinets, Shakers, Shaking Incubator, Water Purification System, pH meter, Course and fine electric balances, Scalpel, forceps, scissors etc., Culture vials (petridishes, test tubes, culture bottles).

3.3 Culture media

Success of any experiment depends on the culture media, hormone combination, tissue and employing cell. Murashige and Skoog (1962) medium were used with different hormone supplements as culture medium for callus induction. Different concentration of salt was added to the basal MS medium supplemented with auxin and cytokinine. Three types of culture media were used in this study, viz,

1. Hormone free basal MS medium for raising of seedling to get explants.
2. MS medium supplemented with 2 mg/l of BAP and 0.2 g/l of NAA for callus induction.
3. MS medium as 2. supplemented with different salt concentrations (0 mM, 50 mM, 100 mM, 150 mM and 200 mM) for salt stress treatment.

Murashige and Skoog (1962) medium were used with different NaCl concentration as culture medium for selection of salt tolerant callus. The composition of MS medium has been presented in Appendix 1. NaCl were added to MS media as per treatment of the experiment. For the preparation of media, stock solutions were prepared at the beginning and stored in the refrigerator at 4±1°C. The respective media were prepared from stock solutions.

3.3.1 Preparation of the stock solutions

The first step in the preparation of the medium is the preparation of stock solutions of the various constituents of the MS medium. As different media constituents were required in different concentrations, separate stock solutions for the macronutrients, micronutrients, Fe-EDTA (Iron stock), vitamins and growth regulators were prepared separately for ready use.

3.3.1.1 Stock solution of macronutrients (stock 1)

Stock solution of macronutrients was prepared with 10 times of the final strength of the medium in one liter of distilled water (DW). Ten times the weight of the salts required for one liter of medium weighted accurately. All the macronutrient were dissolved one by one except $CaCl_2$. The stock solution of $CaCl_2$ should be prepared separately in order to avoid precipitation. And in this way, dissolved all the salts thoroughly in 750 ml of distilled water and final volume was made up to one liter by further addition of (distilled water) DW. The stock solution was poured into a clean sterilized glass container and stored in a refrigerator at 4°C for ready use.

3.3.1.2 Stock solution of micronutrients (stock 2)

A stock solution of all the micronutrients with 100x concentration is generally prepared. Since copper and cobalt are required in very small quantities, it was preferable to first make a separate stock solution of those two salts (100X) and then an appropriate volume were pipetted and put into the main micronutrient stock solution. This stock solution was also stored in refrigerator at 4°C.

3.3.1.3 Stock solution of iron (Fe-EDTA) (stock 3)

Iron-EDTA should be added fresh and it was made 100 times the final strength of the medium in one liter DW. Here, two constituents, $FeSO_4.7H_2O$ and Na_2EDTA, were dissolved in 750 ml of DW in a conical flask by heating in a water bath until the salts dissolved completely and final volume was made up to one liter by further addition of DW. This stock should be stored in an amber color bottle or a bottle covered with an aluminum foil and stored in refrigerator at 4°C.

3.3.1.4 Stock solution of vitamins (stock 4)

The vitamins used in the present study for the preparation of MS medium were, Myo-inositol (Inositol), Nicotinic acid (Vitamin B_3), Pyridoxin HCl (Vitamin B_6), Thiamine HCl (Vitamin B_1) and Glycin. Each of the vitamins except myo-inositol were taken at 100 times of their final strength in measuring cylinder and dissolved in 400 ml of distilled water. The final volume was made up to 1000 ml by further addition of distilled water. This stock solution was also labeled and stored in a refrigerator at 4°C.

3.3.2 MS Media preparation

To prepare one liter of MS medium, the following steps were followed:

1. 500 ml double distilled water was taken into 1 liter beaker
2. 4.4gm MS mixture was added in this 500 ml double distilled water
3. 30g of sucrose was dissolved in this solution with the help of magnetic stirrer
4. 8 gm of agar was added and finally the whole mixture was then made up to 1 liter with further addition of double distilled water.

3.3.2.1 pH of the medium

pH of the medium was adjusted to 5.7±1 by pH meter with the addition of 1 N NaOH or 0.1 N HCl whichever was necessary.

3.3.2.2 Agar

The media was gelled with 8 g/l agar and the whole mixture was gently heated on microwave oven at 250 °C Temperature for 8-10 minutes.

3.3.3 Preparation of NAA (50X)

A stock solution of NAA with 50x concentration was generally prepared. 0.2 mg NAA was dissolved in few drops of 1N NaOH. In this way, this solution was dissolved in 750 ml of distilled water and final volume was made up to one liter by further addition of DW. The stock solution was poured into a clean sterilized glass container and stored in a refrigerator at 4°C for ready use.

3.3.4 Preparation of BAP (10X)

A stock solution of BAP with 10X concentration was generally prepared. 2 mg BAP was dissolved in few drops of 1N NaOH. This solution was dissolved in 750 ml of distilled water and final volume was made up to one liter by further addition of DW. This stock solution was also labeled and stored in a refrigerator at 4°C.

3.3.5 Preparation of 1N NaOH

40 g NaOH pellets were weighed and dissolved in 900 ml. of sterilized distilled water under stirring condition. The flask in a thermostat at 20^0C and maintain for 1 hour and volume with sterilized distilled water up to 1 L.

3.3.6 Preparation of 70% Ethanol

In a 100 ml measuring cylinder 70 ml 99.9% ethanol was poured. Double distilled water was poured up to the level of 100 ml. The solution was stored in a sterilized glass bottle. This solution was made fresh each time before use.

3.3.7 MS medium supplemented with 50 mM NaCl

The molecular weight of NaCl is 58.44 gm. For preparing MS medium supplemented with 1 M NaCl solution, we needed to add 58.44 g NaCl in 1000 ml of distilled water. So for preparing 50 mM concentration, 2.92g of NaCl was added to the MS medium.

3.3.8 MS medium supplemented with 100 mM NaCl

For 100 mM concentration, 5.84 g of NaCl was added to the 1 L of MS medium.

3.3.9 MS medium supplemented with 150 mM NaCl

For 150 mM concentration, 8.78 g of NaCl was added to the 1 L of MS medium.

3.3.10 MS medium supplemented with 200 mM NaCl

For 200mM concentration, 11.68 g of NaCl was added to the 1 L of MS medium.

3.4 Sterilization

3.4.1 Sterilization of culture media

Fixed volume of medium was dispensed into conical flasks. After dispensing the flasks were covered with aluminum foil paper and marked with different codes with the help of a permanent glass marker to indicate specific hormonal supplement. Then flasks were autoclaved at 15 psi pressure at 121°C for 20 minutes. The medium was then transferred into the culture room and cooled at 24°C temperature before used. Marking is also necessary. Fixed volume of medium was aliquoted into petridishes under laminar hood (Plate 1). After dispensing the petridishes were covered with thin polythene (Swaran wrap) and marked with different codes with the help of a permanent glass marker to indicate specific NaCl supplements. The petridishes containing media could be store at 4^0C until use. Marking was done for identification.

3.4.2 Sterilization of glassware and instruments

Glassware, culture vessels, beakers, petridishes, pipettes, slides, plastic caps, other instruments such as forceps, needles, scissor, spatula, surgical blades, brush, cotton, instrument stand and aluminum foil were sterilized in an autoclave at a temperature of 121°C for 20 minutes at 15psi pressure. Before this, all types of glassware instrument was washed properly by liquid detergent, cleaned with running tap water and finally washed with distilled water.

3.4.3 Sterilization of culture room and transfer area

At the beginning, the culture room was sprayed with formaldehyde and then the room was kept closed for one day. Then the room was cleaned through gently washing the floors, walls and rakes with a detergent. This was followed by careful wiping them with 70% ethanol. This process of sterilization of culture room was repeated at regular intervals. The transfer area was also cleaned with detergent and also sterilized twice in a month by 70% ethanol. Laminar air flow cabinet was usually sterilized by switching on the cabinet. The ultra violate ray kills the microbes inside the laminar airflow. It switches on 30 minutes before working in empty condition and for 20 minutes with all the instruments. The working surface was wiping with 70% ethanol, 30 minutes before starting the transfer work.

3.5 Raising of seedling *in vitro*

Seeds of six genotypes (Table 1) of *Solanum lycopersicum* L. were surface sterilized and germinated under *in vitro* conditions as per standard tissue culture procedure. Briefly, seeds were surface sterilized with 70% ethanol for 1 min followed by sodium hypochlorite, NaOCl (10%) for six minutes and then soaked with sterilized distilled water for 30 min. Seeds were inoculated in petridishes for germination in a hormone free MS (Murashige and Skoog, 1962) basal medium containing 30 g sucrose and 0.8% agar adjusted with pH-5.8.

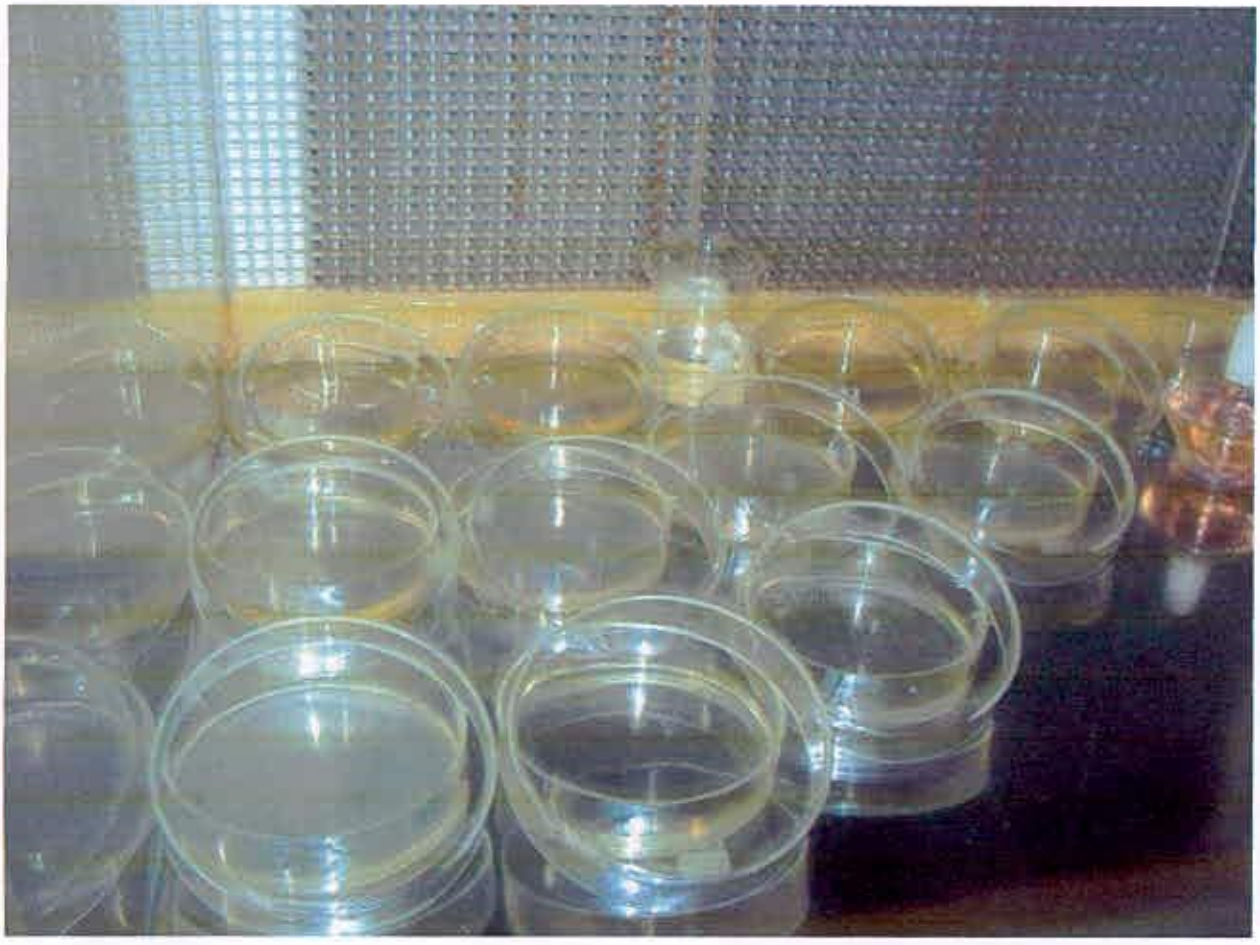


Plate 1. Aliquote of culture media under laminar hood

Twelve seeds were inoculated per petridishes. The cultures were incubated in growth chamber under 16h/8h light/dark photoperiod with the illuminations of white fluorescence lights (50 μmol/ m^2/s^{-1}) at 25±2 °C (Plate 2). Thereafter, three weeks-old seedlings were used for explants source. All of the sterilization and inoculation steps were performed under laminar hood.

3.6 Explants preparation and inoculation

Three weeks after germination, cotyledons and hypocotyls explants were excised by aseptic manipulations. Explants of hypocotyls and cotyledons (0.5 cm) were isolated and inoculated in test tubes containing MS (Murashige and skoog, 1962) medium supplemented with 30 g sucrose, 2 mg/l BAP and 0.2 mg/l NAA. The pH of the medium was adjusted to 5.8 and solidified by 8 g/l agar. The hypocotyls were cut into a lower, middle and upper segment. The explants were placed horizontally on the medium surface, leaf discs explants with the adaxial surface in contact with medium. One explant was cultured in every test tube and test tubes were placed in slope condition. The cultures were incubated in normal growth room conditions (16/8 light/dark regime) having the same light intensity and temperature as above for four weeks.

