IMPACT OF POLYETHYLENE GLYCOL INDUCED WATER STRESS ON CALLI GROWTH AND ACCUMULATION OF PROLINE IN *Capsicum* spp.

MOHAMMAD MAHABUB ALAM LAYLIN

DEPARTMENT OF GENETICS AND PLANT BREEDING
SHER-E-BANGLA AGRICULTURAL UNIVERSITY
DHAKA-1207

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BY

MOHAMMAD MAHABUB ALAM LAYLIN

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Approved by:

_____	_____
(Dr. Naheed Zeba)	**(Dr. Mohammad Saiful Islam)**
Professor	**Professor**
Supervisor	**Co-Supervisor**

_____
(Professor Dr. Md. Sarowar Hossain)
Chairman
Examination Committee

Naheed Zeba, Ph.D
Professor
Department of Genetics and Plant Breeding
Sher-e-Bangla Agricultural University
Sher-e-Bangla Nagar, Dhaka-1207,
Bangladesh

Tel: 88-02-9140770
Mobile: +8801913091772
E-mail: naheed0359@hotmail.com

CERTIFICATE

This is to certify that thesis entitled, "Impact of polyethylene glycol induced water stress on calli growth and accumulation of proline in Capsicum spp." 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 Mohammad Mahabub Alam Laylin, Registration No.: 08-02833 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 by him.

Dated: December, 2014
Place: Dhaka, Bangladesh

_____
(Prof. Dr. Naheed Zeba)
Supervisor

Dedicated to my

First Teachers

Abba Mohammad Gias Uddin

Amma Shalina Begum

Some commonly used abbreviations

Full Word(s)	Abbreviation	Full Word(s)	Abbreviation
2,4-Dichlorophenoxyacetic acid	2,4-D	Microgram per gram	µg/g
Abscisic acid	ABA	Micromol per square meter per second	µmol-m^{-2}-s^{-1}
Agricultural	*Agril.*	Millimole	mM
and others (at elli)	*et al*.	Molar	M
exempli gratia (for example)	e.g.	Murashige and Skoog	MS
Benzyleadenine	BA	Nanometer	nm
Benzyleamino purine	BAP	1-Naphthaleneacetic acid	NAA
Biology	*Biol.*	Negative logarithm of hydrogen ion concentration (-log[H^+])	pH
Biotechnology	*Biotechnol.*	Newsletter	*Newsl.*
Botany	*Bot.*	Normal	N
Cultivar	cv.	Pages	pp.
Culture	*Cult.*	Parts per million	ppm
Days after treatment	DAT	Percent	%
Degree (latitude and longitude)	°	Physiology	*Physiol.*
degree Celsius	°C	Plant growth regulators	PGRs
Distilled water	DW	Polyethylene glycol	PEG
Etcetera	etc.	Potassium chloride	KCl
Food and Agriculture Organization	FAO	Potassium ion	K^+
Gibberellic acid	GA_3	Pounds per square inch	psi
Gram per liter	g/L	Publication	Pub.
Hectare	ha	Reports	*Rep.*
Hour	h	Research	*Res.*
Indole-3-butyric acid	IBA	Science	*Sci.*
Indole-3-acetic acid	IAA	Sodium hydroxide	NaOH
International	*Intl.*	Sodium hypochlorite	NaOCl
Iron(III)Ethylenediaminetetra acetic acid	FeEDTA	Species (Plural)	spp.
Journal	*J.*	Spices Research Center	SRC
Kilometer	Km	Thidiazuron	TDZ
Kinetin	Kin or KIN	Ultraviolet	UV
Liter	L	Weight /Volume	w/v

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The Author

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ABSTRACT

BY

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In this research, *in vitro* studies were apprehended on the *Capsicum* spp. Seedlings of five genotypes of *Capsicum* were grown on agar solidified MS (Murashige and Skoog) medium. Prior to inoculation of seeds in the medium, seeds were pretreated with fungicide 0.1% rekazim (carbendazim) and then surface sterilized with absolute alcohol followed by 10% NaOC1. The cultures of the seeds germinated initially at dark at 27 ± 1 ^{o}C for ten days and then in light under 16 h photoperiod at 25 ± 2^{o}C. Cotyledonary leaf and nodal segment explants from three weeks old seedlings were excised in approximately 0.5 cm of length. These explants were cultured on MS basal medium for callus formation. To find out the effect of water stress or lowering osmotic potential conditions in the medium on the callus formation and response of the biomass induction, polyethylene glycol (PEG 6000) in 0 g/L, 20 g/L, 40 g/L, 60 g/L and 80 g/L concentrations were added to the MS medium. Biomass measurement of calli (size and weight) was realized in three weeks interval from the callus inoculation. In the normal MS medium, biomass formation was very fast but when PEG used, for changing the osmotic potential of the MS medium the biomass formation decreased because of the water stress conditions. Proline content gradually increased as the PEG concentration increased. At short duration (20 days after treatment with PEG) of water stress genotype G3 (SRC02) recovered at high water stress (60 g/L of PEG) in respect of callus size and weight as well as in proline accumulation. At prolonged stress period (40 days after treatment with PEG), G1 (CO611) and G4 (SRC05) showed better performance at severe stress (60 g/L of PEG) which correlates with the accumulation of proline. As G1 (CO611), G3 (SRC02) and G4 (SRC05) showed tolerance to water stress at different duration by showing comparatively better biomass changing of calli and proline accumulation, these genotypes could be selected for getting drought tolerant calli.

CHAPTER I
INTRODUCTION

Drought stress causes adverse effects on the growth and the productivity of crops worldwide (Farooq *et al*., 2012; Mittler, 2006; Peters *et al*., 2004; Hoerling and Kumar, 2003). Due to drought, the rate of photosynthesis is reduced mainly by stomatal closure, membrane damage, and disturbed activity of various enzymes, especially those involved in ATP synthesis (Yuan *et al*., 2015). Because of exceeding drought stress the soils become inconvenient to cultivation. A promising strategy to cope with adverse scenario is to take advantage of the flexibility that biodiversity (genes, species, ecosystems) offers and increase the ability of crop plants to adapt to abiotic stresses especially drought for sustainable use of plant genetic resources for food and agriculture. The Food and Agriculture Organization (FAO) of the United Nations promotes the use of adapted plants and the selection and propagation of crop varieties adapted or resistant to adverse conditions (FAO, 2007). Unfavorable climate resulting in abiotic stresses not only causes changes in agro-ecological conditions, but indirectly affects growth and distribution of incomes, and thus increasing the demand for agricultural production (Schmidhuber and Tubiello, 2007). As has been pointed out, current achievements in crop production have been associated with management practices that have degraded the land and water systems (FAO, 2011).

