IN VITRO SCREENING OF TOMATO (*Solanum lycopersicum* L.) GENOTYPES FOR DROUGHT TOLERANCE USING POLYETHELENE GLYCOL

BY

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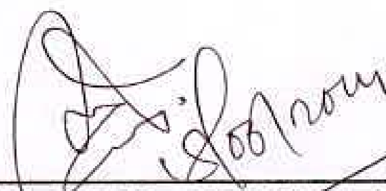

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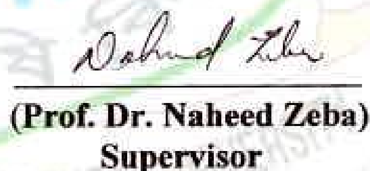
CERTIFICATE

This is to certify that the thesis entitled **"*In vitro* Screening of tomato (*Solanum lycopersicum* L.) genotypes for drought tolerance using Polyethelene Glycol"** submitted to the Department of Genetics and Plant Breeding, 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 **Mallika Rani Roy**, Registration No. **06-2058** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

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



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DEDICATED
TO
MY BELOVED PARENTS

LIST OF ABBREVIATION

Abbreviations	Full word
^0C	Degree Celsius
%	Percentage
1N	1 Normal
BBS	Bangladesh Bureau of Statistics
BARI	Bangladesh Agricultural Research Institute
Cm.	Centimeter
et al.	And others
etc.	Etcetera
FAO	Food and Agricultural Organization
G	Gram
g/L	Gram per liter
i.e	That is
IAA	Indole-3-Acetic Acid
Intl.	International
J.	Journal
Mg	Milligram(s)
mg/L	Milligram per liter
Ml	Milliliter
MS	Murashige and Skoog
PEG	Polyethelene Glycol
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

The experiment was conducted to study genetic diversity analysis of tomato (*Solanum lycopersicum* L.) under drought condition *in vitro*. Fourteen tomato genotypes were used as experimental materials among which BARI Tomato 2, BARI Tomato -11 are parent material and rest of all were lines, and they were BARI Tomato-2, BARI Tomato-11, BD-7260, BD-7290, BD-7295, BD-7286, BD-7269, BD-7258, BD-7289, BD-7292, BD-7291, BD-7302, BD-7301 and BD-7762. Murashige and Skoog medium were used with different PEG concentration as culture medium for root and shoot regeneration. The longest length of root (2.83 cm) was recorded for 0 g PEG, whereas the shortest length (1.88 cm) was observed in 60 g PEG. In an average, among the different genotypes of tomato the highest length of root (5.01 cm) was recorded from BARI-2 and the lowest length of root (0.69 cm) in BARI-11. Among the studied genotypes BARI-2, BD-7258, BD-7301 and BD-7762 produced the highest plant weight in 40 and 60 g PEG compared to 0 and 20 g PEG. In an average among the different genotypes of tomato the highest weight of plant (0.068 g) was recorded from BARI-2 and BD-7290 and the lowest weight of plant (0.003 g) was found in BARI-11 and BARI-7292. Cluster I and IV was the largest cluster comprising of 5 genotypes followed by cluster II with 3 genotypes and cluster III belongs only 1 genotypes of tomato. In considering of clustering mean for initial length the highest mean was 1.49 for cluster IV. In case of length highest cluster mean 6.53 was recorded in cluster III and for plant weight highest cluster 0.066 was observed in cluster III. The results revealed that genotypes chosen for hybridization from clusters with highest distances would give high heterotic F_1 and broad spectrum of variability in segregating generations.

CHAPTER I

INTRODUCTION

Tomato (*Solanum iycopersicum* L.) botanically referred to the family Solanaceae is one of the most important and popular vegetable crop. The centre of origin of the genus *Solanum* is the Andean zone particularly Peru-Ecuador-Bolivian areas (Salunkhe *et al.*, 1987), but cultivated tomato originated in Mexico. Food value of tomato is very rich because of higher contents of vitamins A, B and C including calcium and carotene (Bose and Som, 1990). Tomato adds flavor to the foods and it is also rich in medicinal value. It is widely employed in cannery and made into soups, conserves, pickles, ketchup, sauces, juices etc. More than 7% of total vitamin-C of vegetable origin comes from tomato in Bangladesh. It contains 94 g water, 0.5 g minerals, 0.8 g fibre, 0.9 g protein, 0.2 g fat and 3.6 g carbohydrate and other elements like 48 mg calcium, 0.4 mg iron, 356 mg carotene, 0.12 mg vitamin B-1, 0.06 mg vitamin B-2 and 27 mg vitamin C in each 100 g edible ripen tomato (BARI, 2010).

Tomato ranks top the list of canned vegetables and next to potato and sweet potato in the world vegetable production (FAO, 1997). 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). Now Bangladesh is producing a good amount of tomatoes. In Bangladesh it is cultivated as winter vegetable, which occupies an area of 58,854 acres in 2009-10 (BBS, 2010). The total production of tomato in 2008 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 Egyptin 2008 (FAO, 2010). In Bangladesh in the year of 2009-2010 the total production of tomato was 190 thousand metric tons (BBS, 2010). The average tomato production in Bangladesh is 50-90 tons/ha (BARI, 2010). Nowadays, tomatoes are grown round the year. Due to increasing consumption of

tomato products, the crop is becoming promising. The best tomato growing areas in Bangladesh are Dinajpur, Rajshahi, Dhaka, Comilla and Chittagong.

In Bangladesh, the yield of tomato is not enough satisfactory in comparison with other tomato growing countries of the World (Aditya *et al.*, 1997). 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 and improper irrigation facilities as well as production in abiotic stress conditions especially drought. Generally tomato is grown during Rabi winter season and it is dry and as such, the inadequate soil moisture in this season limits the use of fertilizers, and consequently results in decreased yield. Deficiency of water now a days considered as one of the major constraints to successful upland crop production in Bangladesh (Islam and Noor, 1982). The cultivation of tomato requires proper supply of water and this requirement can be meet by applying irrigation. In spite of its broad adaptation, production is concentrated in a few area and rather dry area (Cuortero and Fernandez, 1999).

Hybridization is one of the major tools for achieving variability aiming at the improvement of a crop. Before hybridization genetic diversity of the existing materials or entries needs to be known. Information about genetic diversity in available germplasm is important for the optimal design of any breeding program. This helps to choose desirable parents for establishing new breeding population. Besides, better knowledge on genetic diversity could help to sustain long term selection gain (Chowdhury *et al.*, 2002). Many researchers have reported about different genetic parameters in tomato based on few traits. As yield is the main object of a breeder, so it is important to know the relationship between various characters that have direct and indirect effect on yield. Diversity in tomato is expected to be immense as the fruits vary greatly in shape and size studies on genetic parameters and character associations provide information about the expected response of various traits to selection and help in developing optimum

breeding procedure under the consideration of different management practices and drought is a remarkable factor in Bangladesh environmental condition.

Multivariate analysis with D^2 technique measures genetic diversity in a given population in respect of several characters (Naidu and Satanarayana, 1991). It is one of the potent techniques for measuring the genetic divergence both in intra and inter cluster level. If a plant breeding program is to be advanced more rapidly and efficiently, knowledge of inter-relationships between yield contributing characters is necessary. The multivariate analysis for measuring the degree of divergence and for assessing the relative contribution of different characters to the divergence has been carried out by several workers (Balasch *et al.*, 1984, Ariyo, 1987, Patil *et al.*, 1987). Precise information about the extent of genetic divergence and on characters used for discrimination among the population is crucial in any crop improvement program, because selection of parents based on genetic divergence has become successful in crops (Ashana and Pandey, 1980; Ananda and Rawat, 1984; De *et al.*, 1988).

Some germplasms were received from Plant Genetic Resource Centre (PGRC) of Bangladesh Agricultural Research Institute (BARI), Gazipur and Lal Teer Seed Company, Dhaka but their genetical and *in vitro* information as well as their identifying characters was unknown in drought condition. So, it is an opportunity to categorize the germplasm morphologically under different species for future utilization in drought prone area of Bangladesh. A study was, therefore, conducted *in vitro* on the performance in relation to growth especially root length and fresh weight under laboratory condition. With conceiving the above scheme in mind, the present research work has been undertaken in order to fulfilling the following objectives:

- To develop a new protocol of tissue culture to regenerate plantlets from tomato seeds
- To identify the best drought tolerent genotypes in vitro
- To identify the characters contributing to genetic diversity
- To assess the magnitude of genetic divergence in genotypes.

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. The crop has received much attention by the researchers on various aspects of its production under different adverse condition especially drought. 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 is an ideal crop for genetic, cytogenetic and *in vitro* studies

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 *in vitro* studies due to its low chromosome no i.e., 2n=2x=24 and due to comprehensive knowledge of tomato genetics. Tomato is also amenable to physiological and cytogenetic investigation due to its ease of *in vitro* handling and genetic uniformity resulting from autogamy (Rick, 1980).

2.1.1 Genetic and Cytogenetic studies in tomato

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 0.95 pg 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. Classical genetics has created one of the largest stocks of morphological mutations induced by radiation (X-rays, UV-light, neutrons) and chemical mutagenesis. A major contributor in the mutagenesis area was Hans Stubbe who developed over 300 *L. esculentum* mutants and 200 in *L. pimpinellifolium*. (Rick and Fobes, 1975). A particularly interesting example of induced mutagenesis was the directed manipulation of fruit size of *L. esculentum* and *L. pimpinellifolium* (Stubbe, 1972). A considerable proportion of these mutations have been mapped onto the classical genetic map. The number of mapped genes in the form of cDNAs has increased considerably with the introduction of RFLP markers. The current tomato RFLP map was constructed using an F_2 population of the interspecific cross *L. esculentum* x *L. pennellii* and contains more than 1030 markers, which were distributed over 1276 cM. (Tanksley *et al.*,1992). An integrated high-density RFLP-AFLP map of tomato based on two independent *L. esculentum* x *L. pennellii* F_2 populations has been constructed (Haanstra *et al.*, 1999).

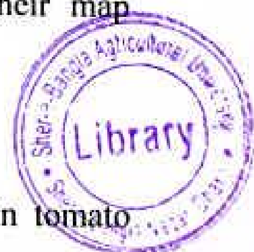
High-resolution genetic and physical mapping around the Tm-2a region, which is located close to the centromere of chromosome 9, indicates that one cM in this area corresponds to more than five million base pairs (Pillen *et al.*, 1996), approximately a sevenfold suppression of recombination over the expected value based on the estimated physical size of the region. In contrast, map-based cloning of the chloronerva gene, which is involved in iron uptake and located in euchromatin of chromosome 1, demonstrated that the ratio of genetic to physical distance in the chloronerva region is 160 kb per 1 cM (Ling *et al.*, 1999) suggesting much higher levels of recombination in this area of the genome. By

determining frequency and distribution of recombination nodules on tomato synaptonemal complexes, Sherman and Stack (1995) observed a much lower frequency of recombination nodules in heterochromatic regions around the centromeres compared to euchromatin. The tomato genome at the DNA level is comprised of approximately 78% single copy sequences, as evaluated under high stringency hybridization conditions (Zamir and Tanksley, 1988). The remaining part of the tomato sequences is repetitive DNA of which four major classes have been characterized (Vallejos *et al.*, 1986; Lapitan *et al.*, 1991).