3.7 Callus induction and subculture

After four weeks, the callus were induced and were the size was optimum to cut into 0.5 cm^2 pieces aseptically and sub-cultured in fresh MS medium with the same ingredients. The calli were sub-cultured as the nutrient media were exhausted and to amplify the calli for salt treatment in different concentrations. The incubation condition was same as mentioned earlier. Within four weeks of sub-culturing, the calli became organized for different salt treatment.

3.8 Salt tolerance assay

The salt tolerance assay was performed as Zeba (2009). Briefly, Four weeks old sub-cultured callus were cut into 0.5 cm^2 pieces under laminar hood and were inoculated in test tubes

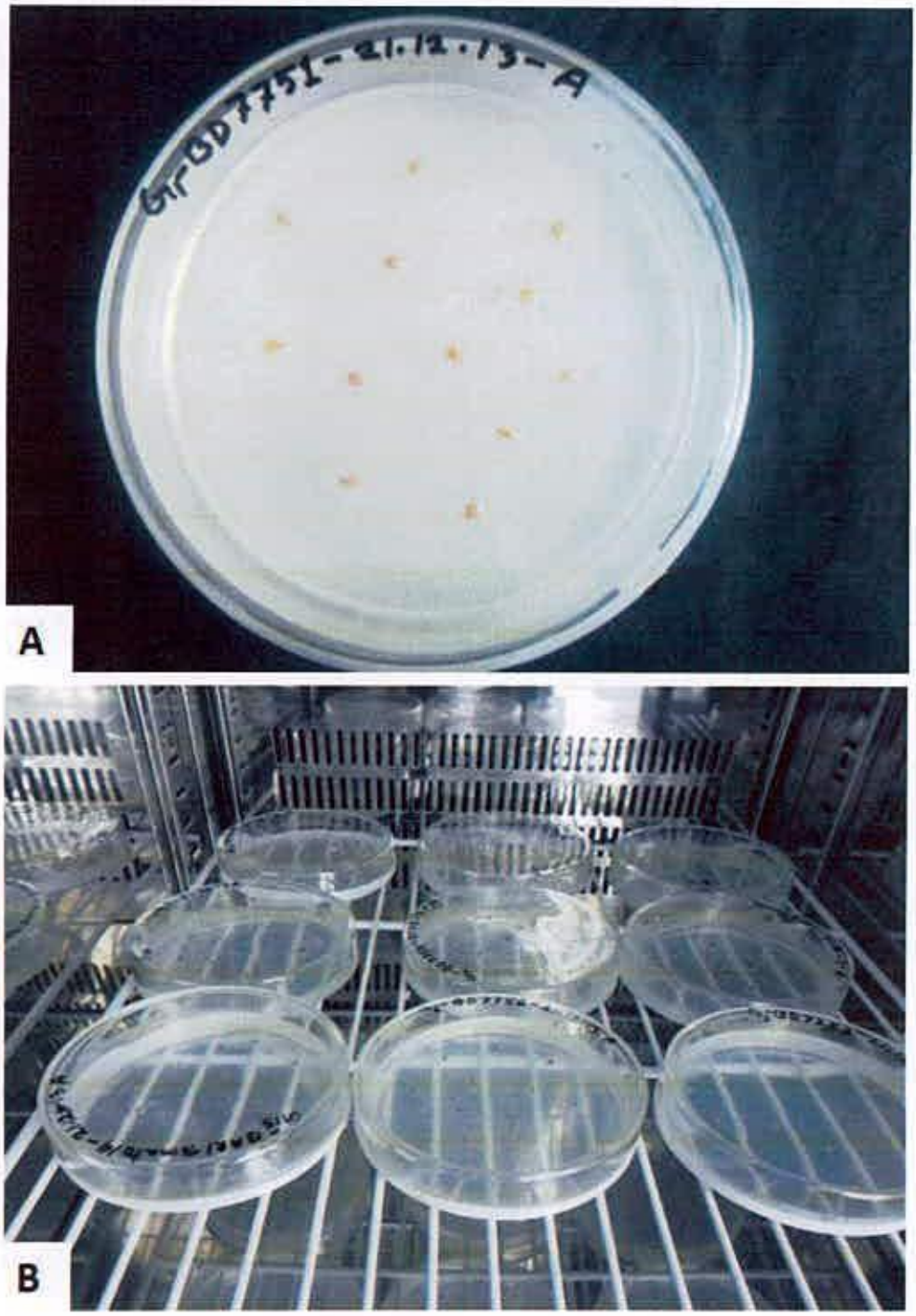


Plate 2: Seed inoculation and Incubation. A. Inoculation of seeds in petridish B. Incubation in growth chamber.

containing MS medium mentioned in section 3.5 supplemented with 0 mM, 50 mM, 100 mM, 150 mM and 200mM of NaCl. One piece of callus of each genotype was inoculated per test tube. The culture plates were kept in the growth chamber in vertical position. The culture environment included, 25±2°C, 60% relative humidity, and a 16-h photoperiod from white fluorescent lights (200 µmol photons/m^2/s^{-1}). The experiment was performed in three replications for each genotype and for each treatment.

3.9 Precautions to ensure aseptic conditions

All inoculation and aseptic manipulations were carried out under laminar air flow cabinet. The cabinet was usually switched on with ultra violet light half an hour before use and wiped with 70% ethanol to reduce the chances of contamination. The instruments like scalpels, forceps, needles, surgical blades, scissor, pipettes, slides, plastic caps, spatula, brush, cotton etc. were pre-sterilized by autoclaving and subsequent sterilization were done by dipping in 70% ethanol followed by flaming and cooling method inside the laminar flow cabinet. While not in use, the instruments were kept inside the laminar airflow cabinet into the instrument stand. Hands were also sterilized by 70% ethanol and wearing of hand gloves. It is also necessary to wear apron and mask to avoid contamination rate. Other required materials like distilled water, culture vessels, beakers, glass plates, petridishes etc. were sterilized in an autoclave following method of media sterilization. The neck of test tubes were flamed before open and also dipping with ethanol with the help of soaked cotton before closing it with the aluminum foil paper. Aseptic conditions were followed during each and every operation to avoid the contamination of cultures.

3.10 Data recorded and statistical analysis

Data were collected and evaluated in terms of the biomass callus weight with digital fine balance and size (diameter) using verneer caliper, after ten days and later after seventeen days of incubation. Tubes were arranged on the shelves of a controlled environment room according to a CRD (completely randomized design). Each tube had a single callus and was considered as an experimental unit. Callus response data were analyzed using MSTAT-C software. The means and the genotype environment interactions were analyzed and the test of significance was performed by DMRT (Duncan's Multiple Range Test).

CHAPTER-IV

RESULTS & DISCUSSION

CHAPTER IV

RESULTS AND DISCUSSION

The experiment was carried out at the Genetics and Plant Breeding Laboratory, Sher-e-Bangla Agricultural University to study the performance of different concentrations of salt on callus initiation and biomass changing of callus in terms of fresh weight and diameter of six genotypes. This study dealt with the *in vitro* selection of different salt tolerant callus lines in tomato. As salinity in soils is variable and plant tolerance depends on the stage of plant development, in this study, calli were phenotyped at several salinity concentrations and at the most sensitive stage (10 days old callus). The genotypes used in this study were, G1 (BD-7755), G2 (BD-7757), G3(BD-9008), G4(BD-9011), G5(BD-10122) and G6(BD-10123) and the salt concentrations were 0 mM (T1), 50 mM (T2), 100 mM (T3), 150 mM (T4) and 200 mM (T5).

4.1 Response of seedlings and callus induction

The seeds of six genotypes were surface sterilized and inoculated in hormone free basal MS medium. The seeds started to germinate within seven days of incubation. Within three weeks of seed inoculation the length of seedlings were in appropriate size to serve as explants source for hypocotyls and cotyledon. The hypocotyls and cotyledon segments from six tomato varieties were used as explants and cultured on MS medium supplemented with NAA and BAP. The hypocotyls and cotyledon were cut into about 0.5 cm^2 size and inoculated in the test tubes. Within 3-4 days the explants became enlarge and start swelling. Within four weeks of culture the swelled explants gradually turned into green callus. For amplification and maintaining, the callus of each genotype cut into pieces aseptically and subculture in the fresh medium. The gradual change of cotyledon and hypocotyls to the callus is presented in (Plate 3). Auxin is NAA is an important media supplements for callus initiation in culture. The combined effect of different tomato varieties and hormone showed significant variation for days required to callus initiation.

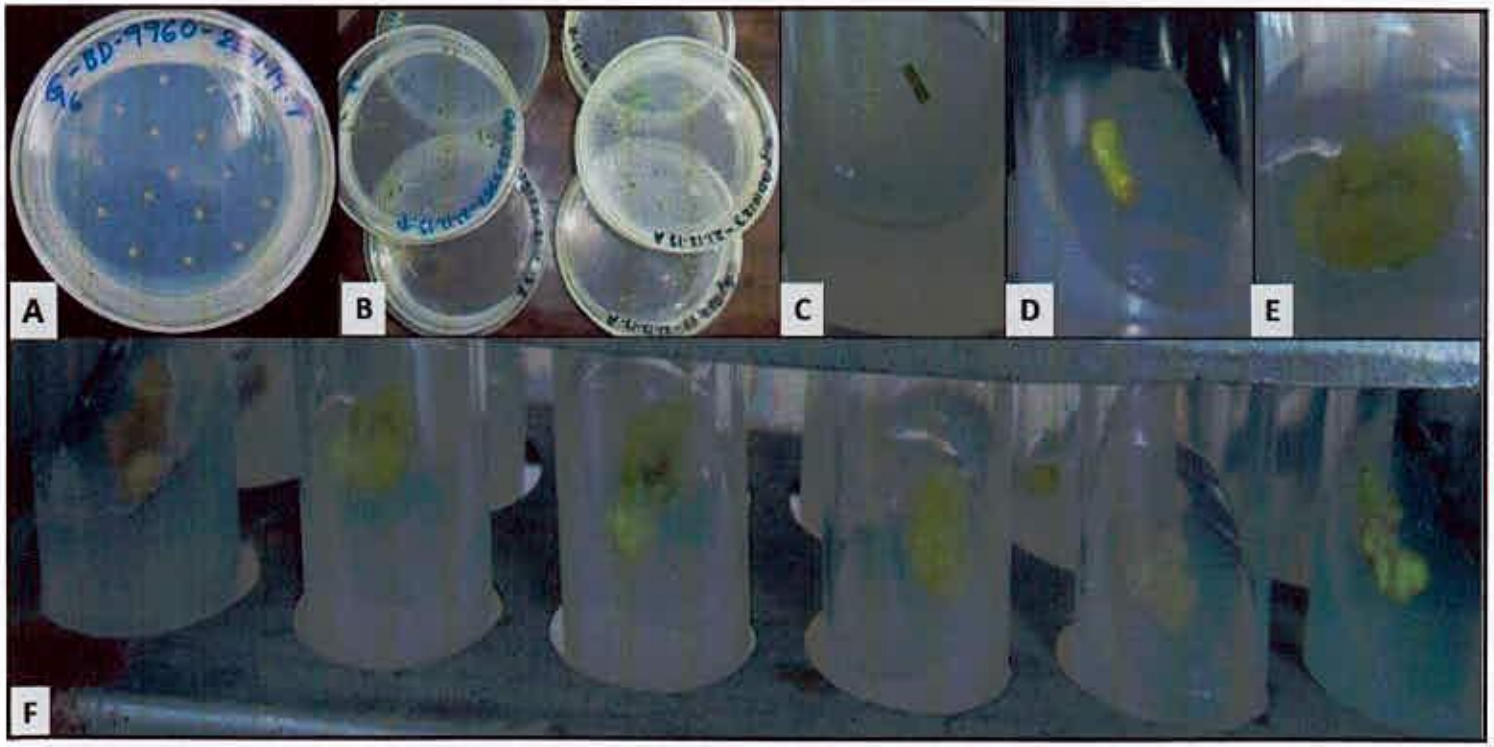


Plate 3. Gradual change of cotyledon and hypocotyls to the callus. A. Inoculation of seed. B. Germination of seedlings. C. Inoculation of hypocotyl. D. Enlargement and swelling of explants.
E. Callus induction. F. Subculture.

4.2 Performance of different genotypes under control and salt stress condition

To investigate the salt tolerance in six genotypes, firstly callus were grown and subcultured and then treated with different salt concentration containing 0 mM, 50 mM, 100 mM, 150 mM and 200 mM NaCl. Callus size and weight is the indicator for salt tolerance. So, the callus diameter and weight were assayed twice after treating with different salt concentrations. Initial weight and diameter of each callus of every genotype were also measured to compare with those data which were taken after 10 days and 17 days after treating NaCl. The callus growth was the highest in control condition (0mM of NaCl) and gradually decreases as the salt stress increases that is 50 mM , 100mM, 150mM and 200mM. The callus diameter and fresh weight were measured and the results obtained from these studies have been presented and discussed separately under different headings. Each of the parameter as influenced by genotypes, treatments and their interactions were discussed below.