Capsicum is one of the most important solanaceous horticultural crops in the world. Capsicum is a model crop for *in vitro* and genetic studies due to its low chromosome number. The basic chromosome number of the genus *Capsicum* is x=12, all the species are diploids, most are 2n=2x=24, including the cultivated ones. A few wild species have 2n=26 which have been found mostly in South America but all domesticated *capsicum* has 24 chromosomes (Pozzobon *et al*., 2005). Wild species of *Capsicum* are important genetic resources for *Capsicum*

breeding (Bosland and Gonzalez, 2000). Not only wild species are useful in breeding for disease resistance, but they can also be used to increase nutritional quality, yield and adaptation to stress (Bosland, 1993).

Fruits of Capsicum are widely used for coloring, flavoring of garnishes, pickles, meats, barbecue sauces, ketchup, snack food, dips, chilli con came, salads, sausages, curry powder, tabasco sauce and herbal remedy while some are cultivated ornamentally, especially for their brightly glossy fruits with a wide range of colours, shape and sizes. It has the highest content of vitamin C among all plants and has important medicinal properties such as prevention of heart disease, actuation of blood ambulation and antioxidant characteristics (Salehi, 2006). Recently, several studies have also demonstrated anti-cancer or anti-mutagenic effect of the chilli extracts. Carotenoids present in chilli extracts were found to have a synergistic anti-mutagenic and *in vitro* anti-tumour-promoting activity. Most *Capsicum* species contain the alkaloid capsaicin, which determines their pungency, an important factor for the food and pharmaceutical industries. Chilli powder is a blend of spices that includes ground chilies. Red or hot peppers from *Capsicum annuum* L. and *Capsicum frutescens* L. are the most pungent peppers and are used extensively in Bangladeshi, Indian, Mexican and Italian foods.

All of the *Capsicum* spp. has economic importance in Bangladesh and some varieties has problem with water stress conditions in some parts of Bangladesh. There is very close relationship between plant growth regulators and effects of drought stress on plant. Endogenous hormone levels in *Capsicum* plant tissues are affected from environmental stresses. Plant growth regulators have very different effects on the plants and because of that these substances are used in a very large amount for field and *in vitro* studies. Some experiments showed us that Naphthalene Acetic Acid (NAA) were stimulated water absorption and cell wall plasticity. In *in vitro* drought stress experiments, Polyethylene glycol (PEG), NaCl, mannitol and sucrose which decrease medium's water potential were used

often. This substances change the osmotic potential of the medium. In this research some useful approaches were used for solution of this problem (drought) in *in vitro* conditions. Plant tissue culture techniques and plant growth regulators were used in different concentrations and combinations. With conceiving the above scheme in mind, the present research work were undertaken in order to fulfill the following objectives:

- To establish a protocol of *in vitro* callus induction of different genotypes of *Capsicum* under control and water stress condition.
- To determine the response of genotypes under control and water stressed condition based on calli size and weight as indicators of tolerance.
- To compare the accumulation of proline in calli of different genotypes as an indicator of water stress tolerance.
- To assess the magnitude of genotype × PEG treatment interaction from initial day to prolonged PEG stress period in cellular level.

CHAPTER II
REVIEW OF LITERATURE

Capsicum 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 Origin and distribution of *Capsicum* spp.

Capsicum L. (pepper) is a member of the Solanaceae family and is cultivated in temperate and tropical regions (Lippert *et al.*, 1966; Eshbaugh, 1993; Pozzobon *et al.*, 2005). Fruits of this plant are used as spice, vegetable and herbal remedy. *Capsicum* has at least 20-30 species, from which five of them have become domesticated by American natives: *Capsicum annuum*, *C. frutescens*, *C. chinense*, *C. pubescens* and *C. baccatum* (Lippert, 1966; Eshbaugh, 1993; Lanteri, 1993; Pozzobon *et al*., 2005; De Teodoro-Pardo *et al*., 2007). After Columbus, they became widely exploited in tropical to temperate regions because of their fruits, which have high nutritional contents, especially in vitamins. They are constituents of the human diet, the pungent cultivars as spice ("ajies," "paprika," "chilies," "hot peppers") and the sweet types as vegetables ("sweet pepper," "bell pepper," "pimiento"). Moreover, the genus has medicinal and ornamental applications (IBPGR, 1983; Pickersgill, 1991; Eshbaugh, 1993; Bosland and Votava, 2000; Hunziker, 2001). Fruit pungency is characteristic of the genus due to substances unique to peppers in a mixture known as "capsaicinoids," which includes more than 20 alkaloids (vanillylamines). The presence of pungency is controlled by a

single dominant major gene (*Pun1* or *C*) demonstrated in the *Capsicum annuum* complex, although its expression varies among species and cultivars depending on other modifier genes epistatically affected by *Pun1* and environmental conditions (Lippert *et al*., 1966; IBPGR, 1983; Bosland and Votava, 2000; Hunziker, 2001; Lefebvre *et al*., 2002; Stewart *et al*., 2005). At least two wild species, *C. lanceolatum* and *C. rhomboideum* (Bosland and Zewdie, 2001), are reported to be completely free of pungency, and pungency is also absent in some accessions of *C. chacoense* (Eshbaugh, 1980), in one of *C. cornutum* (as *C. dusenii* Bitter; Hunziker, 1971), and in cultivars of *C. annuum* var. *annuum* after human selection.

Brazil is considered to be the centre of diversity for the genus and has a richness of germplasm that is reflected in the large numbers of varieties cultivated all over the country, many of which are adapted to specific edapho-climatic micro-regions and have potential for genetic breeding (Buso *et al*., 2001). Brazil also has the largest number of wild *Capsicum* species (Pickersgill, 1984; Bianchetti, 1996), although a comprehensive study of this diversity, especially of the native species, is still lacking.