Zamir and Tanksley (1988) also reported a positive correlation between copy number and rate of divergence of repeats among DNA sequences from related solanaceous species. The more highly repeated sequences evolve more rapidly, whereas single copy coding regions are more conserved among different species. Each tomato chromosome has heterochromatin concentrated around its centromere. Using Feulgen densitometry and SC karyotype data, it was determined that 77% of the DNA in tomato pachytene chromosomes is packaged in heterochromatin, which is similar to an earlier estimate (75.3%) in mitotic metaphase chromosomes (Peterson *et al.*, 1996). In association with findings of Zamir and Tanksley (1988), these data suggest that a large fraction of tomato heterochromatic DNA is composed of single- and/or low-copy sequences and makes tomato heterochromatin unusual and probably genetically active.

The approximate map position of the centromere is now known for each tomato chromosome. For chromosomes 1 and 2, the centromere positions have been identified by RFLP mapping and by in situ hybridization with 5S rDNA and 45S rDNA (Lapitan *et al.*, 1991; Tanksley *et al.*, 1988), respectively. The centromeres of chromosomes 3 and 6 have been located on the integrated molecular-classical map and by deletion mapping (Van Wordragen *et al.*, 1994). Since there is evidence that the potato/tomato inversions on chromosomes 5, 10, 11 and 12 involve entire chromosome arms, the respective centromeres are most likely located at the inversion breakpoints (Tanksley *et al.*, 1992). Map positions of the centromeres of chromosomes 4 and 8 were predicted based on the relationship

among the cytological, genetic and molecular tomato maps. RFLP hybridization and dosage analysis of telo-, secondary and tertiary trisomic stocks (Frary *et al.*, 1996) have achieved a more precise localization of the centromeres of chromosomes 7 and 9. Despite their functional importance, the molecular characteristics of the centromeres of higher eukaryotes remain ill-defined. The copy number and the size of microsatellite containing restriction fragments were highly variable between tomato cultivars (Vosman *et al.*, 1992). The mapping of individual fingerprint bands containing GATA or GACA microsatellites showed predominant association of these repeats with tomato centromeres. Structure, abundance, variability and location were evaluated for a number of different simple sequence repeats isolated from genomic libraries (Broun and Tanksley, 1996). A number of polymorphic microsatellite markers generated from database sequences have been used successfully for genotyping tomato cultivars and accessions (Smulders *et al.*, 1997; Bredemeijer *et al.*, 1998) but their map positions have not been published to date.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2.1.2 *In vitro* studies in tomato

Plant tissue culture techniques are recognized as useful instruments in tomato improvement. Significant advances have been made during the past decades in the development of *in vitro* culture techniques which have been extensively applied to more than 1000 different crop species (Bigot, 1987). 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, Brichkova *et al.*, 2002, Raiziuddin *et al.*, 2004). 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, 2001; Young *et al.*, 1987; Branca *et al.*, 1990; Compton and Veilleux 1991).

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 Pratta *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). Isotopically labeled tomato carotenoids, phytoene, phytofluene, and lycopene, are needed for mammalian bioavailability and metabolism research but are currently commercially unavailable. 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* 13C-labeling. *In vitro* anther culture stands out and is an increasingly powerful tool when integrated into breeding programs (Jose M, 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).

2.2 Effect of drought on developmental stages of plant and crop production

The environmental stresses resulting from drought, temperature, salinity, air pollution, heavy metals, pesticides and soil pH are major limiting factors in crop production (Hernandez *et al.*, 2001; Lawlor and Cornic 2002; Alqudah *et al.*, 2011). Among others, drought stress is a main abiotic stress that limits crop production (Forster, 2004). Drought can be defined as the absence of adequate moisture necessary for a plant to grow normally and complete its life cycle (Zhu, 2002). Drought occurs every year in many parts of the world, often with

devastating effects on crop production (Ludlow and Muchow, 1990). Worldwide losses in crop yields from drought stress probably exceed the losses from all other abiotic stresses combined (Barnabas *et al.*, 2008). Because water resources for irrigating crops are declining worldwide, the development of more drought-resistant or drought-tolerant cultivars and greater water-use efficient crops is a global concern (Ludlow and Muchow, 1990). In the last several decades, the most productive agricultural regions were exposed to drought stress in most years and in occasional years with severe drought. Commonly, drought stress synchronizes with extreme temperature, leading to even greater severity of drought stress (Barnabas *et al.*, 2008).

Drought stress affects crop growth and yield during all developmental stages. The effect of drought on yield is highly complex and involves processes as diverse as reproductive organs, gametogenesis, fertilization, embryogenesis, and seed development stress (Barnabas *et al.* 2008). Reproductive development at the time of flowering is especially sensitive to drought stress (Zinselmeier *et al.* 1995, 1999; Samarah *et al.*, 2009). Therefore, an understanding of how a reproductive process affected by drought is of particular interest for improving drought tolerance (Samarah *et al.*, 2009). The flowering period of a crop is a critical growth stage and a yield determinate factor in normal growing seasons and in drought stressed regions in particular. An understanding of how crop plants respond to drought stress during reproductive stage is important in maximizing yields in water-limited regions.

Drought stress is a main abiotic stress that limits crop pollination by reducing pollen grain availability (Agren 1996; Trueman and Wallace 1999), increasing pollen grain sterility (Schoper 1986; Al-Ghzawi *et al.*, 2009), decreasing pollen grain germination and pollen tube growth (Lee, 1988). Drought stress can also reduce megagametophyte fertility (Young *et al.*, 2004), inhibit the differentiation of young microspores (Satake, 1991), lower the number of dehisced anthers (Sawada, 1987), repress anther development (Nishiyama, 1984), and decrease seed set and seed development (Al-Ghzawi *et al.*, 2009).

Increasing evidence indicates that ovary abortion can account for substantial kernel losses when maize experiences low water potential near the time of pollination (Westgate and Boyer, 1985a; Boyle *et al.*, 1991; Zinselmeier *et al.*, 1999; Andersen *et al.*, 2002). Flowering is one of the most important growth stage affected by drought stress. Drought stress interferes with flower period, flower opening, nectar production, and turgor maintenance of floral organs (Mohan Ram and Rao, 1984). The trend for reduced flower size under drought stress is mirrored in populations of *Clarkia unguiculata* distributed along a natural moisture gradient (Jonas and Geber, 1999). Water stress detrimentally affects flower induction, pollen production and subsequently leads to failure of fertilization and hence grain set (Sheoran and Saini, 1996).

Soil water deficits that occur during the reproductive growth are considered to have the most adverse effect on crop yield (Costa-Franca *et al.*, 2000; Samarah 2004; Samarah *et al.*, 2009a, b). Drought stress imposed on plants leads to decrease yield through reducing seed set (Westgate and Boyer, 1986; Al-Ghzawi *et al.*, 2009). Low seed set percentages are regularly related to several factors such as reducing pollen grain availability (Agren, 1996; Trueman and Wallace, 1999), increase ovary abortion (Boyer and Westgate, 2004), increase pollen grain sterility (Schoper, 1986; Westgate and Boyer, 1986; Al-Ghzawi *et al.*, 2009), slow stigma and style elongation (Westgate and Boyer, 1985b), reducing time of pollination (Westgate and Boyer, 1986), lower pollen grain germination activity, pollen tube growth, and less development of fertilized seeds (Lee, 1988). Many researchers have found that the reduction in number of spikes per plant under drought stress was due to the increase in the number of sterile spikes per plant and the decrease in the number of fertile spikes per plant in six-row barley (Mogensen, 1992; Sanchez *et al.*, 2002; Samarah, 2004; Samarah *et al.*, 2009a). A reduction in number of grains per spike has been reported for barley (Agueda, 1999; Mogensen, 1992; Samarah, 2004; Samarah *et al.*, 2009a) and wheat (Garcia, 2003) under drought stress.

Drought stress not only affects seed production, but many researchers found that drought stress during reproductive growth lowered seed germination and vigor. Seed quality, estimated by standard germination, was lower for seeds harvested from plants grown under drought than seeds harvested from irrigated plants (Smiciklas *et al.*, 1992).

2.3 *In vitro* drought tolerance in tomato

Tomato is one of the popular and most important vegetable crops of Bangladesh and as well as many countries of the world. The crop has received much attention by the researchers on various aspects of its production under different adverse condition especially drought. 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:

2.3.1 Genotypic differences and *in vitro* selection of tomato genotypes for drought tolerance

The existence of genotypic variability reported for differences to drought resistance in solanaceous vegetable (Srinivasa Rao and Bhatt, 1991), tomato (Pillay *et al.*, 1990), Black gram (Geetha *et al.*, 1997) and many researchers worldwide. Declined water contents tend to reduce leaf area in tomato genotypes (Jurekova *et al.*, 2011) which in turn results in reduced shoot lengths (Unyayar *et al.*, 2005). Changing climatic patterns in Bangladesh tend to influence tomato production and quality majorly by water scarcity, elevated saline conditions. Tomato genotypes tend to exhibit limited and inadequate genetic variability for drought tolerance. Hence the best way to mitigate the effects of drought stress involves the crossing of cultivated tomato with drought tolerant lines (Pena and Hughes, 2007). The tomato varieties investigated *in vitro* by George (2013) have shown varying responses for different plant attributes against drought stress which remained significantly different for most of the parameters studied. For a given genotype, the effect of drought *in vitro* was significant for root length and

seedling biomass, whereas it was non-significant in case of germination and shoot length. Kulkarni and Deshpande (2007) evaluated mutant derivatives and hybrids for screening under drought and found better performance in mutant derivatives and hybrids than cultivated genotypes under all levels of water stress. Significant differences were also found between tomato genotypes for their ability for callus formation on the two types of explants on the different types of media. The mass of callus and shoots regenerated directly from explants were evaluated after 60 days of growing on regeneration MS medium supplemented with different concentration of PEG (Abdel-Rahim *et al.*, 2007).

2.3.2 Polyethelene Glycol (PEG) for drought stress *in vitro*

Polyethylene glycol, a non penetrable and nontoxic osmotic, lowers the water potential of the medium and has been used to simulate drought stress in plants. Cells adapted to PEG caused deficit of water have been isolated in *Solanum lycopersicum* (Handa, *et al.*, 1982, 1983; Bressan, *et al.*, 2003). Polyethylene glycol (PEG), a series of polymers that vary from viscous liquids to waxy solids has been used to induce water stress artificially (Larher *et al.*, 1993). PEG induced osmotic stress is found to reduce cell water potential (Govindaraj *et al.*, 2010). An increase in concentration of PEG-6000, resulted a decrease in germination rate, root length, shoot length and seed vigor in certain crop plants (Khodarahmpour, 2011). Tomato has been selected for better growth under PEG simulated water stress (Bressan *et al.*, 2003). *In vitro* selection techniques involving the use of PEG, is one of the reliable methods for screening desirable genotypes and to study further the effects of water scarcity on plant germination indices (Kocheva *et al.*, 2003; Sakthivelu *et al.*, 2008). George *et al.*, (2011) described the effect of artificial drought stress using PEG on different tomato varieties at seedling stage. Effort was also made to screen tomato germplasm under *in vitro* condition using polyethylene glycol (PEG) at four concentrations (0, 20, 40 and 60 g/l) with two replications in factorial CRD (Kulkarni and Deshpande, 2007). Decrease in seedling growth was worth noticed with increasing concentration of PEG indicating precise nature of the *in vitro* screening.