4.2.1 Biomass changing of callus under salt stress at 10 DAT

Size and weight of callus of six genotypes were recorded for 10 days after treatment in different NaCl concentration and significant differences were recorded (Plate 4 and Fig. 1). After 10 days of treatment in case of G6 the highest biomass size was recorded up to 50 mM salt concentration and the lowest was recorded in G5 up to 100 mM salt concentrations. In case of 100 mM the highest biomass size was found in G2 and it was static upto 200 mM salt concentration and the lowest size was found in G1 for both 150 mM and 200 mM salt concentrations. Genotypic variation for biomass size is evident in control (0 mM) and in stressed condition (50 mM, 100 mM, 150 mM and 200 mM) (Fig. 1A). Biomass size reduced gradually as the salt concentration increases. Variable callus size was obtained at 10 DAT in different genotypes.

So, the size of root reduction per treatment was evaluated (Fig. 1B). Callus size reduction is negative in G5 from 50 mM to100 mM, that is root was not reduced at 100 mM rather increased than that of 50 mM. G2 also showed better performance at 50 mM. The biomass size was almost similar to control. G4 size reduced at 50 mM but after that at 100 mM it was similar as in 50 mM (Fig. 1B). After 10 days of treatment in case of G6 the highest biomass weight was recorded upto 50 mM salt concentration whereas biomass size of G5was the lowest from 0 mM to 50 mM salt concentrations In case of 100 mM the highest biomass weight was found in G2 and it was static up to 200 mM salt concentration and the

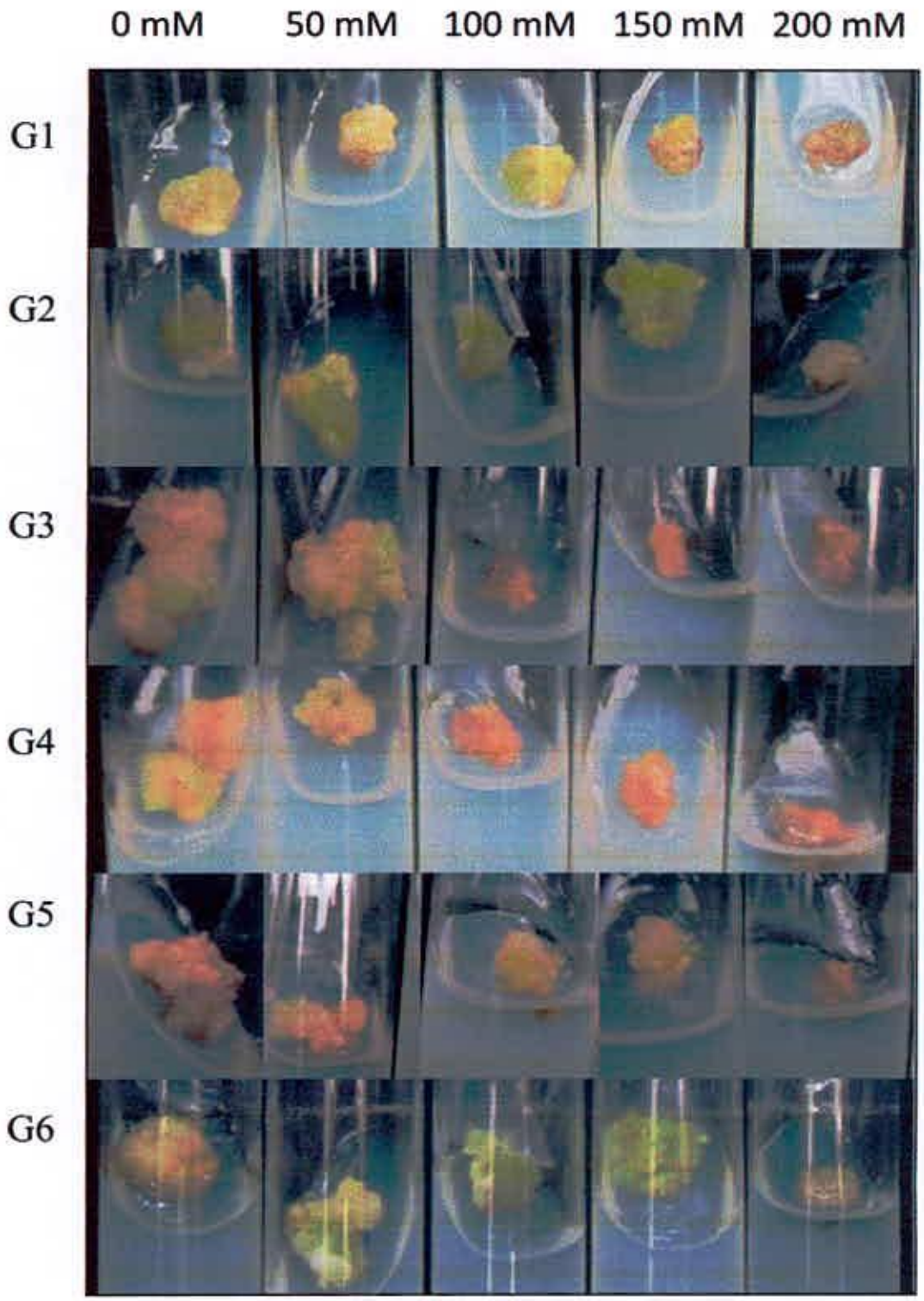


Plate 4: Biomass changing of callus under salt stress at 10 DAT.

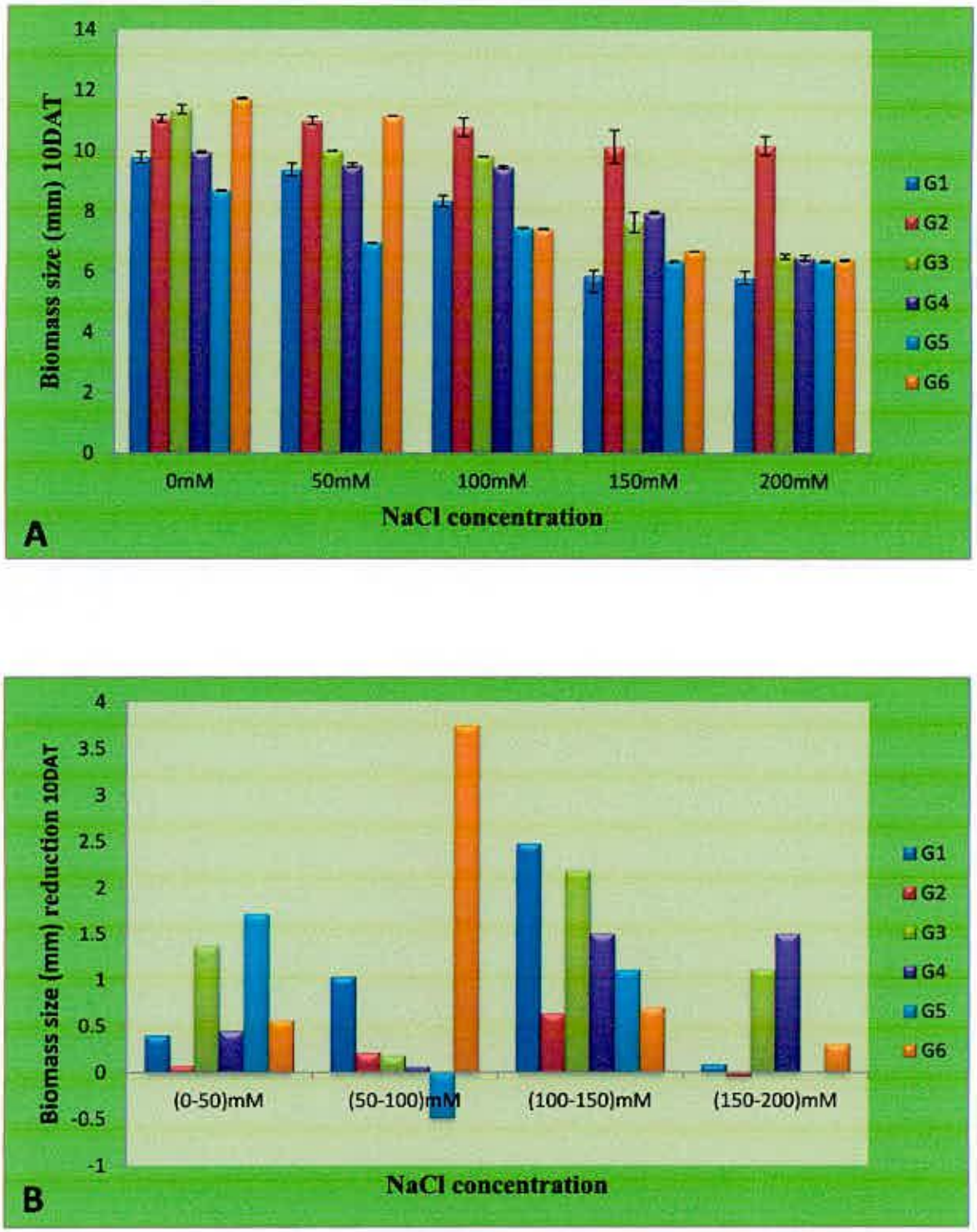


Fig 1. Biomass changing of callus size under salt stress at 10 DAT

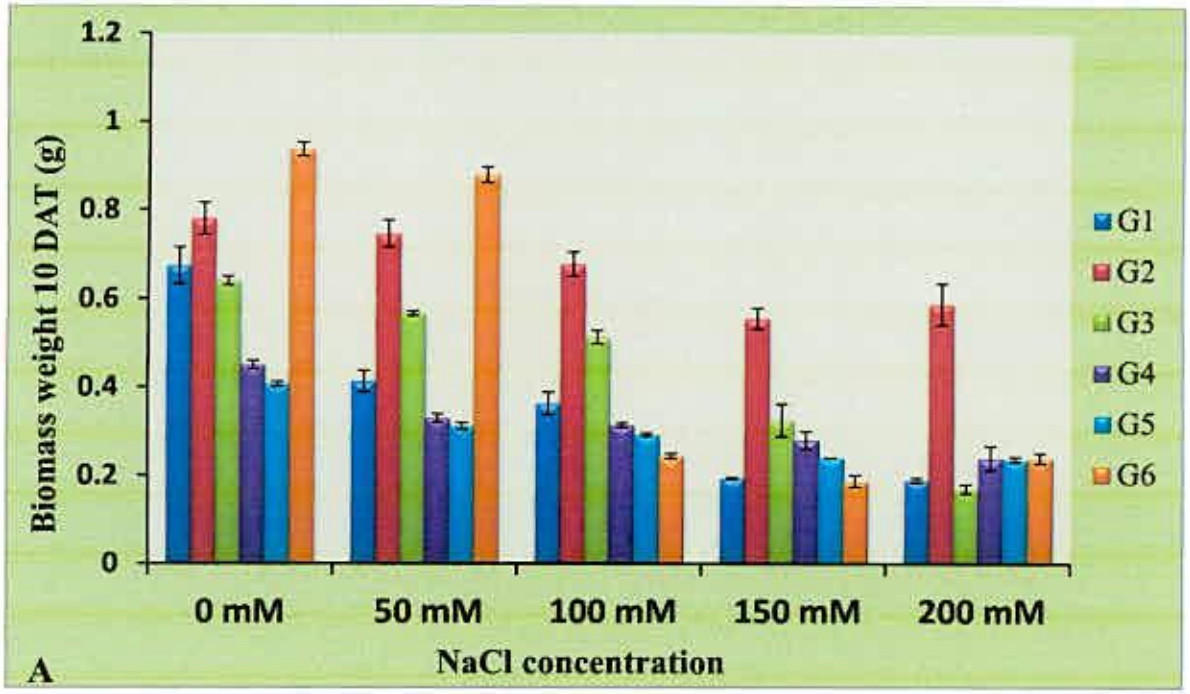
A. Mean Biomass size. B. Mean Biomass size reduction 10DAT

lowest weight was found in G6 for 100 mM and the lowest biomass weight was found in G6 for both 100 mM and 150 mM. In case of 200 mM the lowest weight was recorded for G3. Genotypic variation for biomass weight is evident in control (0 mM) and in stressed condition (50 mM, 100 mM, 150mM and 200 mM) (Fig. 2A). G2 did not loss much weight under 50 mM salt stress (Fig. 2B). Gradually it reduced weight but at severe stress (200 mM) it was showed tolerance to salt (Fig. 2B). Reduction in growth with increasing salinity in growth media may be attributed to water deficit or ion toxicity associated with excessive ion uptake particularly of [Na.sup.+] and [Cl.sup.-] (Satti and Lopez, 1994). Nutrients imbalance as a result of depressed uptake, shoot transport and impaired internal distribution of minerals especially [K.sup.+] and [Ca.sup.+2] may also explained the reduction in plant growth (Munns, 2008).

4.2.2 Biomass changing of callus under salt stress at 17 DAT

Size and weight of callus of six genotypes were recorded for 17 days after treatment in different NaCl concentration and significant differences were recorded (Plate 5 and Fig. 3). After 17 days biomass size was the highest in G4 at 0 mM and the lowest size was found in G3 at 0 mM to 50 mM. From 50 mM to 100 mM the highest size was recorded for G5.The highest size was recorded for G2 from 150 mM to 200 mM whereas the lowest size was recorded for G1 for both of these concentrations (Fig. 3A). The G2 was not significantly reduced in biomass size under 50 mM to 100 mM salt. G6 was significantly reduced under light salt stress (50 mM) but later became stable up to 200 mM salt concentrations. G2 and G3 callus also showed stable condition in size from lower to higher salt stress because size was not significantly reduced from control condition to stress condition (Fig 3B).