According to Hunziker (2001) four centers of distribution can be recognized for *Capsicum:* 1) southern USA and Mexico to western South America (Peru includes 12 spp.), 2) northeastern Brazil and coastal Venezuela (1 sp.), 3) eastern coastal Brazil (10 spp.), and 4) central Bolivia and Paraguay to northern and central Argentina (8 spp.). The greatest number of species (16) is concentrated in Brazil (Barboza and Bianchetti, 2005). It is noteworthy that 16 species are endemics of different regions of South America. Peppers are among the oldest cultivated plants in the America, and archeological remains indicate that *C. annuum* in particular was used by man even before the advent of agriculture (Pickersgill, 1969). The domesticated species include wild populations, except *C. pubescens,* which is known only in cultivation. In the case of *C. annuum* and *C. baccatum,* the wild

forms are distinguished as taxonomic varieties, i.e., *glabriusculum* and *baccatum,* respectively. The spontaneous forms of the *C. annuum* complex intergrade in a way that makes it difficult to separate one from the other and to distinguish between weedy forms escaped from cultivation and true wild forms (Pickersgill *et al*., 1979; Pickersgill, 1988; Eshbaugh, 1993).

Capsicum frutescens L. has wide distribution as a weedy or semi-domesticated plant in the tropics. These are extremely pungent peppers that are called bird chilies. *Capsicum annuum* L. on the other hand is said to be the most widely cultivated and economically important species of *Capsicum* and are commonly known as chilies, red or sweet peppers. They include the sweet and a wide range of pungent peppers (Purseglove, 1968; Heiser, 1976). Research in Mexico on various selections of *Capsicum annuum* has shown many changes in the total length of chromosomes. These changes indicate a very high chromosomal diversity in *Capsicum*, even in different genotypes of the same species (De Teodoro-Pardo, 2007).

Capsicum baccatum L. and *C. chinense* Jacq. are native to tropical and temperate regions in America, and were domesticated by native Americans (Moscone *et al.*, 2007). The domesticated types are found around the world, while wild species have more limited distribution (Lanteri and Pickersgill, 1993). For *Capsicum pubescens,* a mid-elevation region of Bolivia had been proposed as the original place of domestication, as its closely related species grow there and plants with fruits of smaller size occur there also, which approach the more primitive size (McLeod *et al.*, 1982; Eshbaugh, 1993). *Capsicum* phylogenetic relationships, using evidences of chloroplastidic and nuclear DNA showed that, although being close, the species were grouped in different clades. *Capsicum baccatum* was included in the clade *baccatum* while *C. chinense* was included in the clade *annuu*m, and such results agree with groupings obtained based in morphology and

isoenzymes (Choong, 1998). *Capsicum chinense* presents great intraspecific genetic diversity (Fonseca *et al*., 2008).

2.2 Genetic and cytogenetic investigations in *Capsicum* spp.

Interspecific hybridization plays an active role in the introgression of needed genes from wild species to commercial cultivars. Interspecific crossings have been performed in *Capsicum* species to elucidate the genetic relationships between them, and mainly, to permit the genetic improvement of cultivars by introducing valuable alleles from wild accessions that are responsible, for example, for the resistance to pathogens, as obtained by Boiteux *et al*. (1993). Investigations of hybridization compatibility between *Capsicum* species can be helpful in determining the systematic of the genus *Capsicum*, and consequently, the improvement of cultivated varieties. The success of interspecific crossings is related to the genomic homology of parent species. For this kind of analysis, cytogenetic studies in domesticated or semi-domesticated *Capsicum* species and their ancestors have been performed (Lanteri and Pickersgill, 1993). Cytogenetic investigations may reveal the proximity between species and genotypes through the observation of uniformity in chromosome pairing, or the distance between them, as observed in *C. tovarii* x *C. baccatum* hybrids that presented reciprocal translocations certifying the genetic distance between the parent species (Tong and Bosland, 1999).

Nwankiti (1981) carried out hybridization between a mild variety and an extremely pungent variety of *C. annuum* and found normal meiotic behavior in F_1 plant although this hybrid had lower fruit set than the parents. In an earlier work, Nwankiti (1976) indicated that most of the present day strains of *Capsicum* are likely to be of intra-specific hybrid origin. Given the wide range of sizes, from the small chilli to the large, bell-shaped type and the range of pungency in *C.*

frutescens and *C. annuum,* the present day strains of pepper are likely to be of both itra- and inter-specific hybrid origins.

Chromosome number and behavior in meiosis provides critical information for studying *Capsicum* taxonomy, systematic, genetics and breeding. Different chromosome numbers in the parents may cause incompatibility, hybrid abortion or sterility. Cytogenetic studies on *Capsicum* species have shown that they contain 24 chromosomes (2n=2x=24), similar to many species of *Solanaceae* family. There are two distinct groups present in the genus; some species have 24 chromosomes (2n=2x=24) while other species have 26 chromosomes (2n=2x=26). The most common chromosome number in the genus is x=12 (Smith and Heiser, 1957; Lippert *et al.*, 1966; Pickersgill, 1971, 1984, 1991; Limaye, 1989; Moscone, 1993 ; Pozzobon *et al*., 2005).

Rohami *et al.* (2010) conducted an experiment on karyotype analysis of several ecotypes of *Capsicum annuum* L. and they found that all the investigated genotypes of *Capsicum annuum*, had 24 chromosomes and there was a significant chromosomal diversity between the genotypes. Karyotypes in different species with 24 chromosomes are very similar with each other. The species with 24 chromosomes have symmetrical karyotypes. They generally have one pair of acro-centric and the rest of the chromosomes are meta-centric. In contrast with, the species with 26 chromosomes display more asymmetrical complements, with more sub-metacentric (sub-telocentric) chromosomes and often one telo-centric chromosome (Lanteri, 1993; Moscone *et al*., 2007; De Teodoro-Pardo, 2007). Most species of *Capsicum* with 26 chromosomes have been found in South America as wild plants but all domesticated capsicums have 24 chromosomes (Pozzobon *et al*., 2005).

In *Capsicum*, meiotic and pollen grains (PG) stain ability analysis were performed to infer about the genetic relationship between two species (Kumar *et al*., 1988).

Capsicum baccatum and *C. chinense* (2n=24) are considered self-compatible, and usually these species do not present unilateral or crossed incompatibility (Naci Onus and Pickersgill, 2004). However, artificial crossings were performed between *Capsicum baccatum* L. and two accessions of *Capsicum chinense* Jacq. have resulted in very low fertilization rates.