2.4 Genetic diversity

The assessment of genetic diversity using quantitative traits has been of prime importance in many contexts particularly in differentiating well defined populations. The germplasms in a self-pollinated crop can be considered as heterogeneous sets of groups, since each group being homozygous within it. Selecting the parents for breeding program in such crops is critical because, the success of such program depends upon the segregants of hybrid derivatives between the parents, particularly when the aim is to improve the quantitative characters like yield. To help the breeder in the process of identifying the parents, that need better, several methods of divergence analysis based on quantitative traits have been proposed to suit various objectives. Among them, Mahalanobis's generalized distance occupies a unique place and an efficient method to gauge the extent of diversity among genotypes, which quantify the differences among several quantitative traits. In crop improvement programme, genetic divergence has been considered as an important parameter to identity most diverse parents for obtaining highly heterotic F_1 generation through selection. Many scientists have studied genetic divergence of tomato on the basis of Mahalanobis' D^2-statistics based on multivariate analysis. Shashikanth *et al.* (2010) carried out a field experiment to study genetic divergence of 30 tomato genotypes and observed that analysis of variance of the genotypes showed significant differences for all the characters studied indicating the existence of genotypic variation. There was no parallelism between genetic diversity and geographical divergence in tomato and suggested that high diversity among the genotypes belonging to cluster VII and X can be selected in hybridization programmes to obtain good seggregants.

Twelve varieties of tomato were evaluated to estimate heritability by Pandit *et al.* (2010) and reported that high heritability coupled with high genetic advance as percentage of mean for average fruit weight, indicating the control of such character by additive gene. He also recorded that high heritability coupled with low genetic advance as percentage of mean for rest of the characters except pericarp thickness, indicating most of the characters were governed by non-

additive genetic components. On the other hand high heritability and high genetic advance for plant height, moderate for total number of fruit bearing branches, weight per fruit and days to maturity, while the remaining characteristics had low values of genetic advance were estimated (Kumari *et al.*, 2007). Similar results were obtained coupled with high genotypic coefficient of variation and genetic grain for 10-fruit weight, number of locules per fruit and fruit yield, which could be improved by simple selection (Golani *et al.*, 2007)

The effectiveness of selection in the present germplasm of tomato improvement reflects when broad sense heritability is high (Saeed *et al.*, 2007). Broad sense heritability was highest for number of fruits per plant (96.56%), followed by number of flowers per plant (93.45%) (Saeed *et al.*, 2007). The importance of considerable additive gene effects could be attained by giving greater emphasis on some of specific characters while selecting the better genotypes in tomato (Mahesha *et al.* (2006). He estimated heritability and expected genetic advance in 30 genotypes of tomato and observed that fruit weight, fruits per plant and plant height exhibited very high heritability values along with high genetic gain, while Kumar *et al.* (2006) observed low heritability (4.40%) and high genetic advance (35.55) for plant height. Joshi *et al.* (2004) observed moderate heritability and moderate genetic gain for number of fruits per cluster, fruit length, fruit breadth, stem end scar size, number of locules per fruit, whole fruit firmness, ascorbic acid content and plant height indicating additive gene effects. Low heritability and low genetic gain was observed for pericarp thickness. Moderate heritability and low genetic gain for harvest duration suggests the presence of dominance and epistatic effects. High heritability combined with high genetic gain was observed for shelf life indicating additive gene action.

Nineteen genotypes of tomato were evaluated to estimate heritability and high heritability for ascorbic acid content, average weight of fruits, number of leaves per plant, number of locules per fruit, number of fruits per plant, leaf area and dry matter content were observed (Singh *et al.*, 2006). High estimates of heritability with high genetic advance was recorded in case of number of leaves per plant,

average weight of fruits, number of fruits per plant and plant height, whereas high heritability with low genetic advance was recorded for number of locules per fruit, dry matter content, pericarp thickness and yield per plant. Mahesha *et al.* (2006) grouped 30 tomato genotypes into nine clusters studied based on D^2 analysis. The cluster mean indicated that Days to 50% flowering, plant height, number of branches per plant, number of cluster per plant, number of fruit per cluster and fruit yield per plant were reported as chief contributors towards divergence.

Sharma *et al.* (2006) reported Sixty genotypes of tomato were studied for genetic divergence and the genotypes grouped into 10 clusters, maximum divergence within a cluster was exhibited by the cluster VIII (1.531), closely followed by cluster III (1.528) and cluster V (1.460), whereas, cluster VIII and II were the most divergent from each other followed by cluster VII and cluster VIII (Sharma *et al.*, 2006). Veershetty (2004) grouped 32 tomato genotypes into 10 cluster based on D^2 analysis number of fruits per cluster, plant height, number of branches, pericarp thickness, average fruit weight and TSS content of fruit were reported as chief contribution towards divergence. Arun *et al.* (2004) reported that moderate heritability associated with moderate genetic advance for plant height of 37 tomato genotypes of tomato. Kumar *et al.* (2004) estimated heritability and genetic advance in 30 tomato genotypes for the characters like number of primary branches per plant, plant height, number of fruits per plant, fruit yield per plant and average fruit weight. The average fruit weight showed high heritabilities that ranged from 89.10% to 96.50%. The rest of the characters showed moderate heritability and low genetic advance.

The nature and magnitude of genetic divergence in 73 tomato genotypes of different origin were studied for quantitative characters and they grouped genotypes into 15 cluster indicated the presence of wide range of genetic diversity among the genotypes, cluster 5 having 6 genotypes. The mean fruit yield/plant (1034 g/plant) and average fruit weight (102.76 g/plant) were the highest in cluster 5 and 3 respectively. The plant height (135.91 cm), harvest duration (37.77 days) were maximum in cluster 15 and lowest number of leaves (20,280) was

recorded in cluster 9 and cluster 6 consist of highest number of fruits/cluster (4.90) (Arun *et al.*, 2003). Mohanty (2003) observed that high heritability with high genotypic coefficient of variation for fruit weight, plant height, number of fruits and number of branches per plant.

Mohanty (2002) evaluated 18 genotypes of tomato and revealed high heritability with moderate to high genetic gain for average fruit weight, number of fruits per plant and plant height. Singh (2002) reported that heritability was high for all characters except days from fruit setting to red ripe stage and the highest genetic advance was predicted for average fruit weight, followed by shelf life of red ripe fruits. Parthasarathy and Aswath (2002) conducted a study with 23 genotypes of tomato in Meghalaya and observed a considerable diversity among genotypes for 8 morphological characters. Plant height, fruit number, fruit size were contribute to the divergence among them. Crosses involving L-964 and L-154 with Arka Abha and LE-79 were recommended for improved yield and better fruit size. Genetic divergence of 25 cultivars of tomato originating from the area of the former Yugoslavia and recorded the presence of a high degree of genetic divergence in different genotypes consisting of 5 clusters (Markovic *et al.*, 2002).

High degrees of heritability and genetic advance for fruits per plant, individual fruit weight and number of seeds per fruit were observed by Matin (2001). Brar *et al.* (2000) reported that the number of fruits per plant, total yield per plant and marketable yield per plant had low to moderate estimates of heritability and genetic advance and number of marketable fruits per plant had high values of heritability and genetic advance.

A field experiment was carried out in Dharwad, Kamataka, India during 1994-95 to assess genetic diversity in a population of 402 tomato lines by using multivariate analysis based on plant height, number of branches, number of clusters per plant, fruits per cluster, number of fruits per plant, yield per plant, incidence tomato curl viruses and number of whiteflies per plant. They grouped the lines into 4 clusters based on the similarities of D^2 values. Cluster-I was the

biggest having 217 genotypes, which also consisted of commercial ToLCV susceptible genotypes, namely DWD-1, DWD-2, *etc*., cluster-II consisting of 51 genotypes/hybrids with potato leaf type and pink fruit, which exhibited field tolerance to ToLCV and cluster-III and IV had 99 and 35 genotypes respectively. Considerable diversity within and between cluster was noticed (Dharmatti *et al*., 2001).

A study on genetic diversity among 18 indigenous and exotic tomato cultivars were performed for five economic characters (plant height, number of branches per plant, number of fruits per plant, average fruit weight and yield) in Orissa, India during rabi 1998-99 and found considerable variations among the accessions. They could group the genotypes into 5 clusters including two solitary groups and reported that genetic diversity was not associated with geographic distribution. Maximum intercluster distance (D^2=1289.31) was observed between the clusters I and V. The distance between clusters I and III, III and IV, IV and V was moderate. They also reported that number of fruits per plant and average fruit weight contributed predominantly towards the total divergence (Mohanty and Prusti, 2001).

Genetic divergence of 18 genotypes of tomato was studied by Sharma and Verma (2001) and they grouped them into 5 clusters irrespective of geographic divergence indicating no parallelism between genetic diversity and geographical divergence. Fruit yield was one of the three characters which played an important role in divergence between the populations. Nessa *et al*. (2000) reported high heritability for number fruits per plant, plant height and moderate heritability for yield per plant. Prasad *et al*. (1999) estimated heritability in 75 exotic genotypes of tomato and reported very high heritability along with high genetic advance by fruit weight. Genetic divergence of 32 tomato genotypes was studied grouped them into 9 clusters based on D^2 values. The magnitude of inter cluster distances was comparatively lower than that of inter cluster distances Kumar and Tewari, 1999).

Rai *et al.* (1998) studied 37 tomato genotypes and could able to group them into four clusters using a non-heritable clustering approach with the help of Mahalanobis' D^2 statistics for yield and yield contributing characters. The population was grouped into 4 clusters. The clustering pattern indicates that there was no associatior, between geographical distribution of genotype and genetic divergence characters namely number of primary branches, days to first flowering, plant height and average fruit weight contributed to maximum divergence. High heritability and genetic advance in percentage of mean were estimated by Phookan *et al.*, 1998) for fruits per plant and average fruit weight suggesting their importance in selection for tomato improvement. Vikram and Kohli (1998) reported high heritability and genetic advance for mean fruit weight which suggested that improvement for this character should be fairly straight forward. Singh *et al.* (1997) estimated heritability and genetic advance in 23 genotypes of tomato. High values of heritability and genetic advance indicated that effective selection may be made for fruit weight and number of fruits per plant.

High heritability associated with high genetic advance indicates the character, predominantly under the control of additive gene and could be improved through selection. Mittal *et al.* (1996) estimated heritability and genetic advance in 27 genotypes of tomato. Pujari *et al.* (1995) observed high heritability coupled with high genetic advance for number of fruits per plant, plant height and average fruit weight which indicated additive gene action. Aditya *et al.* (1995) reported high heritability (in broad sense) with high genetic advance in percentage of mean for number of fruits per plant, individual. fruit weight and plant height. However, yield per plant showed moderate heritability and low genetic advance but highest genetic advance as percentage of mean under selection. Islam *et al.* (1996) studied heritabiltiy and genetic advance in 26 diverse genotypes of tomato. High heritability and genetic advance was observed in number of fruits per plant, plant height, fruit yield and individual fruit weight. Gadekar *et al.* (1992) obtained high values for heritability along with high genetic advance by fruit weight. Reddy and

Reddy (1992) studied heritability and genetic advance in 139 tomato varieties. Heritability values for yield per plant, number of fruits per fruits per plant and average individual fruit weight were 97.99%, 95.96% and 98.46% respectively.