After 17 days, biomass weight was the highest in G2 from low stress (50 mM) up to the severe stress (200 mM) and size was almost same in control to severe stress condition indicating its tolerance. The lowest weight was found in G3 from 0 mM to 200 mM (Fig. 4A). There was no significant biomass weight reduction in G2 from control (0 mM) to severe stress (200 mM) condition. G6 has the lowest tolerance up to 50 mM regarding its weight but from 150 mM to 200 mM it recovered and showed slight tolerance (Fig. 4B). The addition of NaCl to the culture media decreased the osmotic potential of the media



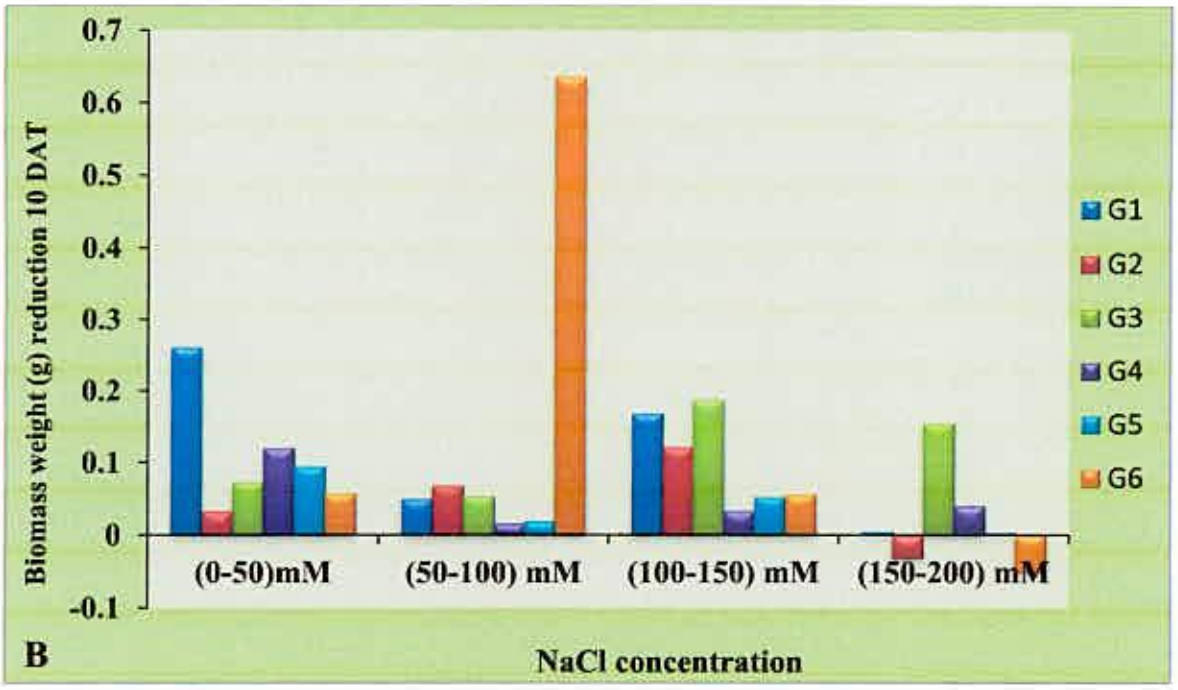


Fig 2. Biomass changing of callus weight under salt stress at 10 DAT

A. Mean Biomass weight. B. Mean Biomass weight reduction at 10DAT

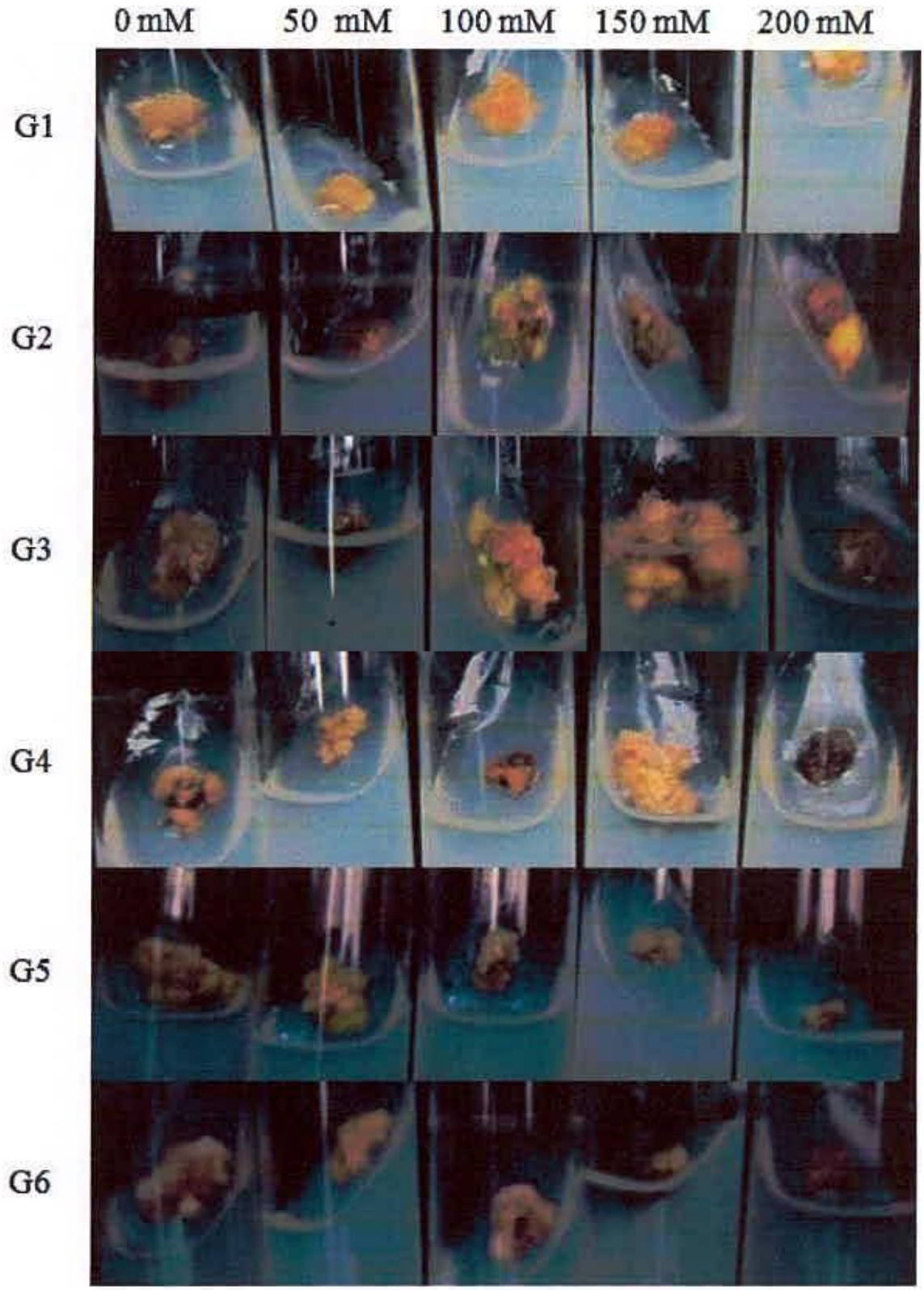
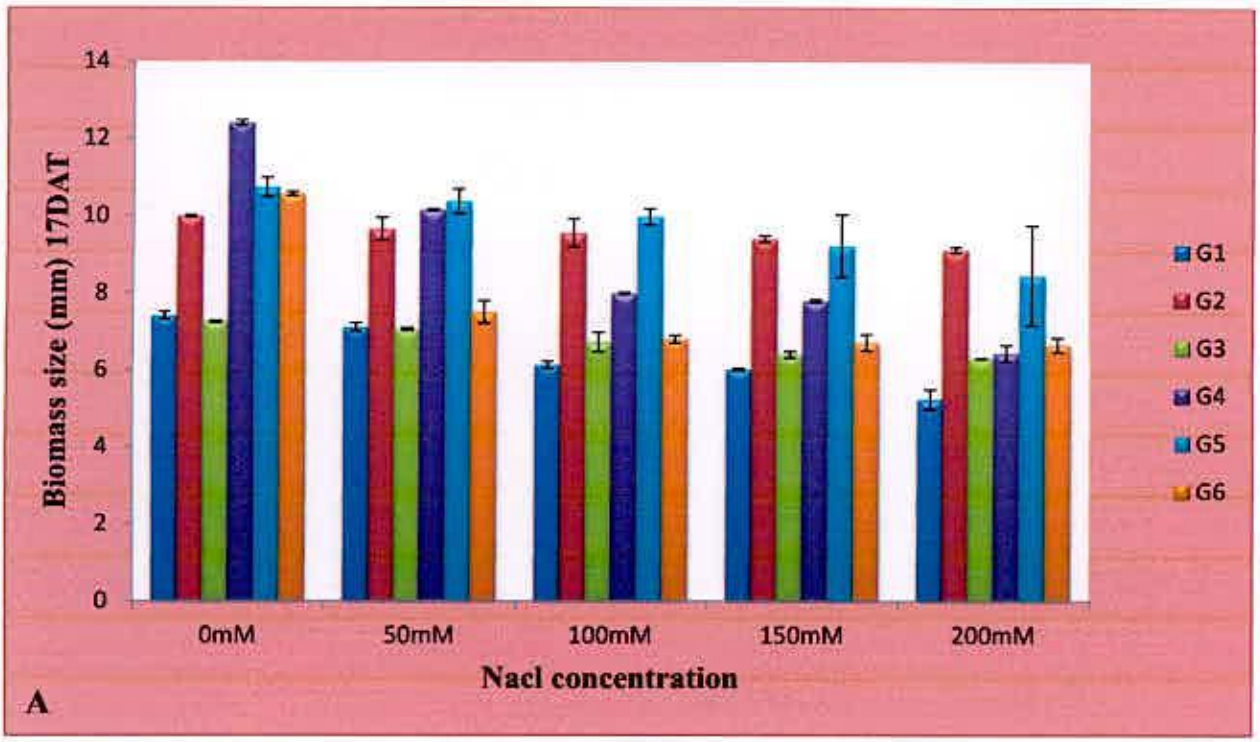


Plate 5: Biomass changing of callus under salt stress at 17 DAT.