Souza *et al.* (2012) conducted an experiment on meiotic irregularities in *Capsicum* L. species where they studied cytogenetic and pollen viability (PV) in pepper accessions, *Capsicum chinense* and *Capsicum baccatum*. They observed irregularities, such as laggard and univalent chromosomes, bridges, problems in the spindle fibers and cytomixis, especially in *C. baccatum* which was the most unstable genotype. In the post-meiotic products, irregularities were observed on average, at 20 % of the microspores in *C. baccatum* and 17 % in *C. chinense*. PV in *C. baccatum* was below 70 %, while in *C. chinense*, it was above 80 %. Meiotic irregularities in *Capsicum*, mainly in *C. baccatum*, considering the low PV estimated were significant but not impeditive for fertilization.

Wild species of *Capsicum* are important genetic resources for *Capsicum* breeding (Bosland and Gonzalez, 2000). A non-pungent species, *C. ciliatum* from South America, has a basic chromosome number n=x=13 (Moscone *et al*., 1993). In addition, *C. campylopodium* Sendt. and *C. mirabile* Mart ex. Sendt. from southern Brazil, and *C. schottianum* Sendt. from Argentina, southern Brazil and southeast Paraguay, have basic chromosome numbers of n=x=3 (Moscone *et al*., 1993). Two wild species for which there is little documentation about hybridization compatibility and cytological investigation are *C. schottianum* and *C. lanceolatum*. *C. buforum* distributed in Brazil and grows in open marshy locations of rain forests (Eshbaugh, 1993; Hunziker, 1969). *C. lanceolatum* is found in Guatemala, Honduras and Mexico (Gentry and Standley, 1974). It was first reported as growing in ‘a wet, damp forest’ (Standley and Steyermark, 1940). The chromosome number and meiotic chromosome pairing of *C. buforum* and *C.*

lanceolatum have not been documented. Interspecific hybridization and meiotic chromosome investigations were conducted with *C. buforum* and *C. lanceolatum* to obtain a better understanding of their genetic and taxonomic relationships within the genus *Capsicum*.

Bosland and Tong (2003) carried out an experiment to observe interspecific compatibility and meiotic chromosome behavior of *Capsicum buforum* and *C. lanceolatum* where the wild species *Capsicum buforum* Hunz. and *C. lanceolatum* (Greenm. ex J.D. Sm.) Morton and Standl. were hybridized to nine different *Capsicum* species to understand their taxonomic and genetic relationships with *Capsicum buforum* as the male parent, the compatibility to the nine species varied from species to species and ranged from producing under-developed embryo, seed coat, seedless fruit, to no fruit set. When *Capsicum buforum* was the female parent, it was incompatible (no fruit set) to the nine *Capsicum* species tested. When *C. lanceolatum* was the female parent, the hybridizations to the other species ranged from aborted embryo, seed coat, seedless fruit or no fruit. As a pollen parent *C. lanceolatum* was incompatible (no fruit set) to the species investigated. In pollen mother cells (PMCs) of *Capsicum buforum*, 24 chromosomes (n=12) paired as 12 bivalents with chromosome lagging at meiotic anaphase-I. Twenty six chromosomes (n=13) were detected in PMCs of *Capsicum lanceolatum*. In *C. lanceolatum* most chromosomes paired as bivalents, but one quadrivalent was observed in some cells. *Capsicum buforum* was found to be self-incompatible, while *Capsicum lanceolatum* was self-compatible.

2.3 *In vitro* culture

''In vitro'' means within a glass, observe in a test tube or in an artificial environment (Cheryl, 2007; Bhuiyan and Hoque, 2008; American Psychological Association, 2010) 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.

In *in vitro* studies, cells or biological molecules are studied outside their normal biological context. For example, proteins are examined in solution, or cells in artificial culture medium. *In vitro* plant regeneration from cells, tissues and organ cultures is a prerequisite for the application of plant biotechnology to plant propagation, plant breeding and genetic improvement. It is the only technology for the production of large quantities of ‘‘elite’’ planting material so as to increase the production and productivity. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds or respond well to vegetative reproduction. It also leads to simultaneous accomplishment of rapid large scale propagation of new genotypes. Colloquially it is called "test tube experiments", these studies in biology and its sub-disciplines are traditionally done in test tubes, flasks, petridishes 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).

2.4 Callus induction and proliferation

Callus induction and prolific 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) and Zang *et al.* (2004). 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 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 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 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) gelled Murashige and Skoog (MS) medium containing 20 g/L 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 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/L NAA and 3 mg/L 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 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.

2.5 *In vitro* studies in *Capsicum* spp.

The conventional method of chilli plant propagation using seeds is restricted by the short span of viability and low germination rate of seeds. Chilli plants are also highly susceptible to fungal and viral pathogens. Since chilli plant lacks natural vegetative propagation, plant tissue culture technique provides an alternative method of propagating novel genotypes. The establishment of efficient and promising protocol for *in vitro* plantlet regeneration of capsicum is required for the application of modern biotechnological tools, such as asexual reproduction of elite stocks, recovery of useful somaclonal variants, germplasm preservation as well as the production of transgenic plants with improved agronomic traits, interspecific hybrids and haploid plants, several attempts have been made on *in vitro* regeneration in the genus *Capsicum*, most of the reports are attempted on *Capsicum annuum* and a few on *Capsicum frutescens*.

Aki (2005) was conducted an experiment where *Capsicum annuum* L. seedlings were grown from seeds which surfaced sterilized with 70% ethanol for 4 minutes and 10% NaOCl (household bleach) solution for 6 minutes. The seeds were sown on the surface of hormone free MS (Murashige and Skoog, 1962) medium, pH adjusted to 5.8, the medium was solidified with 0.8% of bactoagar and 3% of sucrose used for carbon and energy source. After that, the cultures of the seeds germinated less than 16 h photoperiod at 25°C. Cotyledon and hypocotyls explants from 3 weeks old seedlings were excised approximately 1 cm length segments. These explants were cultured on MS basal medium for callus formation. For determination to find what is the effects of drought stress or lowering osmotic potential conditions in the medium on the callus formation and response of the biomass induction. Polyethylene glycol (PEG 3350) in 10% concentration was added to the MS medium and then biomass measurements were realized in 3 weeks interval from the callus. Two cultivars which belonging to the variety *grossum* (bell peppers) showed different responses against the drought stress conditions. In the normal MS medium, biomass formation was very fast but when PEG used for changing the osmotic potential of the MS medium the biomass formation decreased because of the drought stress conditions, after that MS medium was supplemented with auxines (NAA) and cytokinins (KIN) alone and together with PEG 3350 for recovering the biomass formation. As a result, auxines (NAA) and cytokinins (KIN) showed different recovering levels in two cultivars when they used alone or together.