Bai and Devi (1991) evaluated five varieties and nine hybrids of tomato. Heritability estimates of 90% were obtained for plant height, number of fruits per plant and individual fruit weight. Islam and Khan (1991) studied 12 tomato genotypes and reported that heritability values were high for most of the characters but moderate for days to first flowering, maturity and plant height. Sonone *et al.* (1986) reported that heritability estimates for fruit number, plant height and individual fruit weight were high in tomato. He also reported that high genetic advance (>30%) was observed for fruit yield, plant height, individual fruit weight and number of fruits per plant. Estimates of high heritability and high genetic advance for number of fruits per plant, individual fruit weight and plant height indicated control by additive genetic effects. Mallik (1985) reported high genetic advance for plant height, number of fruits per plant, individual fruit weight and yield per plant but low heritability for yield per plant. 55 genotypes in tomato genotypes were grouped into nine cluster based on D^2 analysis. A maximum of 16 genotypes entered cluster I, followed by 15 in cluster IV, 9 in cluster III, 7 in cluster II, 4 in cluster V and the remaining four cluster consisted of solitary genotype Patil, 1984).

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, 2011 to September, 2012 to study the screening of drought tolerant genotypes *in vitro* in Tomato. 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. The place is geographically located at about $24^0 75'$ North latitude and $90^0 50'$ East longitude.

3.2 Tomato genotypes

Fourteen tomato genotypes were used as experimental materials among which BARI Tomato 2, BARI Tomato -11 are parent material and rest of all were lines. The materials were collected from Bangladesh Agricultural Research Institute (BARI), Gazipur. The genotypes used in the study are listed below:

Table1. List of the Tomato genotypes used in the experiment

Genotypes name	
BARI Tomato-2	BARI Tomato-11
BD-7260	BD-7290
BD-7295	BD-7286
BD-7269	BD-7258
BD-7289	BD-7292
BD-7291	BD-7302
BD-7301	BD-7762

3.3 Laboratory preparation

Laboratory preparation was started in early January 2011 by collecting chemical and instruments and presented in Table 2.

Table 2. List of the chemicals and instruments used in the experiment

Chemicals		Instruments	
1.	a) MS medium (powder) (Duchefa, Netherlands) b) MS medium ingredients	1. 2. 3.	Autoclave Hotplate with magnetic stirrer Automatic drying oven
2.	Sterilizing chemicals a. Sodium hypo chloride b. Potassium hypo chloride c. Tween-20	4. 5. 6.	Freezers Autoclave Furnaces
3.	Sucrose	7.	Incubators
4.	Agar	8.	Laminar Air Flow Chamber
5.	NaOH (10 N, 1N)	9.	Microwave oven
6.	HCl	10.	Pipettors
7.	KCl (3M)	11.	Plant Growth Chamber
8.	Poly-ethelene Glycol (PEG)	12.	Safety Cabinets
9.	Sterilized distiled water	13.	Shakers
10	Absolute Ethanol	14.	Shaking Incubator
11	Ethanol (70%)	15.	Water Purification System
12	Methilated spirit	16.	pH meter
		17.	Course and fine electric balances
		18.	Scalpel, forceps, scissors etc.
		19	Culture vials (petridishes, test tubes, culture bottles etc.)

3.4 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 PEG concentration as culture medium for giving stress to the plants.
The composition of MS medium has been presented in Appendix 1. PEG was 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.

3.4.1 Stock solutions preparation

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.

3.4.1.1 Macronutrients stock solution (stock 1)

Stock solution of macronutrients was prepared with 10 times 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. Dissolve all the macronutrient 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 DW. The stock solution was poured into a clean sterilized glass container and stored in a refrigerator at 4°C for ready use.

3.4.1.2 Micronutrients stock solution (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 is preferable to first make a separate stock solution of those two salts (100) and then an appropriate volume can be pipetted and put into the main micronutrient stock solution. This stock solution was also stored in refrigerator at 4°C.

3.4.1.3 Iron (Fe-EDTA) stock solution (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.

3.4.1.4 Vitamins stock solution (stock 4)

The following vitamins were used in the present study for the preparation of MS medium. Myo-inositol (Inositol), Nicotinic acid (Vitamin B_3), Pyridoxin HCl (Vitamin B_6), Thiamine HCl (Vitamin B_1), 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.

Library

3.4.2 Other stock solutions preparation

3.4.2.1 Preparation of IN NaOH:

40 g NaOH pellets were weighed and added to the 800 ml of sterilized distilled water and stirred well until dissolved. Sterilized distilled water was added to make volume 1000ml and mixed the closed bottle.

3.4.2.2 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. Store the solution in a sterilized glass bottle. This solution was made fresh each time before use.

3.4.3 MS Media preparation

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

1. 800 ml double distilled water was taken into 1 liter beaker

2. 100 ml of stock solution of macro-nutrients, 10 ml of stock solution of micro nutrient, 10 ml of stock solution of Fe-EDTA and 10 ml of stock solution of vitamins 800 ml double distilled water
3. 30g of sucrose was dissolved in this solution with the help of magnetic stirrer
4. The whole mixture was then made up to 1 liter with further addition of double distilled water.
5. For control, PEG will not be included but for treatments, 20g/l, 30g/l and 40g/l of PEG will be included

3.4.4 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.

3.4.5 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.

3.5 Sterilization

3.5.1 Sterilization of culture media

One litre of MS medium were divided into two 1 litre conical flasks and capped with aluminium foil. Then the conical flasks were autoclaved at 15 psi pressure at 121^0C for 20 minutes. The medium was then transfer into the culture room and cooled at 24^0C temperature.

Aliquot fixed volume of medium into petridishes (Figure 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 PEG supplements. The petridishes containing media could be store at 4^0C until use. Marking was done for identification.

3.5.2. 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 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 and dried in automatic drying oven.

Plate 1. Aliquot the MS media in petridishes for inoculation. The experiment was carried out at the Genetics and Plant Breeding Laboratory, Sher-e-Bangla Agricultural University, Dhaka.

3.5.3. Sterilization of culture room and transfer area

At the beginning, the culture room was spray 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 is 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.

3.5.4. Sterilization of seed

Seeds were treated with absolute alcohol for 1 minute. After treating, rinsed with sterilized distilled water for 2 times. Then treated with NaOCl/CaOCl (20%) with a drop of tween-20 for 2 minutes. Then rinsed 5 times with sterilized distilled water.

3.6. Inoculation and culture

The sterilized seeds of fourteen genotypes of tomato were inoculated for germination The petridishes were labeled properly. The culture environment was included, 25°C, 60% relative humidity, and a 16-h photoperiod from white fluorescent lamps (200μmol photons/m^2s^{-1}) (Figure 2A).

3.7 Drought tolerance assay

The drought tolerance assay was performed as Zeba et al., (2009). Briefly, Four days old germinated seeds were inoculated in a linear order on MS medium supplemented with 0, 20, 40 and 60 g/L of Poly-Ethelene Glycol (PEG). The culture plates were kept in the growth chamber in vertical position (Figure 2B). The culture environment included, 25°C, 60% relative humidity, and a 16-h photoperiod from white fluorescent lamps (200 μmol photons/m^2/s^{-1}). After seven days, root length and fresh weight of all plants were measured.

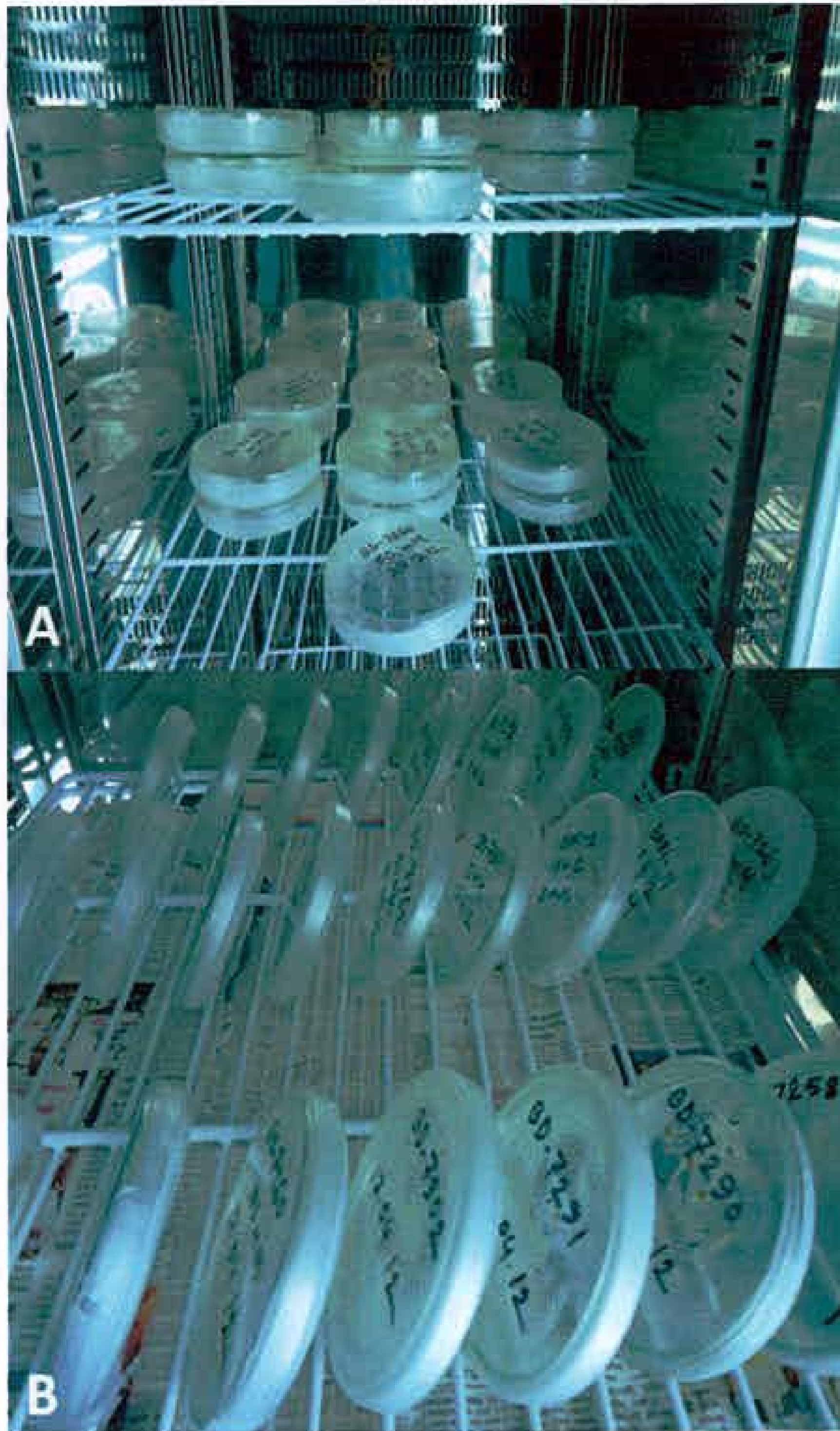


Plate 2. Inoculation and incubation for germination and bioassay. **A.** Inoculation and incubation of sterilized tomato seeds in half strength MS medium without PEG. **B.** Inoculation and incubation of four days old germinated plantlets in half strength MS medium supplemented with 0 g/L, 20 g/L, 30 g/L and 40g/L of PEG. Incubation was done in growth chamber with 25 ± 1°C under 16 h photoperiod at 50 µmol/ m^2/s^{-1} (with white fluorescent lamp)

3.8 Precaution and 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 50 ml conical flasks was flamed during aliquote. Aseptic conditions were followed during each and every operation to avoid the contamination of cultures.