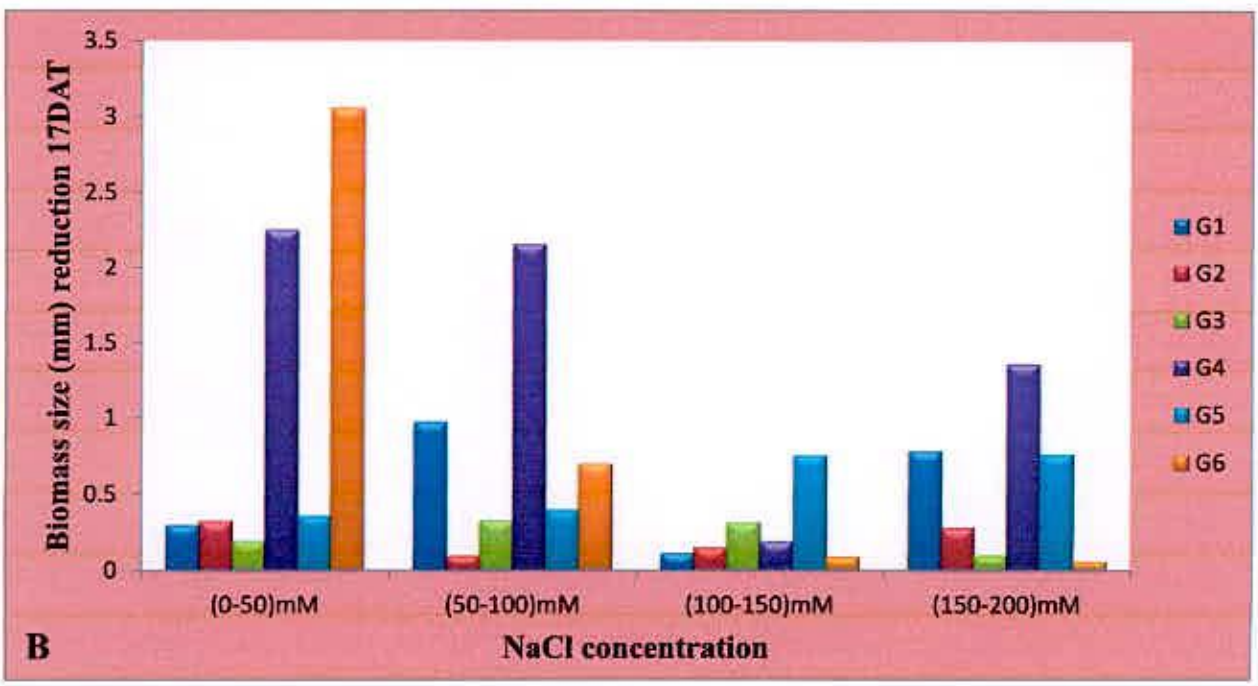


Fig 3. Biomass changing of callus size under salt stress at 17 DAT

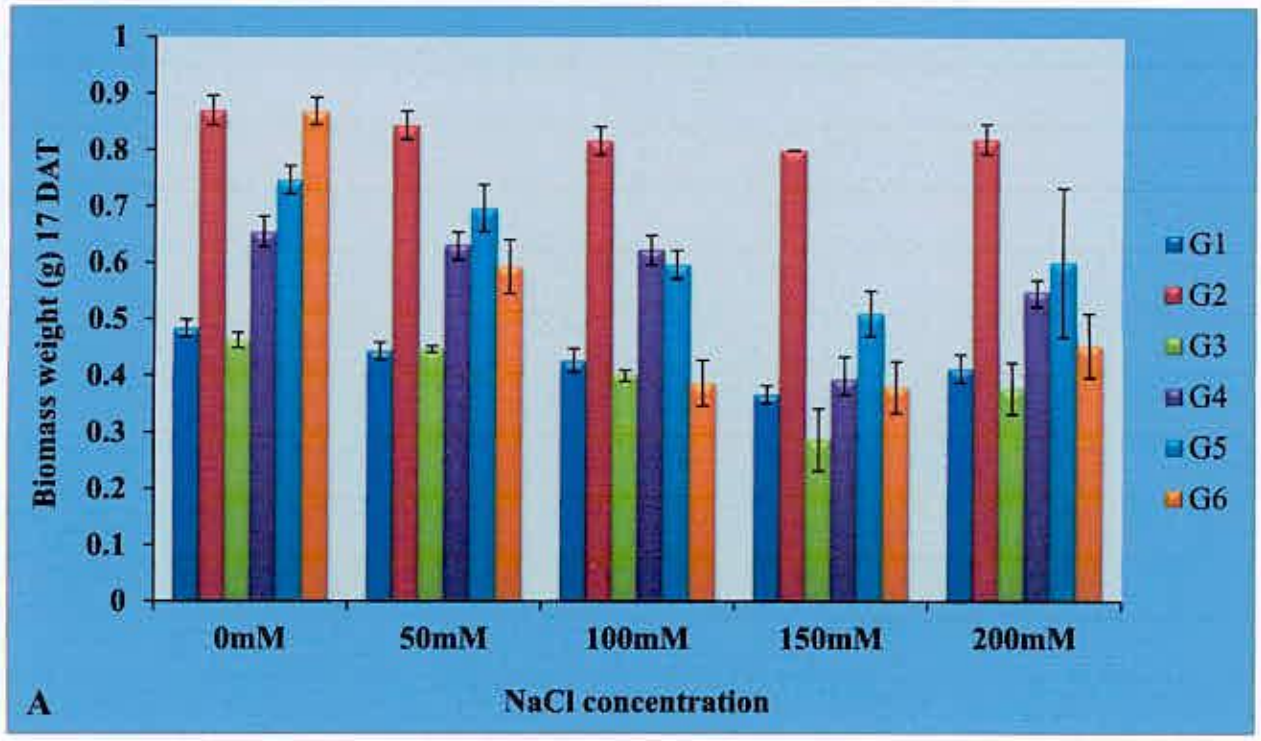
A. Mean Biomass size. B. Mean Biomass size reduction 17DAT

inducing salinity stress that adversely affected the callus growth and in vitro regeneration capacity of tomato cultivars. In this study G2 showed more tolerance than the other genotypes might be due the osmotic adjustment. Yang *et al.* (1990) reported that osmotic adjustment in callus results from both Na + and Cl - accumulation. Similar results have been reported for calli of wheat genotypes (Farrukh, 2002). The tolerance of G2 might also be due to the accumulation of proline under stress condition. Proline protects hydration shell in the cell and helps protect from denaturation of protein. Aazami *et al.* (2010) was also in agreement with this concept. With increasing of NaCl, the proline content of all cultivars significantly increased in the study of Aazami *et al.* (2010). The rates of accumulation were different depending on cultivars and NaCl levels. The results are in agreement with Emilio *et al.* (1998) for *L. esculentum* and *Lecopersicon pennellii*. Marthinez *et al.* (1996) reported a positive relationship between proline accumulation and NaCl tolerance in potato (Mohamed *et al.*, 2007). Salt tolerance also depends on the antioxidative defense. In spite of the large number of publications on the role of antioxidative defense under salt stress, the relative importance of this process to overall plant salt tolerance is still a matter of controversy. More study are needed for the generation and scavenging of reactive oxygen species (ROS) under normal and salt stress conditions in relation to the type of photosynthesis which are directly related to the stress tolerance because they scavenge toxic compounds in the cell.

4.3. Biomass changing in genotype, treatment and their interaction

4.3.1 Biomass changing of callus on genotypes

Salt treatment was given in every genotype and data were taken 10 DAT and 17 DAT. The highest size (diameter) of callus was found in the G2 (10.63 mm) at 10 Days After Treatment (DAT) of callus (Table 2) but it was changed after 17 days and G2 (9.539 mm) and G5 (9.749 mm) both of them were reached in the highest point. The lowest size of callus was found in G5 at 10 DAT (7.153 mm) and at 17 DAT G1 had the lowest size of callus (6.375). The highest weight was found in G2 for both 10DAT (0.6693 g) and 17DAT (0.8100 g). The lowest weight of callus was found in G4 (0.3227 g) and in G5 (0.2980 g) at 10 DAT (Table 2). At 17 DAT, G2 showed the highest callus weight (0.8100 g) and G3 showed the lowest callus weight (0.3593 g). Significant differences were also found



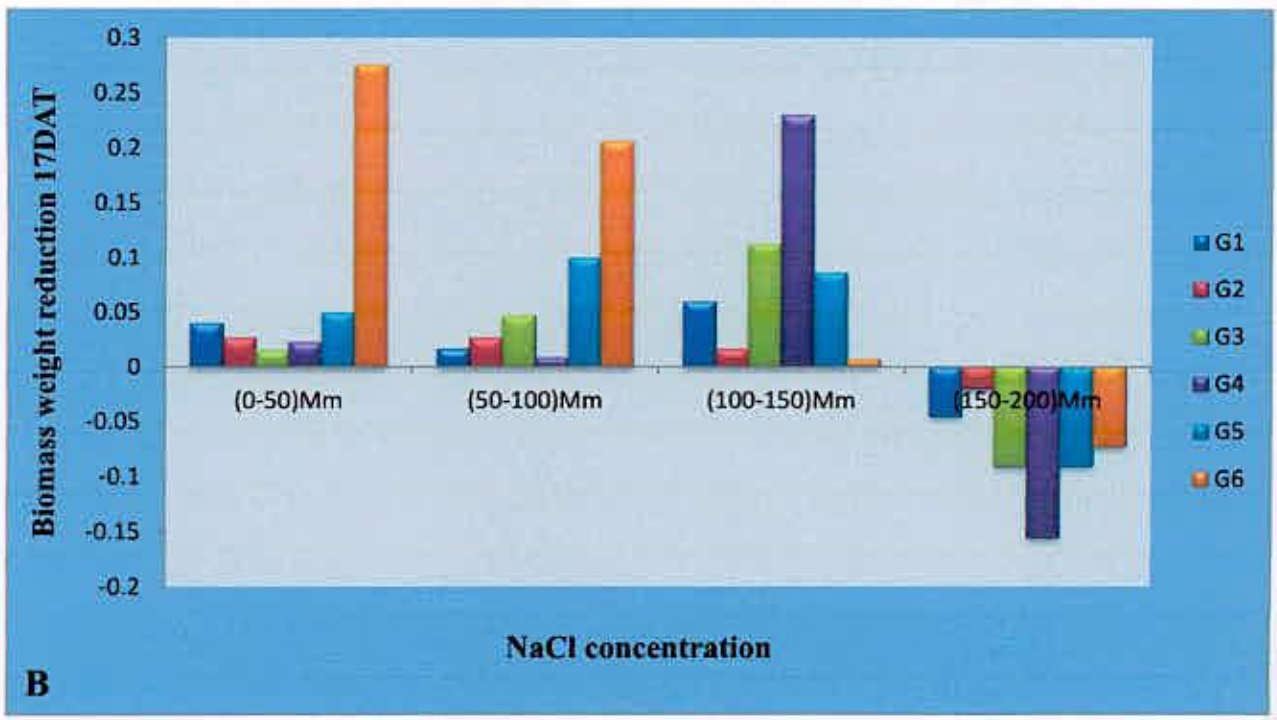


Fig 4. Biomass changing of callus weight under salt stress at 17 DAT

A. Mean Biomass weight. B. Mean Biomass weight reduction at 17 DAT

among cultivars regarding above traits by Aazami *et al.* (2010). Similar results were also found by other researchers (Vijayan *et al.*, 2003; Tewary *et al.*, 2000; Ben-Hayyim, 1987).

4.3.2 Biomass changing of callus on treatment

The main effect of different concentrations of salt showed significant variation on biomass changing of callus at different DAT. In all of the genotypes 0 mM salt treatment showed the best result whereas 200 mM showed the worst result (Table 3). Any increase in salinity levels in the media led to decrease of callus growth was also found by Aazami *et al.* (2010).

4.3.3 Biomass changing of callus on Genotype X Treatment interaction

The combined effect of genotypes and different concentrations (treatments) of salt showed significant variation on biomass changing of callus at different DAT. At 10 DAT the highest callus size was observed in G6T1 (11.73 mm) that is G6 treated with T1 and the lowest callus size was (5.783 mm) in G1T4 and G1T5. At 17 DAT, the highest biomass size was (12.40 mm) in G4T1 and the lowest callus size was in G1T5 (5.233 mm) (Table 4). As the salt concentration increases, the callus size decreases in G1 at 10 DAT as well as in 17 DAT. There was significant interaction effect between the genotypes and salt concentrations on callus weight also at 10 DAT and 17 DAT. In case of weight also all of the genotypes showed gradual decrease of weight from T1 to T5 at 10 DAT and at 17 DAT. In other genotypes also from G2 to G6, the biomass size of callus and weight were decreases gradually from 0 mM (T1) to 200 mM (T5) concentrations at 10 DAT as well as 17 DAT. The addition of NaCl to the culture media decreased the osmotic potential of the media inducing salinity stress that adversely affected the callus growth. Several authors reported the use of NaCl for *in vitro* salinity screening in different plants (Vijayan *et al.*, 2003; Zhao *et al.*, 2009). In this study, the adaption capacity to different salt level varies with the genotype's degree of tolerance. The genotypic variation for salt tolerance in this study agreed with the result of Rus *et al.* (2001) and Perez-Alfocea *et al.* (1994) who detected different salt stress responses among several tomato cultivars, from an halophytic behavior. Callus growing in this study under the increasing NaCl concentrations reduced

Table 2. Biomass changing of callus on genotypes

Genotype	Biomass size after 10 days (mm)	Biomass size after 17 days (mm)	Biomass weight after 10days (g)	Biomass weight after 17days (g)
G_1	7.833 d	6.375 e	0.4633 b	0.4087 d
G_2	10.63 a	9.539 a	0.6693 a	0.8100 a
G_3	9.064 b	6.745 d	0.4433 b	0.3593 e
G_4	8.672 c	8.951 b	0.3227 c	0.5107 c
G_5	7.153 e	9.749 a	0.2980 c	0.5940 b
G_6	8.686 c	7.645 c	0.4973 b	0.5173 c
$s_{\bar{x}}$	**0.04397**	**0.08406**	**0.02828**	**0.01155**

G1= BD-7755, G2= BD-7757, G3= BD-9008, G4= BD-9011, G5= BD-10122, G6= BD-10123.

Table 3. Biomass changing of callus on Treatment

Treatment	Biomass size after 10 days (mm)	Biomass size after 17 days (mm)	Biomass weight after 10days (g)	Biomass weight after 17days (g)
T_1	10.43 a	9.719 a	0.6478 a	0.6811 a
T_2	9.670 b	8.636 b	0.5417 b	0.6094 b
T_3	8.879 c	7.859 c	0.4006 c	0.5417 c
T_4	7.441 d	7.589 d	0.3389 cd	0.4561 d
T_5	6.946 e	7.033 e	0.3161 d	0.3783 e
$s_{\bar{x}}$	**0.04014**	**0.07674**	**0.02582**	**0.01054**

T1= 0 mM, T2= 50 mM, T3= 100 mM, T4= 150 mM, T5= 200 mM.