2.6 Drought stress and its impact on agriculture

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 them, drought stress is a main abiotic stress that limits crop production as it affects 26% of arable area (Roy and Wu, 2001; 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). Dry lands (ca.5.1 billion ha) cover 40% of the world's land surface and serve as the habitat and surface of livelihood for more than 1 billion people. Desertification affects 70% of the world's dry lands, amounting to 3.6 billion ha or one fourth of the land surface (Roy *et al.*, 2009).

Water stress is one of the main environmental stresses responsible for reducing crop productivity in the dry lands as it affects growth through various physiological and metabolic processes of plant (Bray, 1993). Vital biochemical processes including photosynthesis (Boyer, 1976), respiration (Bell *et al.,* 1971), protein synthesis (Good and Zapalachinski, 1994) and assimilation of organic nitrogen (Sprent, 1981) have been demonstrated to be adversely affected by water stress. In rain fed agriculture, the short term water stress (10-20 days) is very common and it reduces productivity (Christiansen, 1982).

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).

The variable and often insufficient rainfalls in extended areas of rain-fed agriculture, the unsustainable groundwater use for irrigated agriculture worldwide, and the fast-growing demands for urban water are putting extreme pressure on global food crop production. The demand for water to sustain the agriculture systems in many countries will continue to increase as a result of growing populations (FAO, 2007). This progressively worsening water scarcity is imposing

hydric stress on both rain-fed and irrigated crops. 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).

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

Drought is a major factor in reducing plants growth, development and productivity. Water deficit stress, permanent or temporary, limits the growth and distribution of natural vegetation and the performance of cultivated plants more than any other environmental factor (Kramer, 1983). Water stress is characterized by reduction of water content, turgor, total water potential, wilting, closure of stomata, and decrease in cell enlargement and growth. Severe water stress may result in arrest of photosynthesis, disturbance of metabolism, and finally dying (Mckersie and Leshem, 1994). When exposed to such condition, plants respond by various mechanisms, ranging from whole plant characteristics such as life cycle timing (maturity) and deep root systems to cellular-level functions such as osmo-regulation. It seems likely that the cellular-level compounds of drought tolerance mechanisms are important and improvement at this level could have a positive impact on whole plant tolerance.

Drought stress affects crop growth and yield during all developmental stages. It is well known that drought stress at the early stages of plant life, shortly after germination, may have devastating impacts as both the root system is not yet fully established, in one hand, and stomatal control is not yet fine tuned. However, drought stress at this early life stage did not attract much research attention, because it is easily overcome by farmers through an accurate choice of seedling dates. Drought stress at later phenological stages received most attention, particularly the comparison between drought effects on the vegetative phases and

in the reproductive phases over grain production. It is now well established that the effects of stress may vary significantly with the phenological stage of plants. Grapevine provides an interesting example of the complexity of the relationships between drought stress and plant phenology. Traditionally, grapevine is a non-irrigated crop that occupies extensive areas in dry lands and semi-arid regions (Cifre *et al.*, 2005). Recently, in the Mediterranean region, irrigation was introduced to increase the low land yield. However, wine quality is strongly dependent on the organoleptic characteristics of grapes which, in turn, particularly in what concerns soluble sugar contents, are dependent on moderate drought stress during berry expansion (i.e. in the phases from fruit set to veraison). The irrigation strategy must therefore maximize the vineyard production without decreasing berry quality, an objective suitable for deficit irrigation programs (DRI).

Furthermore, a deep understanding of plant carbon assimilation and partitioning mechanisms under different water regimes will be required in the frame of precision agriculture, as, in fact, these mechanisms play a key role in the fine tuning of the balance between berry yield and quality. Hopefully, this will lead to the adoption of criteria for irrigation scheduling based on vine physiology (Cifre *et al.*, 2005).

The effect of drought on yield is highly complex and affects diverse processes including growth, reproductive development (gametogenesis), fertilization, embryogenesis, fruit and seed development (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). Reproductive stages are generally more sensible to stress than vegetative ones, but differences can also be made between different phases of the reproductive stage. 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. Mouhouche *et al*. (1998) found in *Phaseolus vulgaris* that periods of flowering were more sensitive than pod elongation and grain filling phases. Casanovas *et al.* (2003) reported a decrease of both leaf physiology and grain yield in maize subjected to drought during flowering. Boonjung and Fukai (1996) reported that when drought occurred during vegetative stages, it had only a small effect on subsequent development and grain yield. The effect of water stress on yield was most severe when drought occurred during panicle development.

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 mega-gametophyte 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 is not only affects seed production, but also lowered seed germination and vigor during reproductive growth. 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).

2.8 Drought stress mechanism

One of the most important constraints for agriculture is water limitation. More recently, global warming may be worsening this situation in most agricultural regions. Thus, it is quite relevant to understand the mechanisms that enable plants to cope with water deficit. Indeed, plants show a wide range of adaptations, at

different levels, to drought stress. Water deficiency stress induces a wide range of physiological and biochemical alterations in plants; arrestment of cell growth and photosynthesis and enhanced respiration are among the early affects. Genome expression is extensively remodeled, activating and repressing a variety of genes with diverse functions (Hong *et al.*, 2004; Shinozaki and Yamaguchi, 2007).

Sensing water deficit and activation of defense mechanisms comes through chemical signals in which abscisic acid (ABA) plays a central role. ABA accumulates in tissues of plants subjected to hydric stress and promotes transpiration reduction via stomatal closure. Through this mechanism, plants minimize water losses and diminish stress injury. ABA regulates expression of many stress-responsive genes, including the late embryogenesis abundant (LEA) proteins, leading to a reinforcement of drought stress tolerance in plants (Aroca *et al.*, 2008).

One of the main mechanisms by which plants cope with water deficits is osmotic adjustment. These adjustments maintain a positive cell turgor via the active accumulation of compatible solutes. Traditionally, the analysis of metabolic responses to drought stress was limited to analysis of one or two classes of compounds considered as “role players” in the development of tolerance.

Osmotic adjustment is considered as an important physiological mechanism of drought adaptation in many plants (Subbrao *et al.,* 2000). Osmotic adjustment is a mechanism to maintain water relations under osmotic stress. It requires regulation of intracellular levels of several compounds, collectively known as osmolytes (Janardhan and Bhojraj, 1999). It involves the accumulation of a range of osmotically active molecules or ions including soluble sugars, sugar alcohols, proline, glycinebetaine, organic acids, calcium, potassium, chloride ions, etc. (Farooq *et al.*, 2009).