3.9 Analysis of genetic divergence

Genetic divergences among the genotypes studied were assessed by using Mahalanobis' D^2 statistics and its auxiliary analysis. Both techniques estimate divergences among a set of genotypes on multivariate scale.

Mahalanobis' D^2 statistics

First the variation among the materials were tested by Wilkin's criteria '^'.

$$\text{'}\wedge\text{'} = \frac{|W|}{|S|} = \frac{|\text{Determination of error matrix}|}{|\text{Determination of error + variety matrix}|}$$

Now, $\text{'v'}_{(stat)} = -m \log_{e^\wedge} = -\{n-(p+q+1)/2\}\log_{e^\wedge}$

Where,

$m = n-(p+q+1)/2$

p = number of variables or characters

q = number of varieties – 1 (or df for population)

n = df for error + varieties

e = 2.7183

Data were then analysed for D^2 statistics according to Rao (1952). Error variance and covariance matrix obtained from analysis of variance and covariance were inverted by pivotal condensation method. Using the pivotal elements the original means of the characters (X_1, X_2---------X_8) were transformed into a set of uncorrelated variables (Y_1, Y_2---------Y_8).

Now, the genetic divergence between two varieties/lines (suppose Vi and Vj was calculated as –

$$D^2ij = \sum_{k=1}^{8} (Vik - Vjk)^2$$

Where,

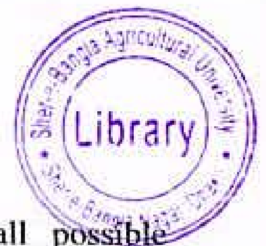
D^2ij = Genetic divergence between 'i' th and 'j' th genotypes

Vik = Transformed mean of the 'i' th genotype for 'k' th character

Vjk = Transformed mean of the 'j' th genotype for 'k' th character

The D^2 values between all varieties were arranged in order of relative distances from each other and were used for clusters formation, as suggested by Rao, 1952.

$$\text{Average intra-cluster } D^2 = \frac{\sum D^2 i}{n}$$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Where,

$\sum D^2 i$ = Sum of distances between all possible combinations (n) of the genotypes included in a cluster.

N = All possible combinations.

CHAPTER IV

RESULTS AND DISCUSSION

The experiment was conducted to study the screening of drought tolerant genotypes *in vitro* in tomato. Four days old germinated seeds were transferred on MS medium containing 0 g, 20 g, 30 g and 60 g/L of PEG for root assay and weight assay. After 7 days of inoculation, roots were increased in length and shoots were also developed (Figure 3A, B and C). The results from the root length and plantlet weight have been presented and discussed with the help of table and graphs and possible interpretations given under the following headings:

4.1 Mean performance of different tomato genotypes

4.1.1 Root length

Statistically significant variation was recorded for root length due to PEG level of 0, 20, 40 and 60 g under the present trial (Table 3). For different mean PEG root length varied from 1.88 cm to 2.83 cm. The longest root length (2.83 cm) was recorded for 0 g PEG which was followed (2.38 cm) by 20 g PEG, whereas the shortest length (1.88 cm) was observed in 60 g PEG which was statistically similar (2.04 cm) with 40 g PEG. Data revealed that in the application of 0 g PEG produced comparatively the longest root which was generally followed by 20 g PEG. It was also found that 20 g PEG have minimum adverse effect on root length for the genotypes that were used in this trial. On the other hand, in 40 and 60 g PEG produced the shortest length of root indicated that the tomato genotypes exhibited adverse effect for root length due to the effect of PEG. Among the studied genotypes BD-7301 produced the longest root in 40 and 60 g PEG compared to 0 and 20 g PEG. Figure 1 and 2 showed the root length for 0 and 40 g PEG. In an average among the different genotypes of tomato the highest length of root (5.01 cm) was recorded from BARI-2 which was followed (4.02 cm and 3.86 cm) by BD-7258 and BD-7290. On the other hand, the lowest length of root (0.69 cm) was found in BARI-11 which was statistically identical (0.70 cm, 0.76 cm and 0.94 cm) with BD-7292, BD-7260 and BD-7295, respectively which was closely followed (1.18 cm) by BD-7302 (Table 3).

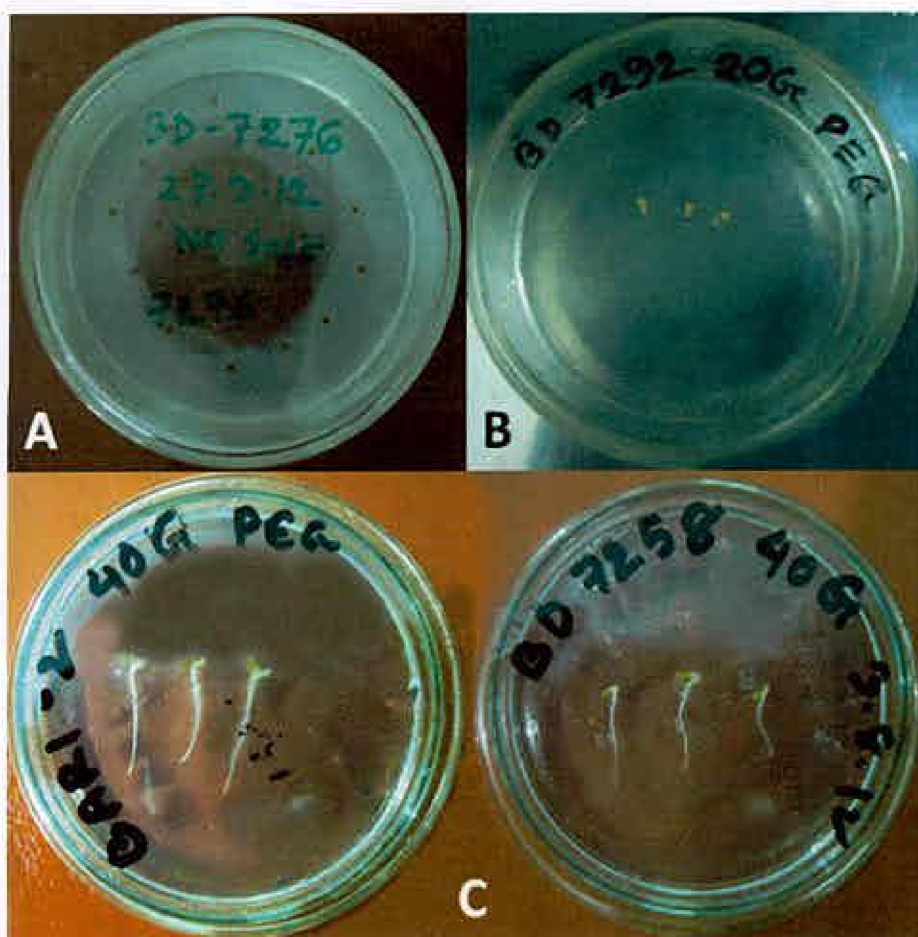

Plate 3. Enhanced drought tolerance using PEG of some genotypes of tomato. **A.** Inocultion of sterilized seed in half strength MS media without salt. **B.** Germination of seeds. **C.** Inoculation of four days old germinated plantlets in half strength MS medium supplemented with 0g , 20g , 40g and 60g/L of PEG and grown in vertical position for 7 days.

Table 3. Root length of different tomato genotypes under different level of PEG

Genotypes	Mean root length (cm) under different level of PEG				Mean
	0 g PEG	20 g PEG	40 g PEG	60 g PEG	
BARI-2	6.53	6.00	4.17	3.33	**5.01 a**
BARI-11	1.23	0.87	0.40	0.27	**0.69 h**
BD-7260	1.30	0.97	0.43	0.33	**0.76 gh**
BD-7290	4.13	3.67	3.87	3.77	**3.86 b**
BD-7295	1.60	1.27	0.50	0.40	**0.94 gh**
BD-7286	2.63	2.33	3.17	3.03	**2.79 d**
BD-7269	3.50	2.83	1.17	1.00	**2.13 ef**
BD-7258	4.30	3.83	4.17	3.77	**4.02 b**
BD-7289	2.20	1.67	1.67	1.57	**1.78 f**
BD-7292	1.20	0.80	0.43	0.37	**0.70 h**
BD-7291	3.17	2.67	1.67	1.90	**2.35 e**
BD-7302	1.77	1.30	0.87	0.77	**1.18 g**
BD-7301	2.27	1.80	3.00	2.90	**2.49 de**
BD-7762	3.80	3.33	3.00	2.87	**3.25 c**
Mean	**2.83 a**	**2.38 b**	**2.04 c**	**1.88 c**	

In a column, means having similar letter(s) are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05 level of probability

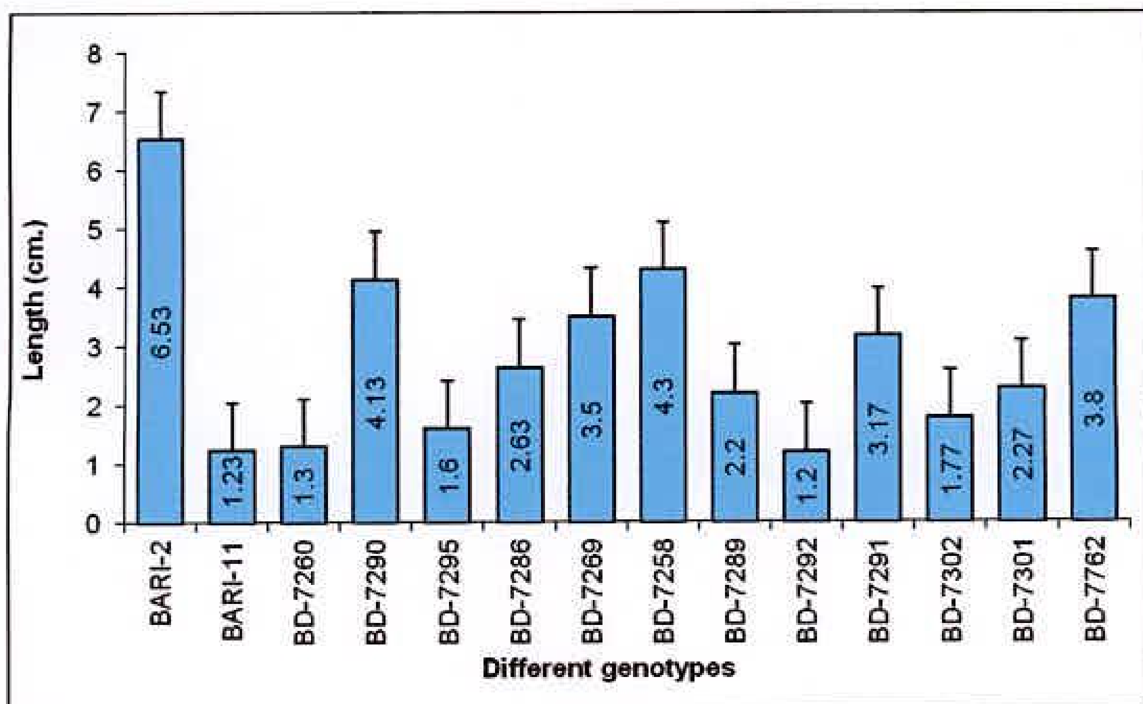

Figure 1. Root length of different tomato genotypes under control condition (0 g PEG). Vertical bars indicate the $LSD_{(0.05)}$ value