Table 4. Interaction effect of genotype and treatment

Interaction	Biomass size after 10 days (mm)	Biomass size after 17 days (mm)	Biomass weight after 10days (g)	Biomass weight after 17days (g)
G_1T_1	9.783 fg	7.400 jk	0.6733 b-d	0.4833 gh
G_1T_2	9.383 h	7.100 kl	0.4133 f-i	0.4433 g-j
G_1T_3	8.347 j	6.127 no	0.3633 g-j	0.4267 h-k
G_1T_4	5.867 p	6.013 o	0.4433 e-i	0.3667 j-l
G_1T_5	5.783 p	5.233 p	0.4233 f-i	0.3233 l-n
G_2T_1	11.06 cd	9.97 d-f	0.7800 ab	0.8700 a
G_2T_2	10.99 cd	9.65 e-g	0.7467 a-c	0.8433 a
G_2T_3	10.78 d	9.55 e-g	0.6767 b-d	0.8167 ab
G_2T_4	10.14 e	9.400 fg	0.5567 c-g	0.8000 ab
G_2T_5	10.19 e	9.117 g	0.5867 b-f	0.7200 cd
G_3T_1	11.37 b	7.2 j-l	0.6400 b-e	0.4633 g-i
G_3T_2	9.997 ef	7.050 kl	0.5667 c-g	0.4467 g-j
G_3T_3	9.817 f	6.7 l-n	0.5133 d-h	0.4000 h-l
G_3T_4	7.630 l	6.4 m-o	0.3267 h-j	0.2867 mn
G_3T_5	6.510 no	6.303 m-o	0.1700 j	0.2000 o
G_4T_1	9.97 ef	12.40 a	0.4500 e-i	0.6533 d-f
G_4T_2	9.520 gh	10.1 c-e	0.3300 h-j	0.6333 ef
G_4T_3	9.460 h	7.987 hi	0.3133 h-j	0.6233 ef
G_4T_4	7.957 k	7.793 ij	0.2800 ij	0.3933 i-l
G_4T_5	6.45 no	6.4 m-o	0.2400 ij	0.2500 no
G_5T_1	8.680 i	10.73 b	0.406 f-i	0.7467 bc
G_5T_2	6.960 m	10.37 b-d	0.313 h-j	0.6967 c-e
G_5T_3	7.450 l	9.970 c-f	0.293 ij	0.5967 f
G_5T_4	6.337 o	9.217 g	0.240 ij	0.5100 g
G_5T_5	6.33 o	8.457 h	0.236 ij	0.420 h-k
G_6T_1	11.73 a	10.56 bc	0.9367 a	0.8700 a
G_6T_2	11.17 bc	7.500 i-k	0.8800 a	0.5933 f
G_6T_3	7.41 l	6.800 lm	0.2433 ij	0.3867 i-l
G_6T_4	6.710 mn	6.710 l-n	0.186 j	0.3800 i-l
G_6T_5	6.403 o	6.653 l-n	0.24 ij	0.3567 k-m
$s_{\bar{x}}$	**0.09832**	**0.1880**	**0.06325**	**0.02582**
CV%	**1.98**	**3.99**	**24.21**	**7.33**

G1= BD-7755, G2= BD-7757, G3= BD-9008, G4= BD-9011, G5= BD-10122, G6= BD-10123, T1= 0 mM, T2= 50 mM, T3= 100 mM, T4= 150 mM, T5= 200 mM

relative growth rate in all tomato cultivars as in the study of Ben-Hayyim, 1987, Zahang *et al.* 2004 and Amini and Ehsanpour, 2006. *In vitro* plant tissue culture is useful and quick tool to evaluate plant tolerance to salt stress. Many studies were carried out through using different tissue culture methods (Bhatia *et al.*, 2008). The callus induction potential was decreased with increasing NaCl levels in this study. A similar observation was found by Yusuf *et al.* (1994), Cano *et al.* (1998) and Mercado *et al.* (2000) in tomato using tissue culture techniques for *in vitro* selection for salinity tolerance. Cultivated tomato is generally classified as being moderately salt-sensitive. Different genotypes of tomato displayed widely different degrees of salinity tolerance (Ghoshal and Bajaj, 1984). Marked differences in the behavior of both susceptible and tolerant tomato genotypes were evident (Cano *et al.*, 1998; Rus *et al.*, 2001). Yet, an understanding of the mechanisms that plants use to cope with high salinity is necessary to select and develop tomato plants that are more tolerant to salinity.

CHAPTER-V

SUMMARY & CONCLUSION

SUMMARY AND CONCLUSION

High salinity is one of the major stress factors among the abiotic stresses. In the world, about 400 million hectares of land are affected by high salinity. In Bangladesh about 1 million hectares of land are affected by high salinity in the coastal regions and it is increasing day by day with the expansion of shrimp culture. Salinity affects almost every aspect of the physiology and biochemistry of plants and significantly reduces yield. As saline soils and saline waters are common around the world, great effort has been devoted to understanding physiological aspects of tolerance to salinity in plants, as a basis for plant breeders to develop salinity-tolerant genotypes. In spite of this great effort, only a small number of cultivars, partially tolerant to salinity, have been developed. Further effort is necessary if the exploitation of saline soils and saline waters that are not currently usable is to be achieved. Salinity affects yield quality and quantity, so that yield characters must be taken into account when breeding for salinity tolerance. But not only yield-related characters are important. As salinity affects almost every aspect of the physiology and biochemistry of the plant, the enhancement of crop salt tolerance will require the combination of several to many physiological traits not simply those directly influencing yield. As salinity in soils is variable and plant tolerance depends on the stage of plant development, plants should be phenotyped at several salinity concentrations and at the most sensitive plant stage(s). For *in vitro* selection of callus line, six genotype of tomato was taken for this study. They were collected from Bangladesh Agricultural Research Institute (BARI). The seeds of six genotypes were cultured for cullus formation, subcultured and treated with different concentration of salt in the Genetics and Plant Breeding laboratory of Sher-e-Bangla Agricultural University, Dhaka. Seeds were processed and stored at 4°C. Evaluation of response of these six genotypes to normal and high salt conditions has been carried out in controlled environment with 25°C, 60% relative humidity, and a 16-h photoperiod from white fluorescent lamps (200 µmol photons/m^2/s^{-1}). Callus diameter assay and fresh weight assay were performed to compare the tolerance response of these genotypes. Two genotypes viz., G2 and G6 showed excellent performance of tolerance up to 50 mM of NaCl. G6 showed better performance under high salt concentrations i.e., at 100 mM and 200 mM but not at low salt stress. It

indicates the expression of functional gene occurs at high salt stress. However it is possible to select callus line tolerant to elevated levels of NaCl stress by sudden exposure to high of NaCl, accordingly a NaCl tolerant cell line was selected from hypocotyls and cotyledon derived callus of tomato which proved to be a true cell line variant. It can also be concluded that, the salt tolerant callus line could overcome the adverse effect of NaCl and maintained better growth on NaCl supplemented medium when compared to salt sensitive callus lines (controls). This conclusion is based on the observation of cells which have been removed from the selection pressure for at least four passages retained tolerance to NaCl after transferring to NaCl (150 mM or 200 mM) medium. As proline plays a significant role in protecting the cells from oxidative stress and the enzymes such as Super oxide dismutase (SOD) Ascorbate peroxidase (APX) and Catalase (CAT) protect cells and sub cellular systems from the effects of reactive oxygen species (ROS), in future, it is required to assess the accumulation of proline, the activity of anti-oxidant enzymes like SOD, APX and CAT from the control and stressed callus as they are the indicator of salt tolerance. This study dealt with the *in vitro* selection of salt tolerant callus lines in tomato and could be made the progress of regeneration followed by gene expresssion analysis and thereby identify and isolate the genes involved in the process of salt tolerance for future genetic transformation.

CHAPTER-VI

REFERENCES

REFERENCES

Aazami, M.A., Torabi, M. and Shekari, F. (2010). Response of some tomato cultivars to sodium chloride stress under *in vitro* culture condition. *African J. Agril. Res.* **5**(18): 2589-2592.

Aditya, T.L., Rahman, L., Shah-E-Alam, M. and Ghosh, A.K. (1999). Correlation and Path Coefficient Analysis in Tomato. *Bangladesh Agril. Sci.* **26**(1):119-122.

Afroz, A., Chaudhry, Z., Rashid, U., Khan, M.R., and Ali, G.M. (2010). Enhanced regeneration in explants of tomato (*Lycopersicon esculentum* L.) with the treatment of coconut water. *African J. Biotec.* **9**: 3634-3644.

Aftab, F., Zafar, Y., Malik, K.A. and Iqbal, J. (1996). Plant regeneration from embryogenic cell suspensions and protoplasts in sugarcane (*Saccharum* spp. *hybrid* cv. CoL-54). *Plant Cell, Tissue and Organ Cult.* **44**: 71-78.

Ahmed, K. U. (1976). "Phul Phal O Shak Shabji (in Bangla)". 2nd Edition, Alhaj Kamaluddin Ahmed, Banglow No. 2, Farm Gate, Dhaka, Bangladesh. p.470.

American Psychological Association (2010), "4.21 Use of Italics", *The Publication Manual of the American Psychological Association* (6th ed.), Washington, DC, USA: APA,ISBN 978-1-4338-0562-2.

Amini, F., Ehsanpour, A.A. (2006). Response of tomato (*Lycopersicon esculentum* Mill.) cultivars to MS, water agar and salt stress in *in vitro* culture. *Asian J. Plant Sci.*, **9**(1): 170-175.

Amtmann, A. and Sanders, D. (1999). Mechanisms of Na^+ uptake by plant cells. *Adv. Bot. Rev.* **29:** 75–112.

Apse, M.P., Aharon, G.S., Snedden, W.A. and Blumwald, E. (1999). Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in Arabidopsis. *Science* **285:** 1256–1258.

Arumuganathan, K. and Earle, E.D. (1991). Nuclear DNA content of some important plant spedies. Plant Mol. Biol. Rep. 9: 208-218.

Baksha, R., Alam, R., Karim, M.Z., Paul, S.K., Hossain, M.A., Miah, M.A.S. and Rahman, A.B.M.M. (2002). *In vitro* shoot tip culture of sugarcane (*Saccharum officinarum*) Variety Isd 28. *Biotechnol.* **1**: 67-72.

BARI. (2010). Krishi Projukti Hatboi, Bangladesh Agricultural Research Institute, Joydevpur, Gazipur. p. 304.

Baroncelli, S., Buiatti, M. and Bennici, A. (1973). Genetics of growth and differentiation *in vitro* of *Brassica oleracea var. botrytis*. 1. Differences between 6 inbred lines. *Z. Pflanzenzuchtung* **70**: 99-107.

Barton, D.W. (1950). Pachytene morphology of the tomato chromosome complement. *Amer. J. Bot.* **37**: 639-643.

Bayliss, M.W. and Dunn, S.D.M. (1979). Factors affecting callus formation from embryos of barley (*Hordeum vulgare*). *Plant Sci. Lett.* **14**: 311-316

BBS, 2005. Yearbook of Agricultural Statistics of Bangladesh. Planning Division, Ministry Ministry of Planning, Govt. of the Peoples Republic of Bangladesh, Dhaka. p. 163.

BBS. (2010). Year Book of Agricultural Statistics of Bangladesh. Bangladesh Bureau of Statistics, Planning Division, Ministry of Planning, Govt. of the Peoples Republic of Bangladesh, Dhaka. p. 163.

Ben-Hayyim, G (1987). Relationship between salt tolerance and resistance to polyethylene glycol-induced water stress in cultured citrus cells. *Plant Physiol.* **85**: 430-433.

Bhansali, R.R. and Singh, K. (1982). Callus and shoot formation from leaf of sugarcane in tissue culture. *Phytomorphology* **32**: 167-170.

Bhatia P., and Ashwath, N. (2008). Improving the quality of *in vitro* cultured shoots of tomato *(Lycopersicon esculentum* Mill. cv. Red Coat. *Biotechnol.* 7(2): 188-193.

Bhattarai, S, de la Pena, R, Midmore, D., and Palchamy, K. (2009). *In vitro* culture of immature seed for rapid generation. *Euphytica,* **167**(1): 23-30.

Blumwald, E. and Poole, R.J. (1985). Na^+/H^+ antiport in isolated tonoplast vesicles from storage tissue of Beta vulgaris. *Plant Physiol.* **78:** 163–167.

Branca, C., Bucci, G., Domiano, P., Ricci, A., and Bassi, M. (1990). Auxin: structure and activity on tomato morphogenesis *In vitro* and pea stem elongation. *Plant Cell Tiss. Org. Cult.*, **24**: 105-114.

Brichkova, G.G., Maneshina, T.V., and Kartel, N.A. (2002). Optimization of the nutrient medium for effective regeneration of tomatoes (*Lycopersicon esculentum*) *In vitro.Vestsi-Natsyyanal'nai-Akademii-Navuk-Belarusi.-Seryya-Biyalagichnykh-Navuk,* **2**: 47-52.

Buiatti, M., Tognoni, F., Lipucci, M., Marcheschi, G., Pellegrini, G., Grenci F., Scala, A. Bettini, P. P., Bogani, P, Betti, L., and Collina, G. (1984). Genetic variability in tomato (*Lycopersicon esculentum*) plants regenerated from *in vitro* cultured cotyledons. *Genetica Agraria* **38**: 325-331.

Cano, E.A., Perez, A., Moreno, V., Caro, M., and Bolarin, M. (1998). Evaluation of salt tolerance in cultivated and wild tomato species through in vitro shoot apex culture. *Plant Cell Tiss. Organ Cul.*, **53** (1): 19-26.

Chandler, S.F Paek, K.Y., Pua, E.C., Ragolsky, E. Mandal, B.B, and Thrope, T.A. (1988). The effectiveness of selection for salinity tolerance using in vitro shoot culture. *Bot Gaz.,* **149**:166-172.

Chaudhry, Z., Afroz, A., and Rashid, H. (2007). Effect of variety and plant growth regulators on callus proliferation and regeneration response of three tomato cultivars (*Lycopersicon esculentum*). *Pakistan J. Bot.*, **39**: 857-869.

Chen, R., Gyokusen, K. and Saito, A. (2001). Selection, regeneration and protein profile characteristics of NaCl-Tolerant callus of Robinia pseudoacaia L. *J. For. Res.* **6**: 43-48.

Chen, W.H., Davey, M.R., Power, J.B. and Cocking, C.E. (1988). Control and maintenance of plant regeneration in sugarcane callus cultures. *J. Exp.Bot.* **39**: 251-261.