The production of reactive oxygen species (ROS) is linear with the severity of drought stress, which leads to enhanced peroxidation of membrane lipids and degradation of nucleic acids, and both structural and functional proteins. Various organelles including chloroplasts, mitochondria and peroxisomes are the seats as well as first target of reactive oxygen species produced under drought stress (Farooq *et al*., 2009). Stomata closure, reduction of photosynthesis and osmotic adjustment are typical responses of plants to water stress at the first stage (Tanaka *et al.*, 1990). The photosynthetic apparatus is sensitive to water deficit. Closure of stomata and direct inhibition of Calvin cycle enzymes result in exposure of cells to excess excitation energy (Smirnoff, 1995). This excess energy may be diverted to activate molecular oxygen. Excess accumulation of reactive oxygen species damages various macromolecules, resulting in lipid peroxidation and enzyme inactivation (Elstner, 1982). The active oxygen induced damage to the cell may be minimized or prevented by increased antioxidant activity (Doulis, 1994). The severity of this damage largely depends on the status of antioxidant systems, since plants develop antioxidants to remove toxic reactive oxygen species and protect the plant cells from lipid peroxidation and inactivation of enzymes that occur under stress (Smirnoff, 1993).

2.9 Plant responses to drought stress

Abiotic stress leads to a series of morphological, physiological, biochemical and molecular changes in plants that adversely affect growth and productivity. A frequent result is protein dysfunction. Understanding the mechanisms of protein folding stability and how this knowledge can be utilized is one of the most challenging strategies for aiding organisms undergoing stress conditions. Stresses also affect the biosynthesis, concentration, transport, and storage of primary and secondary metabolites. As a more comprehensive view of these processes evolves, applications to reducing plant stress are emerging.

Through the history of evolution, plants have developed a wide variety of highly sophisticated and efficient mechanisms to sense, respond, and adapt to a wide range of environmental changes. When in adverse or limiting growth conditions, plants respond by activating tolerance mechanisms at multiple levels of organization (molecular, tissue, anatomical, and morphological), by adjusting the membrane system and the cell wall architecture, by altering the cell cycle and rate of cell division, and by metabolic tuning (Atkinson and Urwin, 2012). At a molecular level, many genes are induced or repressed by abiotic stress, involving a precise regulation of extensive stress-gene networks (Shinozaki and Yamaguchi, 2007; Delano *et al.*, 2011; Grativol *et al.*, 2012). Products of those genes may function in stress response and tolerance at the cellular level. Proteins involved in biosynthesis of osmoprotectant compounds, detoxification enzyme systems, proteases, transporters, and chaperones are among the multiple protein functions triggered as a first line of direct protection from stress. In addition, activation of regulatory proteins (e.g., transcription factors, protein phosphatases, and kinases) and signaling molecules are essential in the concomitant regulation of signal transduction and stress-responsive gene expression (Wang *et al.*, 2009; Krasensky and Jonak, 2012). Early plant response mechanisms prevent or alleviate cellular damage caused by the stress and re-establish homeostatic conditions and allow continuation of growth (Peleg *et al.*, 2011). Equilibrium recovery of the energetic, osmotic, and redox imbalances imposed by the stressor are the first targets of plant immediate responses.

Observed tolerance responses towards abiotic stress in plants are generally composed of stress specific response mechanisms and also more general adaptive responses that confer strategic advantages in adverse conditions. General response mechanisms related to central pathways are involved in energy maintenance and include calcium signal cascades (Reddy *et al.*, 2011; Pan *et al.*, 2012), reactive oxygen species scavenging or signaling elements (Ahmad *et al.*, 2010; Loiacono

and De Tullio, 2012) and energy deprivation (energy sensor protein kinase, SnRK1) signaling (Baena and Sheen, 2008). Induction of these central pathways is observed during plant acclimation towards different types of stress. For example, protein kinase SnRK1 is a central metabolic regulator of the expression of genes related to energy-depleting conditions, but this kinase also becomes active when plants face different types of abiotic stress such as drought, salt, flooding, or nutrient depravation (Lovas *et al.*, 2003; Umezawa *et al.*, 2004; Hey *et al.*, 2007; Ghillebert *et al.*, 2011; Cho *et al.*, 2012). SnRK1 kinases modify the expression of over 1000 stress-responsive genes allowing the re-establishment of homeostasis by repressing energy consuming processes, thus promoting stress tolerance (Baena, 2010; Cho *et al.*, 2012). The optimization of cellular energy resources during stress is essential for plant acclimation; energetically expensive processes are partially arrested, such as reproductive activities, translation, and some biosynthetic pathways. For example, nitrogen and carbon assimilation are impaired in maize during salt stress and potassium deficiency stress; the synthesis of free amino acids, chlorophyll, and protein are also affected (Good and Zaplachinski, 1994; Holcik and Sonenberg, 2005; Qu *et al*., 2011). Once energy expensive processes are curtailed, energy resources can be redirected to activate protective mechanisms. This is exemplified by the decrease in *de novo* protein synthesis in *Brassica napus* seedlings, *Glycine max*, *Lotus japonicas*, and *Medicago truncatula* during heat stress accompanied by an increased translation of heat shock proteins (Dhaubhadel *et al.*, 2002; Soares *et al.*, 2012).

2.9.1 Metabolic adjustments during stress conditions: osmolyte accumulation

A common defensive mechanism activated in plants exposed to stressing conditions is the production and accumulation of compatible solutes. The chemical nature of these small molecular weight organic osmoprotectants is diverse; these molecules include amino acids (asparagine, proline, serine), amines (polyamines and glycinebetaine), and γ-amino-N-butyric acid (GABA). Furthermore,

carbohydrates, including fructose, sucrose, trehalose, raffinose, and polyols (myo-inositol, D-pinitol) (Banu *et al.*, 2010; Krasensky and Jonak, 2012), as well as pools of antioxidants such as glutathione (GSH) and ascorbate (Phang *et al.*, 2008; Shabrawi *et al.*, 2010), accumulate in response to osmotic stress. Accumulation of compatible solutes in response to stress is not only observed in plants, it is a defense mechanism triggered in animal cells, bacteria, and marine algae, indicative of an evolutionarily conserved trait (Grant, 2004; Empadinhas and Costa, 2008). Scavenging of reactive oxygen species (ROS) to restore redox metabolism, preservation of cellular turgor by restitution of osmotic balance, and associated protection and stabilization of proteins and cellular structures are among the multiple protective functions of compatible osmoprotectants during environmental stress (Rathinasabapathi, 2000; Yancey, 2005; Mittler, 2006).