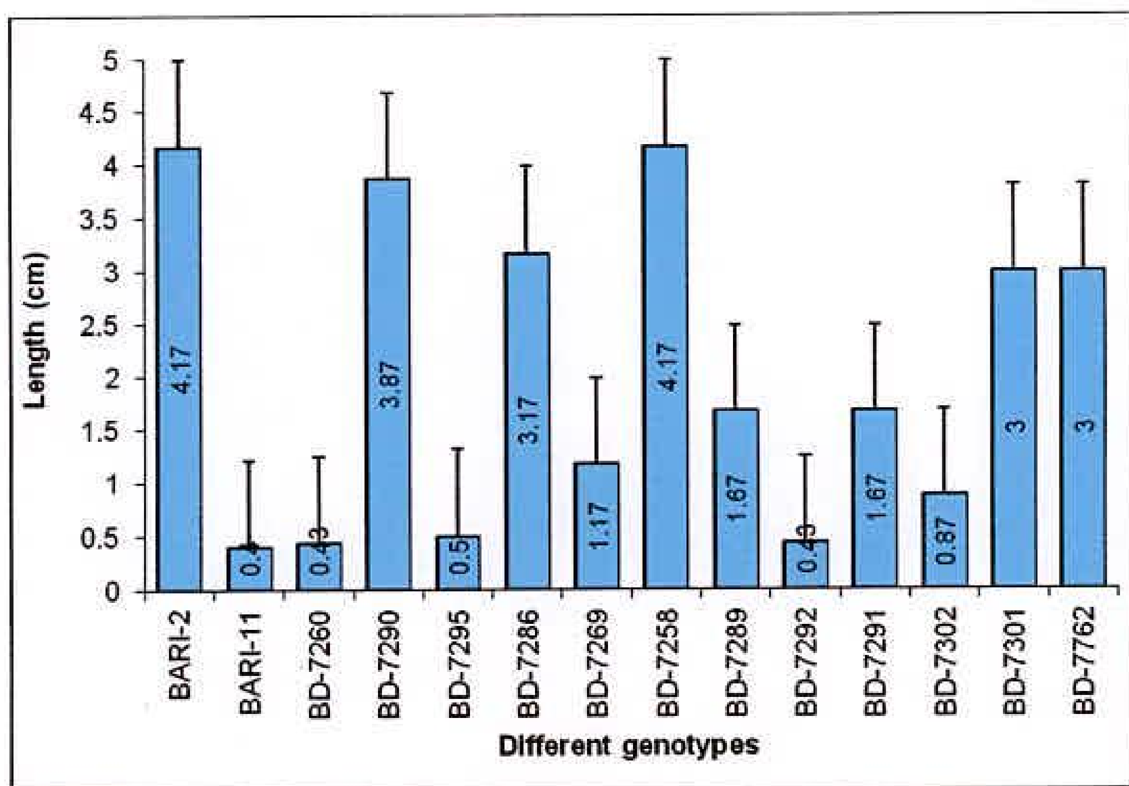

Figure 2. Root length of different tomato genotypes under stress condition (40 g PEG). vertical bars indicate the $LSD_{(0.05)}$ value

4.1.2 Relative root length over control

Statistically significant variation was recorded for relative root length due to PEG level of 20, 40 and 60 g under the present trial (Table 4). For different PEG the average relative root length was 0.80, 0.71 and 0.66, respectively for 20, 40 and 60 g PEG. The highest relative root length over control (0.80) was recorded for 20 g PEG, while the lowest relative root length (0.66) was observed in 60 g PEG which was closely followed (0.71) by 40 g PEG. Data revealed that with the increase of level of PEG relative root length decreases and for 40 and 60 g PEG the relative root length followed more or less similar decreasing trend. From the data of relative root length over control BD-7290, BD-7286, BD-7258, BD-7289, BD-7301 and BD-7762 gave the increasing trend with the increasing level of PEG. It was also found that PEG from 40 to 60 g relative root length showed decreasing trend that men with the increase of PEG level relative root length decreases for the studied genotypes. Figure 3 showed the relative root length of different genotypes for 40 g PEG.

In an average among the different genotypes of tomato the highest relative root length over control (1.20) was recorded from accession BD-7301 which was followed (1.07 and 1.01) by BD-7286 and BD-7290. On the other hand, the lowest relative length of root (0.42) was recorded in BARI-11 which was statistically identical (0.45 and 0.46) with accession BD-7260 & BD-7292 and BD-7295 & BD-7269, respectively which was closely followed (0.54) by BD-7302 (Table 3) under the present trial. Markovic *et al.* (2002) studied genetic divergence of 25 cultivars of tomato originating from the area of the former Yugoslavia and recorded the presence of a high degree of genetic divergence in different genotypes consisting of 5 clusters. Sharma and Verma (2001) reported genetic divergence of 18 genotypes of tomato and grouped them into 5 clusters irrespective of geographic divergence indicating no parallelism between genetic diversity and geographical divergence.

Table 4. Relative root length over control (0 g PEG) of different tomato genotypes under different level of PEG

Genotypes	Relative root length over control (0 g PEG) of different level of PEG			Mean
	20 g PEG	40 g PEG	60 g PEG	
BARI-2	0.91	0.65	0.52	**0.69 e**
BARI-11	0.70	0.33	0.22	**0.42 g**
BD-7260	0.75	0.33	0.25	**0.45 fg**
BD-7290	0.87	1.10	1.07	**1.01 bc**
BD-7295	0.80	0.32	0.25	**0.46 fg**
BD-7286	0.89	1.18	1.13	**1.07 b**
BD-7269	0.79	0.32	0.26	**0.46 fg**
BD-7258	0.89	0.98	0.89	**0.92 cd**
BD-7289	0.74	0.88	0.83	**0.81 d**
BD-7292	0.67	0.36	0.31	**0.45 fg**
BD-7291	0.80	0.55	0.71	**0.69 e**
BD-7302	0.73	0.48	0.42	**0.54 f**
BD-7301	0.77	1.58	1.52	**1.29 a**
BD-7762	0.84	0.93	0.90	**0.89 d**
Mean	**0.80 a**	**0.71 b**	**0.66 c**	

In a column, means having similar letter(s) are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05 level of probability

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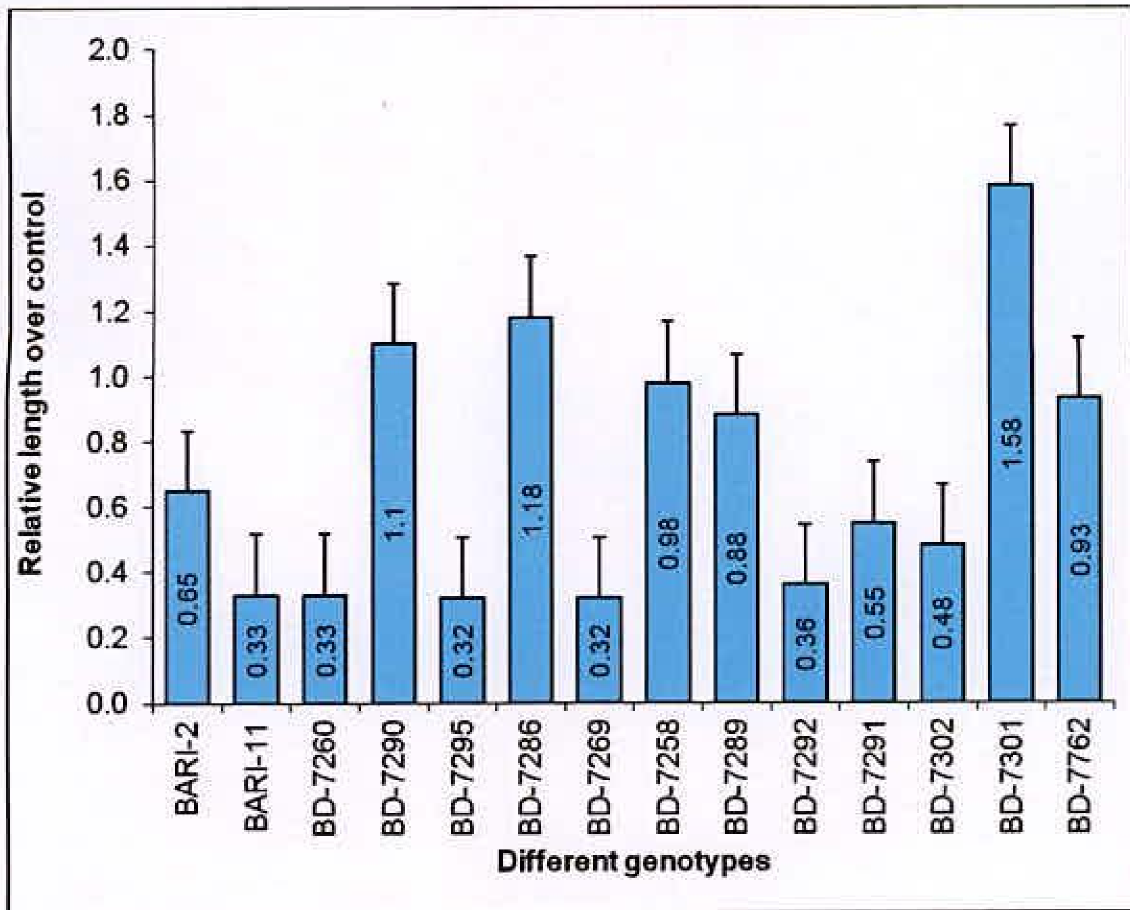

Figure 3. Relative root length of different tomato genotypes under stress condition (40 g PEG). Vertical bars indicate the $LSD_{(0.05)}$ value

4.1.3 Comparison of root reduction rate under control and *in vitro* drought condition

To investigate the drought tolerance in the genotypes of tomato, the root assay was performed with the seeds grown on media containing 0, 20, 40 and 60 g/L of PEG. Figure 4 illustrates the comparison of mean root length of different genotypes under different concentrations of PEG. As the root lengths of different genotypes under control condition (0g/L of PEG) are not of same sizes, it was necessary to obtain root reduction rate under different level of water stress. Figure 5 illustrates how inhibition of root elongation by PEG was significantly alleviated in the genotypes in comparison to the control condition. The length of control roots were reduced upto 0.67 cm. (BD-7269) (Figure 5.). The lowest root reduction rate was observed in BD-7286 (0.3 cm.) under 20g/L of PEG indicating increased tolerance in drought stress as compared to all other genotypes. Accession BD-7286 also showed increased tolerance to more severe stresses i.e., 20, 40 and 60 g/L of PEG and found negative root reduction rate indicating much better recovery under high drought stress (40g/L PEG). Some other genotypes also exhibited less root reduction rate under different concentration of PEG such as BD-7290, BD-7258 and BD-7301 under 40g/L of PEG (Figure 4. and Figure 5). At 60 g/L of PEG stress, the plants almost died, as such authentic data could be obtained. From the above mentioned discussions, it can be inveterate that four tomato genotypes viz. BD-7286, BD-7290, BD-7258 and BD-7301 had no drought effect. Exposure of plants to drought condition results in many physiological, biochemical and molecular changes, including massive changes in the profile of gene expression (Shinozaki and Yamaguchi, 2000). During drought stress some genes might induced and produce some proteins which might confer tolerance to these four genotypes of tomato. Similar results are obtained previously where several different genes such as *CaRZFP1*, *AGP*, *DREB1*, *NtHSP70 etc.* (Zeba *et al.*, 2009) confer tolerance to drought to plants. So expression pattern of those genes could be executed and analyzed to know the underlying molecular genetic mechanism of drought tolerance. Some physiological and biochemical studies are also necessary to support those results.