Cheryl, I. (2007). "12.1.1 Use of Italic". *AMA Manual of Style* (10th *ed.*). Oxford, Oxfordshire: Oxford University Press. ISBN 978-0-19-517633-9.

Chowdhury, B. (1979). Vegetables. 6th Edition, The Director, National Book Trust, New Delhi, India. p.46.

Compton, M.E., and Veillux, R.E. (1991). Shoot root and flower morphogenesis on tomato inflorescence explants. *Plant Cell Tiss. Org. Cult.*, **24**: 223-231.

Croughan, T. P., S. Stavarek and D. Rains. (1981). *In vitro* development of salt resistant plants. *Environ. Exp. Bot.* **21**: 317-324.

Cuartero, J., and Ferna´ndez-Mun˜oz, R. (1999). Tomato and salinity. *Scientia Horticulturae*, **78**: 83–125.

Cuartero, J., Boları´n, M.C., Ası´ns, M.J., and Moreno, V. (2006). Increasing salt tolerance in the tomato. *J. Exp. Bot.* **57(5):** 1045–1058.

Davenport, T.G., Jerome-Majewska, L.A., and Papaioannou, V.E. (2003). Mammary gland, limb and yolk sac defects in mice lacking Tbx3, the gene mutated in human ulnar mammary syndrome. *Development* **130**: 2263-2273.

Debez, A., Saadaoui, D., Ramani, B., Ouerghi, Z., Koyro, H.W., Huchzermeyer, B., and Abdelly, C. (2006) Leaf H - ATPase activity and photosynthetic capacity of Cakile maritima under increasing salinity. *Environ. Exp. Bot.*, **57**: 285-290.

Emilio, A.C., Francisco, P.A., Vicente, M., Manuel, C., Maria, C.B. (1998). Evaluation of salt tolerance in cultivated and wild tomato species through in vitro shoot apex culture. *Plant Cell Tiss Org. Cult.*, **53**: 19- 26

Engelmann, N.J., Campbell, J.K., Rogers, R.B., Indumathie, S. (2010). Screening and Selection of High Carotenoid Producing *in vitro* Tomato Cell Culture Lines for [13C]-Carotenoid Production. *J. Agric. Food. Chem.*, **58**(18): 9979–9987.

FAO. (2010). The State of Food Insecurity in the World: Addressing food insecurity in protracted crises. Food and Agriculture Organization of the United Nations. Rome.

Farrukh, J. (2002). *In vitro* salt tolerance in wheat. III. Water relations in callus. *Int. J. Agri. Biol.* **4**(4): 665-667.

Fitch, M.M.M. and Moore, P.H. (1993). Long term culture of embryogenic sugarcane cells. *Plant Cell, Tiss. Organ Cult.* **32**: 335-343.

Flowers, T.J., and Yeo, A.R. (1995). Breeding for salinity resistance in crop plants: where next? *Aust. J. Plant Physiol.*, **22**: 875–884.

Foranzier, R.F., Forreira, R.R., Pereira, G.J.G., Molinqa, S.M.G., Smith, R.J., Lea, P.J. and Azeudo, R.A. (2002). Cadmium stress in sugarcane callus cultures: Effect of antioxidant enzymes. *Plant Cell, Tissue and Organ Cult.* **71**: 125-131.

Gallo-Meagher, M., English, R.G. and Abouzid, A. (2000). Thidiazuron stimulates shoot regeneration of sugarcane embryogenic callus. *In Vitro Cell. Develop. Biol. Plant* **36**:37-40.

Gandonou, C.h., Errabii, T., Abrini, J., Idaomar, M., Chibi, F. and Skali-Senhaji, N. (2005). Effect of genotype on callus induction and plant regeneration from leaf explants of sugarcane (*Saccharum* sp.). *African J.Biotechnol.* **4**: 1250-1255.

Garbarino, J., DuPont, F.M. (1988). NaCl induces a Na^+/H^+ antiport in tonoplast vesicles from barley roots. *Plant Physiol.* **86**: 231–236.

Ghosal, S.S., and Bajaj, Y.P.S. (1984). Isolation of sodium chloride resistant cell lines in some grain legumes. *Indian J. Exp. Biol.*, **22**: 209-214.

Gomez-Cadenas, A., Tadeo, F.R., Primo-Millo, E. and Talon, M. (1998). Involvement of abscisic acid and ethylene in the response of citrus seedlings to salt shock. *Physiol. Plant* **103**:475–484.

Gu, R., Liu, Q., Pie, D. and Jiang, X. (2004). Understanding saline and osmotic tolerance of Populus euphratica suspended cells. *Plant Cell Tissue Organ Cult.* **78**: 261-265.

Guiderdoni, E. (1986). Somatic embryogenesis of foliar explants of sugarcane (*Saccharum* sp.) cultured *in vitro*. *Agronomia Tropicale* **41**: 50-59.

Gulati, A. and Jaiwal, P.K. (1994). *In vitro* selection and characterization of a callus line of Vigna radiate resistant to NaCl, KCl and Na2SO4. *Biologia Plantarum* **36**(1) 21-28.

Gupta, P., Sharma, S. and Saxena, S. (2014). Effect of salts (NaCl and Na2CO3) on callus and suspension culture of Stevia rebaudiana for Steviol glycoside production. *Appl. Biochem. Biotechnol.* **172**(6):2894-906.

Hernandez, J.A., Jimenez, A. Mullineaux, P. and Sevilla, F. (2000) Tolerance of pea (*Pisum sativum* L.) to long term salt stress is associated with induction of antioxidant defenses. *Plant Cell Environ.* **23**: 853-862.

Himanshu, S., Gill, M.S., Gosal, S.S. and Sinha, H. (2000). Regulation of somatic embryogenesis and plant regeneration in sugarcane (*Sacchanarum officinarum*). *Indian J. Agric. Sci.* **70**:181-183.

Hossain, M. A., Goffer, M. A., Chowdhury, J. C. S., Rahman, M. S. and Hossain, M. I. (1999). A Study on Postharvest Practices and Loss of Tomato in Some Selected Areas of Bangladesh. *Bangladesh J. Agril. Res.* **24**(2): 299.

Hossain, M. M., Karim, M. K., Haque, M. M. and Hassain, A. K. M. A. (1986). Performance of Some Promising Tomato Lines Planted at Different Dates. Annual

Report, Vegetable Section, Division of Horticulture, Bangladesh Agricultural Research Institute, Gazipur. pp. 9-12.

Hu, H., and Zeng, J.Z. (1984). Development of new varieties via anther culture. *In:* Ammirato, P.V., Evans, D.A., Sharp, W.R., *(eds)*. Handbook of Plant Cell Culture. New York: Macmillan, **3**: 65–90.

Islam, R., Haider, S.R., Alam, M.A. and Joarder, O.I. (1996). High frequency somatic embryogenesis and plant regeneration in sugarcane. *Rice Biotechnol. Quarterly* **25**: 8.

Jaiswal, R. and Singh, N.P. (2001). Plant Regeneration from NaCl Tolerant Callus/Cell Lines of Chickpea. *ICPN* **8**: 21-23.

Jarret, R.I., Hasegawa, P.M. and Erickson, H.T. (1980). Effects of medium components on shoot formation from cultured tuber discs of potato. *J. American Soc. Hort. Sci.* **105**: 238-242.

Jatoi, S.A., Ahmad, M., and Suppal, H.U. (1999). Manipulation of internodal segments of F1 tomato hybrids raised *In vitro* under different regimes of plant growth regulators. *Pakistan J. Bot.*, **31**: 37-40.

Jose,´ M., Seguı´-Simarro, and Nuez, F. (2007).Embryogenesis induction, callogenesis, and plant regeneration by in vitro culture of tomato isolated microspores and whole anthers. *J.Exp.Bot.*, **58**: 1119–1132.

Karim, M.Z., Amin, M.N., Hossain, M.A., Islam, S., Hossin, F. and Alam, R. (2002). Micropropagation of two sugarcane (*Saccharum officinarum*) varieties from callus culture. *J. Biol. Sci.* **2**: 682-685.

Kartha, K.K., Gamborg, O.L., Shyluk, J.P. and Constabel, F. (1976). Morphogenetic investigations on *in vitro* leaf culture of tomato (*Lycopersicon esculentum* Mill., cv. Starfire) and high frequency plant regeneration. *Z. Pflanzenphysiol.* **77**: 292-301.

Kulkarni, S.D. (1989). Premilling aspects of pulse processing: a review. *J. Ins. Eng.* **69**: 73-76.

Kurtz, S.M. and Lieneberger, R.D. (1983). Genotypic differences in morphogenic capacity of cultured explants of tomato. *J. American Soc. Hort. Sci.* **108**: 710-714.

Kut, S.A. and Evans, D.A. (1982). Plant regeneration from cultured leaf explants of eight wild tomato species and two related *Solanum* species. *In vitro* **18:** 593-598.

Liu, M.C. and Chen, W.H. (1974). Histological studies on origin and process of plantlet differentiation in sugarcane callus mass. International Society of Sugarcane Technologists Proceedings, Durban, South Africa. pp. 118-128.

Liza, L.N., Nasar, A.N.M., Zinnah, K.M.A., Chowdhury, M. A. N. and Ashrafuzzaman, M. (2013). In Vitro Growth Media Effect for Regeneration of Tomato (*Lycopersicon esculentum*) and Evaluation of the Salt Tolerance Activity of Callus. *J. Agric. Sus.* **2:** 132-143.

Locky, R.D. (1983). Callus formation and organogenesis by explants of six *Lycopersicon* species. *Canadian J. Bot.* **61**: 1072-1079.

Lopez-Climent, M.F., Arbona, V., Perez-Clemente, R.M., Gomez-Cadenas, A (2008). Relationship between salt tolerance and photosynthetic machinery performance in citrus. *Env. Exp. Bot.* **62**:176–184

Lutts, S., Majerus, V. and Kinet, J.M. (1999). NaCl effects on proline metabolism in rice (*Oryza sativa*) seedlings. *Physiol. Plant* **105**: 450-458.

Mamun, M.A., Sikdar, M.B.H., Paul, D.K., Rahman, M.M. and Islam, M.R. (2004). *In vitro* Micropropagation of Some Important Sugarcane Varieties of Bangladesh. *Asian J. Plant Sci.* **3**: 666-669.

Marcano, A.K., Guevara, M.P., Oropeza, M. and De-Garcia, E. (2002). Improvement of somatic embryogenesis process in sugarcane Venezuelan cultivars. *Acta Cientifica Venezolana* **53**: 251-257.

Martinez, C.A., Maestri, M., and Lani, E.G. (1996). *In vitro* salt tolerance and proline accumulation in andean potato (Solanum spp.) differing in frost resistance. *Plant Sci.*, **116**: 177-184.

Matin, M. A., Huq, A. S. M. A., Karim, M. R. and Baksha, E. (1996). Farm Level Yield Analysis of Tomato Cultivation in Selected Areas of Bangladesh: An Economic Profile. *Bangladesh J. Agril. Res.* **21**(1): 50-57.

Mercado, J.A., Sancho, Jimenez, B.S., Peran, U.R., Pliego, A.F., and Quesada, M.A. (2000). assessment of *in vitro* growth of apical stem sections and adventitious organogenesis to evaluate salinity tolerance in cultivated tomato. *Plant Cell Tiss. Organ Cul.*, **62**: 101-106.

Messai, A., Hannachi, C., and Zid, E. (2007).Evaluation of Salt-tolerant Genotypes of two Processing Tomato Derived from in vitro Regeneration under Salt stress. *Acta Hort.* **758**: 51-58

Miki, Y., Hashiba, M. and Hisajima, S. (2001). Establishment of salt stress tolerant rice plants through step-up NaCl treatment in vitro. *Biol. Plant* **44**: 391-395.

Miller, J.C., and Tanksley, S.D. (1990). Effects of restriction enzymes, probe source, and probe length on detecting restriction fragment length polymorphism in tomato. *Theor. Appl. Genet.* **80:** 385-389.

Moghaieb, R., Saneoka, H., and Fujita, K. (2004). Shoot regeneration from Gus-transformed tomato (*Lycopersicon esculentum) hairy* root. *Cell. Mol. Biol. Lett.*, **9**: 439-449.

Mohamed, A.N., Ismail, M.R., Kadir, M.A. and Saud, H.M. (2011). *In vitro* performances of hypocotyl and cotyledon explants of tomato cultivars under sodium chloride stress. *African J. Biotechnol.* **10**(44): 8757-8764.

Mohamed, A.N., Rahman, M.H., Alsadon, A.A. and Islam, R. (2007). Accumulation of proline in NaCl-treated callus of six tomato (*Lycopersicon esculentum* Mill.) cultivars. *Plant Tissue Cult. Biotech.* **17**(2): 217-220.

Moraes, F. (1990). Obtenção de plantas haplóides através da cultura da anteras. In: Torres AC, Caldas LS (eds). Técnicas e aplicações da cultura de tecidos de plantas. Brasília: EMBRAPA, Centro Nacional de Pesquisas de Hortaliças, 311-332.

Moya, J.L., Gomez-Cadenas, A., Primo-Millo, E., Talon, M. (2003). Chloride absorption in salt-sensitive Carrizo citrange and salt-tolerant Cleopatra mandarin citrus rootstocks is linked to wateruse. *J. Exp. Bot.* **54**:825–833.