A large amount of research has been done on the beneficial effects of compatible solutes on plant tolerance to environmental stress. Correlation between amino acid accumulation (mainly proline) and stress tolerance was described in the mid-1960s in Bermuda grass during water stress (Barnett and Naylor, 1966). Since then, extensive work has proven that proline serves as an osmoprotectant, a cryoprotectant, a signaling molecule, a protein structure stabilizer, and an ROS scavenger in response to stresses that cause dehydration; including salinity, freezing, heavy metals, and drought (low water potential) (Verslues, 2006; Verbruggen and Hermans, 2008). Proline oxidation may also provide energy to sustain metabolically demanding programs of plant reproduction, once the stress has passed (Mattioli *et al.*, 2009).

Proline metabolism and its regulation are processes well characterized in plants. Proline is synthesized from glutamate in the cytoplasm or chloroplasts: Δ-1-pyrroline-5-carboxylate synthetase (P5CS) reduces glutamate to glutamate semialdehyde (GSA). Then GSA spontaneously cyclizes into pyrroline-5-carboxylate (P5C), which is further reduced by P5C reductase (P5CR) to proline.

Conversely, proline is catabolized within the mitochondrial matrix by action of proline dehydrogenase (ProDH) and P5C dehydrogenase (P5CDH) to glutamate. In an alternative pathway, proline can be synthesized from ornithine in a pathway involving ornithine δ-aminotransferase (OAT). Core enzymes P5CS, P5C, P5CR, ProDH, and OAT are responsible for maintaining the balance between biosynthesis and catabolism of proline. Regulation comes at transcriptional level of genes encoding the key enzymes. Transcriptional up-regulation of genes for P5CS and P5C to increase proline synthesis from glutamate and down-regulation of genes for P5CR and ProDH to arrest proline catabolism is observed during dehydration or osmotic stress (Verslues and Sharma, 2010). Also, post-translational regulation of core enzymes is closely associated with proline levels and environmental signals. For example, the Arabidopsis P5CS1 enzyme is subjected to feedback inhibition by proline, controlling the carbon influx into the biosynthetic pathway (Yoshiba *et al.*, 1997; Hong *et al.*, 2000). Considering that proline accumulation is associated with stress tolerance, that core enzymes regulate proline biosynthesis, and that these core enzymes are likely rate-limiting steps for its accumulation, logic dictates that overexpression of biosynthetic proline enzymes might increase the levels of the compatible solute and thus improve the tolerance in plants against abiotic stress. Several studies have tested this by overexpressing genes for P5CS or P5C enzymes in different plant species, reporting the expected rise in proline levels and the associated resistance to dehydration, salinity, or freezing (Kishor *et al.*, 1995; Zhu *et al.*, 1998; Sawahel and Hassan, 2002; Su and Wu, 2004; Parvanova, 2004; Gleeson *et al.*, 2005; Yamada *et al.*, 2005; Vendruscolo *et al.*, 2007). Furthermore, deletion of genes coding ProDH (Nanjo *et al.*, 1999) or P5CDH (Borsani *et al.*, 2005; Deuschle *et al.*, 2004) expression of a feedback-insensitive P5CS (Hong *et al.*, 2000), or the overexpression of OAT (Roosens *et al.*, 2002; Wu *et al.*, 2005) increase the cellular levels of proline and osmoprotection to some abiotic stresses.

Comparable extensive work has been done for other compatible solutes such as γ-aminobutyric acid (Akcay *et al.*, 2012), glycine betaine (Giri, 2011), trehalose (Fernandez *et al.*, 2010), mannitol, and sorbitol (Rathinasabapathi, 2000); these solutes are efficient protectors against some abiotic stressors. Metabolic pathways for biosynthesis and catabolism of compatible solutes, their regulation, participant enzymes, and compartmentalization are well characterized in most important plant species. This knowledge has led to strategies for improvement of plant tolerance involving the accumulation of those protective osmolytes in plants by expression of core biosynthetic enzymes or their improved derivatives, expression of related transporters, and deletion of osmolyte consuming enzymes. These numerous studies have provided evidence that enhanced accumulation of compatible solutes correlates with reinforcement of plant resistance to adverse growth conditions.

2.9.2 Engineering polyamine accumulation

Polyamines (PAs) are small (low molecular weight), positively charged, aliphatic amines that are found in all living organisms. The major forms of PAs are putrescine (Put), spermidine (Spd) and spermine (Spm), although plants also synthesized a variety of other related compounds. Arginine (Arg) and ornithine (Orn) are the precursors of plant PAs. Ornithine decarboxylase (ODC) converts Orn directly into Put. The other biosynthetic route to Put, via arginine decarboxylase (ADC), involves the production of the intermediate agmatine (Agm) followed by two successive steps catalysed by agmatine iminohydrolase (AIH) and N-carbamoylputrescine amidohydrolase (CPA). In animals and fungi Put is synthesized primarily through the activity of ODC while in plants and bacteria the main pathway involves ADC. Aminopropyl groups, donated by decarboxylated S-adenosyl methionine (dcSAM), must be added to convert Put into Spd and Spm in a reaction catalysed by spermidine synthase (SPDS) and spermine synthase (SPMS), respectively (Alcazar *et al.*, 2010).

Polyamines levels in plants increase under a number of environmental stress conditions, including drought and salinity (Flores, 1991; Bouchereau *et al.*, 1999; Kasinathan and Wingler, 2002). Several biological roles were proposed for polyamines action in stress situations; PAs could act as osmoprotectants, as scavengers of active oxygen species (AOS) or by stabilizing cellular structures, such as thylakoid membranes (Tiburcio *et al.*, 1997; Bouchereau *et al.*, 1999; Martin, 2001). The first reports of transgenic approaches using genes responsible for PA biosynthesis were conducted in two species, tobacco and rice (Burtin and Michael, 1997; Masgrau *et al.*, 1997; Capell, 1998; Roy and Wu, 2001; Capell *et al.,* 2004). Recently, new role and regulatory function of polyamines in plant abiotic stress tolerance have been achieved, with several abiotic (salt, drought, freezing, heat) stress tolerant transgenic plants overproducing polyamines being described in several reviews (Kuznetsov *et al.,* 2006; Groppa and Benavides, 2008; Alcazar *et al.,* 2010; Gill and Tuteja, 2010).