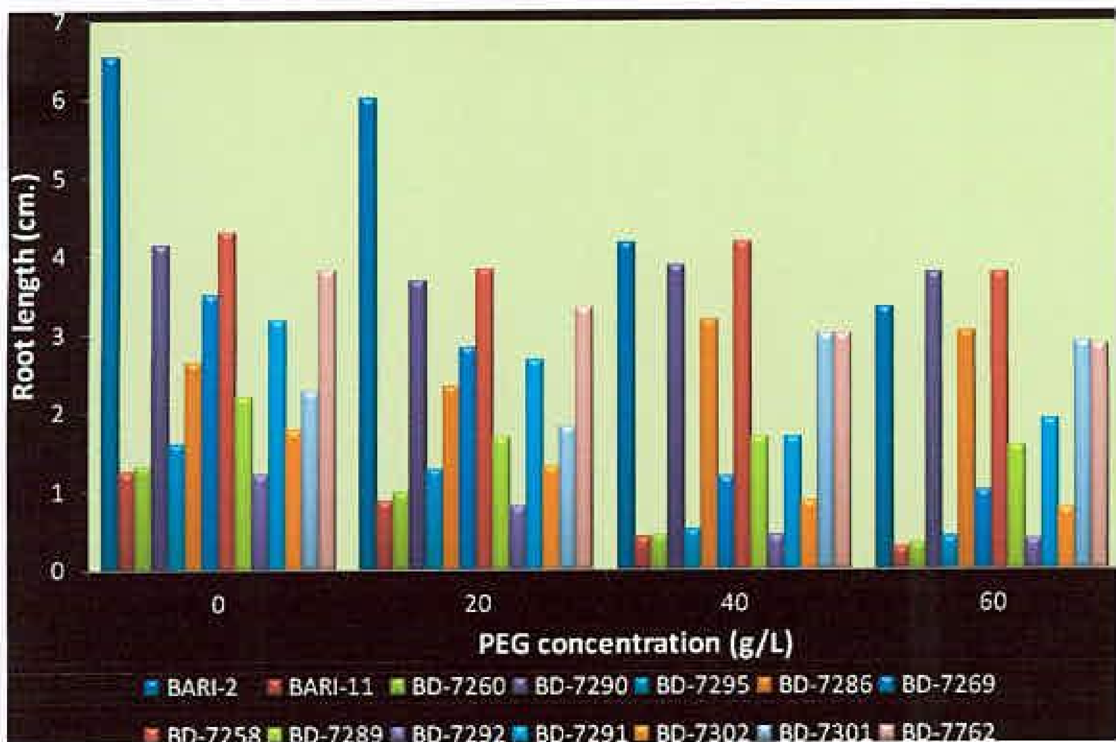

Figure 4. Comparison of root length of different genotypes of tomato in different concentrations of PEG. Data are mean values ±SD of three individual experiments.

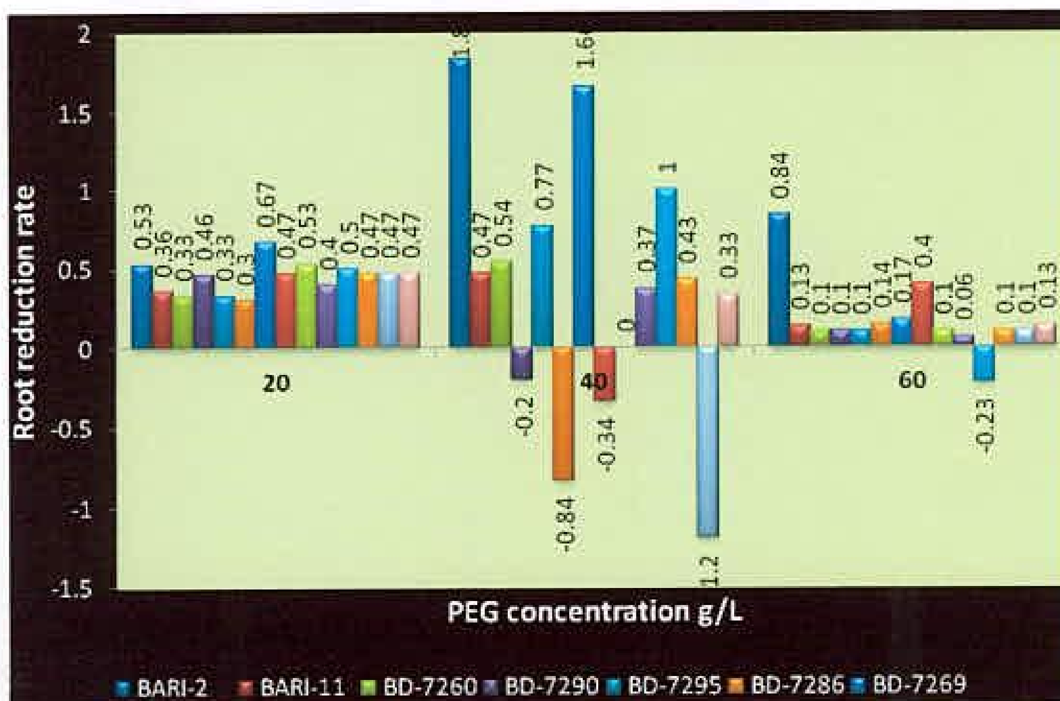

Figure 5. Comparison of root reduction rate in different genotypes of tomato under different concentrations of PEG. Data are mean values ±SD of three individual experiments.

4.1.4 Plant weight

Statistically significant variation was recorded for plant weight due to PEG level of 0, 20, 40 and 60 g under the present trial (Table 5). For different PEG plant weight varied from 0.036 g to 0.043 g. The highest plant weight (0.043 g) was recorded for 0 g PEG which statistically similar (0.039 g) with 20 g PEG, whereas the lowest plant weight (0.036 g) was observed in 60 g PEG which was statistically similar (0.037 g) with 40 g PEG. Data revealed that in the application of 0 g PEG produced comparatively the highest plant weight which was generally followed by 20 g PEG. It was also found that 20 g PEG had minimum adverse effect on plant that produced significant weight of plant for the genotypes that were included under this trial. On the other hand, in 40 and 60 g PEG produced the lowest weight of root that mean in this level of PEG tomato genotypes exhibited adverse effect in consideration of plant weigh. Among the studied genotypes BARI-2, BD-7258, BD-7301 and BD-7762 produced the highest plant weight in 40 and 60 g PEG compared to 0 and 20 g PEG.

On an average among the different genotypes of tomato the highest plant weight (0.068 g) was recorded from BARI-2 and BD-7290 which was statistically identical (0.062 g and 0.061 g) with BD-7286 and BD-7258. On the other hand, the lowest weight of plant (0.003 g) was found in BARI-11 and BARI-7292 which was statistically identical (0.012 g) with BD-7260 (Table 5). Mittal *et al.* (1996) estimated heritability and genetic advance in 27 genotypes of tomato. High heritability associated with high genetic advance was observed by them indicating the character, predominantly under the control of additive gene, could be improved through selection.

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Table 5. Plant weight of different tomato genotypes under different level of PEG

Genotypes	Weight (g) under different level of PEG				Mean
	0 g PEG	20 g PEG	40 g PEG	60 g PEG	
BARI-2	0.066	0.062	0.072	0.070	**0.068 a**
BARI-11	0.005	0.004	0.002	0.002	**0.003 f**
BD-7260	0.022	0.016	0.005	0.004	**0.012 ef**
BD-7290	0.085	0.082	0.052	0.051	**0.068 a**
BD-7295	0.025	0.021	0.004	0.003	**0.013 e**
BD-7286	0.063	0.058	0.064	0.063	**0.062 ab**
BD-7269	0.063	0.056	0.025	0.023	**0.042 d**
BD-7258	0.049	0.045	0.076	0.075	**0.061 ab**
BD-7289	0.048	0.045	0.048	0.047	**0.047 cd**
BD-7292	0.004	0.002	0.003	0.003	**0.003 f**
BD-7291	0.054	0.050	0.045	0.043	**0.048 cd**
BD-7302	0.029	0.026	0.010	0.009	**0.019 e**
BD-7301	0.033	0.031	0.058	0.056	**0.045 cd**
BD-7762	0.052	0.048	0.057	0.055	**0.053 bc**
Mean	**0.043 a**	**0.039 ab**	**0.037 b**	**0.036 b**	

In a column, means having similar letter(s) are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05 level of probability

4.1.4 Relative plant weight over control

Statistically significant variation was recorded for relative plant weight due to PEG level of 20, 40 and 60 g under the present trial (Table 6). For different PEG the average relative plant weight was 0.86, 0.92 and 0.86, respectively for 20, 40 and 60 g PEG. The highest mean relative plant weight over control (0.92) was recorded for 40 g PEG, while the lowest relative plant weight (0.86) was observed in 0 and 60 g PEG. Data revealed that 40 g PEG gave the average highest relative weight of plant and for 0 g PEG the relative plant weight was same for 0 and 60 g PEG. From the data of relative plant weight over control BARI-2, BD-7286, BD-7258, BD-7289, BD-7292, BD-7301 and BD-7762 gave the increasing trend with the increasing level of PEG.

In an average among the different genotypes of tomato the highest relative plant weight over control (1.81) was recorded from BD-7301 which was followed (1.38) by BD-7258. On the other hand, the lowest relative plant weight (0.41) was recorded in BD-7260 which was statistically identical (0.51) with BD-7269 under the present trial (Table 6).

Table 6. Relative plant weight of different tomato genotypes under different level of PEG

Genotypes	Relative weight over control (0 g PEG) of different level of PEG			Mean
	20 g PEG	40 g PEG	60 g PEG	
BARI-2	0.94	1.11	1.06	**1.04 cd**
BARI-11	0.79	0.48	0.36	**0.54 fg**
BD-7260	0.72	0.31	0.20	**0.41 h**
BD-7290	0.96	0.63	0.62	**0.74 e**
BD-7295	0.78	0.42	0.30	**0.50 gh**
BD-7286	0.91	1.12	1.09	**1.04 cd**
BD-7269	0.90	0.34	0.30	**0.51 gh**
BD-7258	0.92	1.62	1.59	**1.38 b**
BD-7289	0.94	1.02	0.99	**0.98 d**
BD-7292	0.53	0.70	0.63	**0.62 efg**
BD-7291	0.92	0.97	0.91	**0.93 d**
BD-7302	0.87	0.56	0.49	**0.64 ef**
BD-7301	0.91	2.30	2.23	**1.81 a**
BD-7762	0.91	1.27	1.22	**1.13 c**
Mean	**0.86 b**	**0.92 a**	**0.86 b**	

In a column, means having similar letter(s) are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05 level of probability

4.2 Genetic Diversity Analysis

Study of genetic diversity among 14 genotypes of tomato evaluated through Mahalanobis' D^2 statistics in 0 g PEG and 60 g PEG and which has been discussed below:

Mahalanobis D^2 statistics was used to measure the degree of diversification among the genotypes in 0 g PEG. Using this technique, grouping of genotypes was done in four clusters where genotypes grouped together were less divergent than the ones placed in different clusters. The clusters separated by greatest statistical distance exhibited maximum divergence. Composition of different clusters with their corresponding genotypes and their source are shown in Table 7. Cluster I and IV was the largest cluster comprising of 5 genotypes followed by cluster II with 3 genotypes and cluster III belongs only 1 genotypes of tomato (Table 7). In consideration of clustering mean for initial length the highest mean of 1.49 was found in cluster IV. In case of length the highest cluster mean 6.53 was recorded in cluster III and for plant weight the highest cluster mean 0.066 was observed in cluster III. Shashikanth *et al.* (2010) reported significant differences for all the characters studied indicating the existence of genotypic variation; there was no parallelism between genetic diversity and geographical divergence in tomato and suggested that high diversity among the genotypes belonging to cluster VII and X can be selected in hybridization programmes to obtain good seggregants.