Muhling, K.H., and Lauchli, A. (2003) Interaction of NaCl and Ca stress on compartmentation pattern of cations, antioxidant enzymes and proteins in leaves of two wheat genotypes differing in salt tolerance. *Plant Soil*, **253**: 219-231.

Munns, R. and Tester, R. (2008) Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. **59**:651–681.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473-497.

Nabors, M.W., Gibs, S.E., Bernstein, C.S., and Mise, M.E. (1980). NaCl-tolerant tobacco plants from cultured cells. *Z. pflanzen-physiol.* **97**: 13-17.

Nadar, H.M., Soepraptopo, S., Heinz, D.J. and Ladd, S.L. (1978). Fine structure of sugarcane (*Saccharum* spp.) callus and role of auxin in embryogenesis. *Crop Sci.* **18**: 210-216.

Nagai, C., Ahloowalia, B.S., and Tew, Y.L. (1991). Somaclonal variants from an intergeneric hybrid: *Saccharum* spp. *Hybrid* x *Erianthus arundinaceum*. *Euphytica* **53**: 193-199.

Naik, G.R. and Harinath Babu, K. (1987). *In vitro* response of sugarcane to salinity tolerance. 37th convention *D.S.T.A.* 187-194.

Niaz, F. and Quraishi, A. (2002). Effect of growth regulators on regeneration potential of two sugarcane cultivars SPF-213 and CPF-237. *Pakistan J. Biol. Sci.* **5**: 1081-1083.

Niu, X., Bressan, R.A., Hasegawa, P.M., Pardo J.M. (1995). Ion homeostasis in NaCl stress environments. *Plant Physiol.* **109:** 735–742.

Nublat, A., Desplans, J., Casse, F. and Berthomieu, P. (2001). *sas1*, an *Arabidopsis* Mutant Overaccumulating Sodium in the Shoot Shows deficiency in the control of the root radial transport of sodium. *Plant cell* **13**(1): 125-137

Nurhidayah T, Horn R, and Rocher T. (1996). High regeneration rates in anther culture of interspecific sunflower hybrids. *Plant Cell Reports*, **16**: 167–173.

Ochatt, S. J, Marconi, P.L., Radice, S., Arnozis, P.A., Caso, O.H. (1999). *In vitro* recurrent selection of potato: production and characterization of salt tolerant cell lines and plants. *Plant Cell Tissue Organ Cul.* **55**: 1-8.

Osman, M. G., Elhadi, E. A., and Khalafalla, M.M. (2011). *In vitro* screening of some tomato commercial cultivars for salinity tolerant. *Int. J. Biotec. Biochem.*, 3946.

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

Perez-Alfocea, F., Guerrier, G., Estañ, M.T. and Bolarin, M.C. (1994) Comparative salt responses at cell and whole-plant levels of cultivated and wild tomato species and their hybrid. *J. Hort. Sci.* **69**: 639-644

Pratt, L.H., Cordonnier-Pratt, M. M., Kelmenson, P.M., Lazarova G.I., Kubota T. and Alba R.M. (1997) The phytochrome gene family in tomato (Solanum lycopersicum L.). *Plant, Cell Environ.* **20:** 672–677.

Priya, A.M., Pandian, S.K.and Ramesh, M. (2011). Effect of NaCl on in vitro plant regeneration from embryogenic callus cultures of 'cv IR 64' indica rice *(Oryza sativa L.). African J. Biotech.* **10**(36): 6947-6953

Rains, D.W., Croughan, S.S and Croughan, T.P. (1986). Isolation and characterization of mutant cell lines and plants: salt tolerance. *In*: Cell Culture and Somatic Cell Genetics of Plants. *Ed.* Vasil, I.K. Academic Press Inc., Orlando, FL, pp 537-547.

Ramanand, Kureel, N., Subhanand, N., Lal, M. and Singh, S.B. (2006). Plantlet regeneration through leaf callus culture in sugarcane. *Sugar Tech.* **8**: 85-87.

Rao, S. and Patil, P. (2012). *In vitro* Selection of Salt Tolerant Calli Lines and Regeneration of Salt Tolerant Plantlets in Mung Bean (*Vigna radiata L.* Wilczek). *Biotechnol. Mol. Studies Novel App. Improved Quality Human Life* **1**(1):197-212.

Raziuddin, S.S., Chaudhry, H.J., Mohammad, T., and Ali, S. (2004). Hormonal effect on callus induction in tomato. *Sarhad J. Agric.*, **20** :223-225.

Rick, C. M. (1980) Tomato. *In*: Hybridization of Crop Plants. ASA-CSSA, Madison. pp669- 680.

Rubio, F., Gassmann, W. and Schroeder, J.I. (1995). Sodium driven potassium uptake by the plant potassium transporter HKT1 and mutations conferring salt tolerance. *Science* **270:** 1660–1663.

Rus, A.M., Estañ, M.T., Gisbert, C., Garcia-Sogo, B., Serrano, R., Caro, M., Moreno, V., and Bolarín, M.C. (2001). *Expressing the yeast HAL1 gene in tomato increases fruit yield and enhances K^+/Na^+ selectivity under salt stress. Plant Cell Environ.*, **24:** 875–*880.*

Satti, S.M.E., and Lopez, M. (1994). Effect of increasing potassium levels for alleviating sodium chloride stress on the growth and yield of tomato. *Commun. Soil Sci. Plant Anal.* , **25:**2807-2823.

Sharfudin, A. F. M. and Siddique, M. A. (1985). Sabji Bigyan. 1st Edition, Bangladesh Agricultural University, Mymensingh. p.4.

Shatnawi, M.A. (2006). Micropropagation and germplasm storage of Prunus amygdalus by the vitrification method. *Jordan J. Agric. Sci.* **6**(4): 446-450.

Silva, J.V, Lacerda, C.F, Costa, P.H.A, Enéas Filho, J, Gomes-Filho, E. and Prisco, J.T. (2003). Physiological responses of NaCl stressed cowpea plants grown in nutrient solution supplemented with CaCl2 . *Braz. J. Plant Physiol.* **15**:99-105.

Simarro, J.M.S., Nuez F. (2007). Embryogenesis induction, callogenesis, and plant regeneration by in vitro culture of tomato isolated microspores and whole anthers. *J. Exp. Bot.*, **58**(5): 1119–1132.

Smolik, M., Kram, P., Krupa-Małkiewicz, M., Smolik, B., and Malinowska, K. (2011). Response of tomato genotypes to salinity stress assessed at the seedlings stage. *Elec tronic j. Polish Agric. Univ.*, **14**(4):17.

Snyman, S. J., Hucket, B.I., Botha, F.C. and Watt, M.P. (2001). A comparison of direct and indirect somatic embryogenesis, the production of transgenic sugarcane. *African J. Bot.* **62**: 105-107.

Snyman, S.J., Watt, M.P., Hucket, B.I. and Botha, F.C. (2000). Direct somatic embryogenesis for rapid, cost effective production of transgenic sugarcane (*Saccharum* spp. *Hybrid*). *Proc. South African Sugar Technol.Asso.* **74**: 186-187.

"Soil salinity" (2010) in Water Wiki, the on-line Knowledge and Collaboration Tool) of the Community of Practice (CoP) on Water- and UNDP-related activities in Central and South-Eastern Europe, Caucasus and Central Asia.

Tal, M, Heikin H and Dehan K (1978) Salt tolerance in the wild relatives of the cultivated tomato: responses of callus tissues of Lycopersicon esculentumand Solanum pennellito high salinity. *Z. Pflanzenphysiol.* **86**: 231-240.

Tal, M. (1996) Somaclonal variation for salt tolerance in tomato and potato. *In*: Bajaj YPS (*ed*) Biotechnology in Agriculture and Forestry. 36: Somaclonal Variation in Crop Improvement II. Springer-Verlag, Berlin. pp. 132–145.

Tal, M., Dehan, K. and Heiken, H. (1977). Morphogenetic potential of cultured leaf sections of cultivated and wild species of tomato. *Ann. Bot.* **41**: 937-941.

Tewary, P.K., Sharma, A., Raghunath, M. K. and Sarkar, A. (2000). *In vitro* response of promising mulberry (*Morus* sp.) genotypes for tolerance to salt and osmotic stresses. *Plant Grow. Regul.* **30**: 17-21

Uozumi, N., Kim, E.K., Rubio, F., Yamagushi, T., Muto, S., Tsuboi, A., Bakker, E.P., Nakamura, T. and Schroeder, J.I. (2000). The Arabidopsis HKT1 gene homolog mediates inward Na^+ currents in Xenopus laevis oocytes and Na^+ uptake in *Saccharomyces cerevisiae. Plant Physiol.* **122:** 1249–1259.

Vaziri, A., Ahmadi, A.R., Fahimi, H., Bernard, F. and Shaker, H. (2004). The Effect of Different Concentrations of NaCl on the Callus Induction and Lipids of Soybean. *Plant Tissue Cult.* **14**(21): 75-77.

Venkataiah, P., Christopher, T. & Subhash K. (2004). Selection and characterization of sodium chloride and mannitol tolerant callus lines of red pepper (*Capsicum annuum* L.). *Plant Physiol.* **9**(2): 158-163.

Vidal, M., Almudena , M., Climent, L., Fernanda, M., Vicent, M., Clemente, P., Maria, R., Cadenas, G. and Aurelio. (2009). A novel in vitro tissue culture approach to study salt stress responses in citrus. URI: http://hdl.handle.net/10234/23921. DOI: http://dx.doi.org/10.1007/s10725-009-9401-0.

Vijayan, K., Chakraborti, S.P. and Ghosh, P.D. (2003). *In vitro* screening of mulberry (*Morus* spp.) for salinity tolerance. *Plant Cell Rep.* **22**: 350-357.

Walker, N.A., Sanders, D. and Maathuis, F.J.M. (1996). High affinity potassium uptake in plants. *Science* **273:** 977–979.

Winicov, I. (1996). Characterization of rice (*Oryza sativa*) plants regenerated from salt-tolerant lines. *Plant Sci.* **113**: 105-111.

Yang, Y.W., Newton, R. J. and Miller, F. R. (1990). Salinity tolerance in sorghum. II. Cell culture response to sodium chloride in *S. bicolor* and *S. halepense. Crop sci.* **30:** 781-785.

Yeo, A.R. and Flowers, T.J. (1986). Salinity resistance in rice (*Oryza sativa* L.) and a pyramiding approach to breeding varieties for saline soils. *Aust. J. Plant Physiol.* **13:** 161–173.

Young, R., Kaul, V., and Williams, E.G. (1987). Clonal propagation *in vitro* from immature embryos and flower buds of *Lycopersicon peruvianum* and *L. esculentum. Plant Sci.*, **52**: 237-242.

Yusuf, A., Li, S.J., and Li, S.X. (1994). *In vitro* flowering, fruiting and differentiation of callus in different genotypes of tomato in the presence of NaCl. *Sarhad J. Agric.*, **10**(1): 59-62.

Zeba, N., (2009). Functional analysis of abiotic stress-inducible genes encoding a RING Zinc Finger Protein and an Adenylate kinase from *Capsicum annuum*. PhD. Thesis, Seoul National University, Seoul, Korea.

Zhang, F., Yang, Y.L., He, W.L., Zhao, X., Zhang, L.X. (2004). Effects of salinity on growth and compatible solutes of callus induced from *Populus euphratica. In vitro Cell Dev. Biol.*, **40**(5): 491-494.

Zorzoli, R., Bueno, M.S. and Picardi, L.A. (1993b). Regeneración *in vitro* a partir de anteras y folíolos en genotipos de tomate (*Lycopersicon esculentum* Mill). *Hort. Arg.* **8-12**: 1-7.

Zorzoli, R., Cointry, E., Prado, E., Mroginski, L. and Picardi, L. (1993a). Influencia del citoplasma sobre la capacidad de regeneración *in vitro* en híbridos y sus generaciones segregantes en tomate (*Lycopersicon esculentum* Mill.). *Agriscientia* **10**: 15-19.

CHAPTER-VII

APPENDICES

APPENDICES

APPENDIX 1. Composition and stock solution of MS (Murashige and Skoog, 1962) medium

Stock-1	**Major salts (10*)**	**mg/L**	**g/L**
	KNO_3	1900	19.00
	NH_4NO_3	1650	16.50
	$MgSO_4.7H_2O$	370	3.370
	$CaCl_2.2H_2O$	440	4.40
	KH_2PO_4	170	1.70
Stock-2	**Minor salts (100*)**		
	KI	0.83	83
	H_3BO_3	6.2	620
	$MnSO_4.4H_2O$	22.3	2230
	$ZnSO_4.7H_2O$	8.6	860
	$Na_2MoO_4.2H_2O$	0.25	25
	$CuSO_4.5H_2O$	0.025	2.5
	$CoCl_2.6H_2O$	0.025	2.5
Stock-3	**Iron EDTA soln. (100*)**		
	$FeSO_4.7H_2O$	27.8	2.78
	$Na_2EDTA.2H_2O$	37.3	3.73
Stock-4	**Organics (100*)**		
	Myo-inositol	100	10000
	Nicotinic acid	0.5	50
	Pyridoxin HCl	0.5	50
	Thiamine HCl	0.1	10
	Glycin	2.0	200

APPENDIX 2. Working in the Genetics and Plant Breeding Laboratory using sterile environment.