Table 7. Clustering pattern of 14 tomato genotypes by Tocher's method

Cluster	Members	Genotypes No.
I	5	BARI-11, BD-7260, BD-7295, BD-7292, BD-7302
II	3	BD-7290, BD-7258, BD-7762
III	1	BARI-2
IV	5	BD-7286, BD-7269, BD-7289, BD-7291, BD-7301

Table 8. Clustering mean of 14 tomato genotypes

Parameters	Cluster			
	I	II	III	IV
Initial length	0.48	0.92	1.00	1.49
Root length	1.42	4.08	6.53	2.75
Plant Weight	0.017	0.062	0.066	0.052

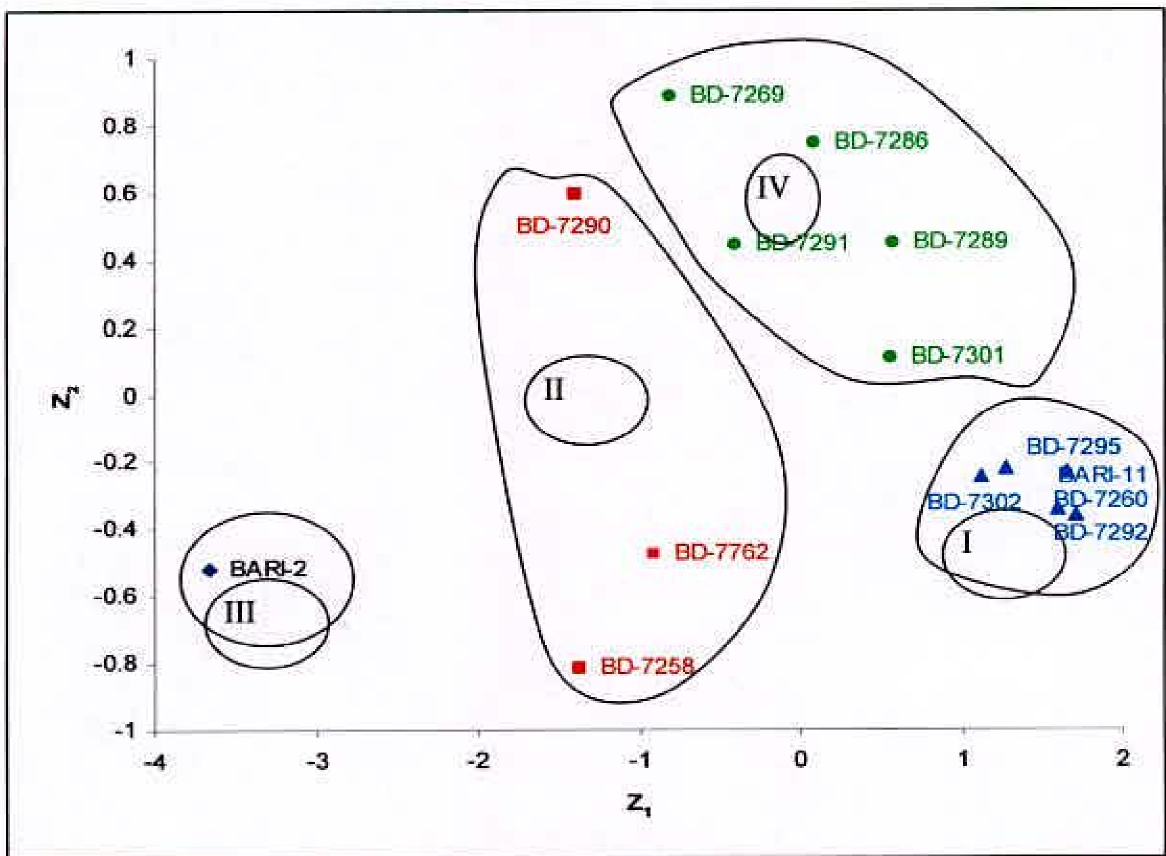

Figure 6. Scatter diagram of 14 genotypes on the basis of principal component analysis.

Cluster distances denoted by the average inter and intra-cluster distances are the approximate measure of the cluster divergence in 60 g PEG (Table 9). Inter cluster distance was maximum (13.288) between clusters I and III, followed by clusters III and IV (10.744). The intra and inter cluster distance presented in Figure 5. The results revealed that genotypes chosen for hybridization from clusters with highest distances would give high heterotic F_1 and broad spectrum of variability in segregating generations. Sharma *et al.* (2006) reported that genotypes grouped into 10 clusters, the maximum divergence within a cluster was exhibited by the cluster VIII (1.531), closely followed by cluster III (1.528) and cluster V (1.460), whereas, cluster VIII and II were the most divergent from each other followed by cluster VII and cluster VIII.

Table 9. Average intra (bold) and inter-cluster D^2 and D values of 4 clusters for 14 tomato genotypes formed by Torcher's method

Cluster	I	II	III	IV
I	**0.2013**			
II	7.034	**0.4809**		
III	13.288	6.324	**0.0000**	
IV	3.409	5.051	10.744	**0.3117**

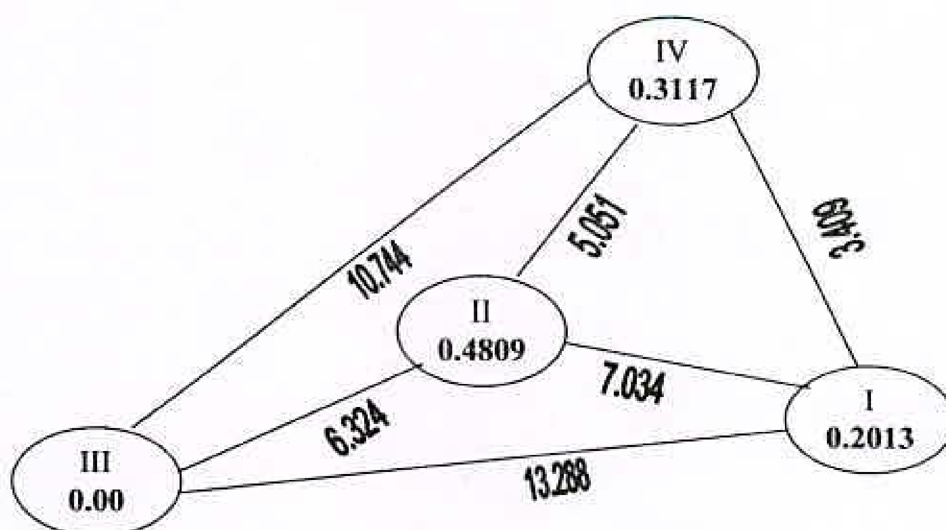

Figure 7. Intra and inter cluster distance between different cluster

CHAPTER V

SUMMARY AND CONCLUSION

The experiment was conducted to study genetic diversity analysis of tomato (*Solanum lycopersicum* L.) under drought condition *in vitro*. Fourteen tomato genotypes were used as experimental materials among which BARI Tomato 2, BARI Tomato -11 are parent material and rest of all were lines, and they were BARI Tomato-2, BARI Tomato-11, BD-7260, BD-7290, BD-7295, BD-7286, BD-7269, BD-7258, BD-7289, BD-7292, BD-7291, BD-7302, BD-7301 and BD-7762. Murashige and Skoog medium were used with different PEG concentration as culture medium for root and shoot regeneration.

Statistically significant variation was recorded for root length due to PEG level of 0, 20, 40 and 60 g. For different PEG root length varied from 1.88 cm to 2.83 cm. The longest length of root (2.83 cm) was recorded for 0 g PEG, whereas the lowest length (1.88 cm) was observed in 60 g PEG. In an average, among the different genotypes of tomato the highest length of root (5.01 cm) was recorded from BARI-2 and the lowest length of root (0.69 cm) in BARI-11.

For different PEG the average relative root length was 0.80, 0.71 and 0.66, respectively for 20, 40 and 60 g PEG. The highest relative root length over control (0.80) was recorded for 20 g PEG, while the lowest relative plant weight (0.66) was observed in 60 g PEG. Relative root length over control BD-7290, BD-7286, BD-7258, BD-7289, BD-7301 and BD-7762 gave the increasing trend with the increasing level of PEG. In an average among the different genotypes of tomato the highest relative length of root over control (1.20) was recorded from BD-7301 and the lowest relative length of root (0.42) in BARI-11.

For different PEG plant weight varied from 0.036 g to 0.43 g. The highest weight of plant (0.43 g) was recorded for 0 g PEG, whereas the lowest weight of plant (0.36 g) was observed in 60 g PEG. Among the studied genotypes BARI-2, BD-

7258, BD-7301 and BD-7762 produced the highest plant weight in 40 and 60 g PEG compared to 0 and 20 g PEG. In an average among the different genotypes of tomato the highest weight of plant (0.068 g) was recorded from BARI-2 and BD-7290 and the lowest weight of plant (0.003 g) was found in BARI-11 and BARI-7292.

For different PEG the average relative plant weight was 0.86, 0.92 and 0.86, respectively for 20, 40 and 60 g PEG. The highest relative plant weight over control (0.92) was recorded for 40 g PEG, while the lowest relative plant weight (0.86) was observed in 0 and 60 g PEG. From the data of relative plant weight over control BARI-2, BD-7286, BD-7258, BD-7289, BD-7292, BD-7301 and BD-7762 gave the increasing trend with the increasing level of PEG. In an average among the different genotypes of tomato the highest relative plant weight over control (1.81) was recorded from BD-7301 and the lowest relative plant weight (0.41) was recorded in BD-7260. Based on the comparison of drought stressed genotypes and control genotypes, BD-7286, BD-7290, BD-7258 and BD-7301 found promising and could be use for further breeding program or for further gene expression analysis.

The clusters separated by greatest statistical distance exhibited maximum divergence. Cluster I and IV were the largest clusters comprising of 5 genotypes followed by cluster II with 3 genotypes and cluster III belonged only 1 genotypes of tomato. In considering of clustering mean for initial length the highest mean was 1.49 for cluster IV. In case of length highest cluster mean 6.53 was recorded in cluster III and for plant weight highest cluster 0.066 was observed in cluster III.

The results revealed that genotypes chosen for hybridization from clusters with highest distances would give high heterotic F_1 and broad spectrum of variability in segregating generations.

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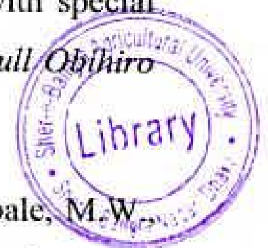

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