Plants respond to changes in water status by accumulating low molecular weight osmolytes including Polyamines (PAs). Polyamines may have a primary role of turgor maintenance but they may also be involved in stabilizing proteins and cell structures. The polycationic nature of PAs at physiological pH is believed to mediate their biological activity, since they are able to bind to several negatively charged molecules, such as DNA, membrane phospholipids, pectic polysaccharides and proteins (Martin, 2001).

In respect to the antioxidant activity of PAs, the research data is contradictory; on the other hand, PAs have been suggested to protect cells against active oxygen species (AOS) and on the other hand, their catabolism generates AOS (Groppa and Benavides, 2008). PA catabolism produces H_2O_2, a signaling molecule that can act promoting activation of antioxidative defense response upon stress, but can also act as a peroxidation agent. In a recent study, the effect of increased putrescine (Put) accumulation was found to negatively impact the oxidative state of poplar

cells in culture due to the enhanced turnover of Put (Gill and Tuteja, 2010). Gill and Tuteja (2010) stated that, while increase Put accumulation may have a protective role against AOS in plants, enhanced Put turnover can actually make them more vulnerable to increased oxidative damage. The higher polyamines, Spd and Spm are believed to be most efficient antioxidants and are considered scavengers of oxyradicals (He *et al.,* 2008).

As plants with elevated putrescine (Put) contents are able to tolerate drought stress because Put has a direct protective role in preventing the symptoms of dehydration, higher PAs (Spd and Spm) appear to play an important in role in stress recovery (Peremarti *et al.,* 2009). Recently, transgenic rice plants overexpressing samdc (S-Adenosyl methionine decaboxylase gene), with increased Spd and Spm levels, were considered to be non drought tolerant, but showed a more robust recovery from drought compared to wild type (Peremarti *et al.,* 2009). The *de novo* synthesis of Spd and Spm in transgenic plants under drought stress, at the expenses of Put, was responsible for the stress tolerance observed in these plants.

2.10 Polyethylene glycol (PEG) for drought stress *in vitro*

Polyethylene glycol of high molecular weights has long been used to simulate water stress in plants (Rao and Jabeen, 2013; Ruf *et al.*, 1967; Kaufman and Eckard, 1971; Corchete and Guerra, 1986). It is a non penetrable and nontoxic osmotic, lowers the water potential of the medium. It appears to be better suited as an external osmoticum to analyze water retention in plants. It is 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, resulted a decrease in callus induction, germination rate,

root length, shoot length and seed vigor in certain crop plants (Rao and Jabeen, 2013; Khodarahmpour, 2011).

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). Most of the researchers have used polyethylene glycol because of its high molecular weight and this feature of the molecules inhibits the entry to the plasmalemma and symplast with this way metabolic product does not affect directly (Cay and Yurekli, 1995).

C. annuum L. is drought sensitive species. Some of the researches have been using PEG on *in vitro* studies for *C. annuum* varieties to increase the tolerance against drought sensitivity (Van Der Beck and Ltifi, 1991). Concentration of polyethylene glycol from 15 to 30% were increased the drought tolerance of cell line but this tolerance has been lost in subcultured cell line which adapted to polyethylene glycol medium which doesn't include osmotic conditions (Bressan *et al*., 1982). The effect of polyethylene glycol on the development of cell colony, osmotic potential and ion change in red pepper were realized. One of the cell colonies have been developed in concentration of 20 and 25% PEG. The other cell colonies have been highly developed biomass in 5 and 10% PEG (Santos-Diaz and Alejo-Ochoa, 1994). PEG treatment in *C. annuum* L. cell colony has been changed ion balance (Santos-Diaz and Alejo-Ochoa, 1994). According to some of the researcher's osmotic potential increasing which medium supported with 5-10% polyethylene glycol were stimulated *nicotiana* callus growing, protein and chlorophyll content. When polyethylene glycol concentration increased to 30% inhibition of callus growing and the other parameters were realized. During all subculture period biomass increasing have been inhibited when MS medium supported with 10% PEG.

In an experiment, callus of bell pepper (*Capsicum annuum* L.) was initiated from hypocotyl on MS medium supplemented with NAA (0.5 mg/L) and BAP (0.2 mg/L). For proliferation of callus the hormone concentrations were reduced to half. Cell clumps of about 1 mm diameter were exposed to increasing concentration of polyethylene glycol (PEG) ranging from 10 g/L to 100 g/L for water stress tolerance. Upon incubation for 30 days, the cells, which could tolerate this concentration of PEG, grew to form calli. Selected calli were further subcultured on to the selective medium (100 g/L) PEG for 8 weeks and then transferred to normal MS medium for proliferation. The selected calli when transferred from the normal to the selective medium, were capable of growing on it. Although, there was difference in their growth, the pattern was sigmoidal in both the cell lines. Compared to the control, selected cells contained significantly higher levels of soluble proteins, total sugars, reducing sugar, and free amino acids. The water stress tolerant cells also revealed enhanced activities of enzymes, malate dehydrogenase, alkaline invertase, NADP+ -isocitrate dehydrogenase, aspartate amino transferase, glutamate pyruvate transaminase and acid phosphatase (Nath *et al*., 2005).

Aki (2005) reported that biomass changing percentage of *C. annuum* Var. *grossum* cultivar Kandil Dolma and cultivar Yaglik 28 have shown differences in *in vitro* conditions because of differences their endogenous hormone levels. MS medium which supported with auxin and cytokinin together have negative effect on biomass increasing comparing with auxin alone in Kandil Dolma cultivar. This show that cytokinin which added to the MS medium have synergistic effect with PEG for inhibition of biomass formation in Kandil Dolma cultivar. But in Yaglik 28 cultivar when MS medium supported with auxin and cytokinin together biomass increasing were realized. In this case synergistic effect of PEG and NAA were inhibited with adding KIN to the MS medium. Endogenous hormone levels in both cultivars are affected from drought stress conditions differently and

independently. Biomass decreasing has observed in drought stressed tissues. Thus, it has been thought that singly or together external applications of NAA and KIN would optimize physical metabolic conditions favorable for drought stress. While the growth regulator NAA stimulated biomass increasing in Kandil dolma cultivar, growth regulator KIN stimulated biomass increasing in Yaglik 28 cultivar.

Joshi *et al*. (2011) also used PEG for screening of drought tolerant genotypes in rice. They studied the *in vitro* somatic embryogenesis and screened the calli for drought tolerance using mature embryos as explants. Mature embryos of three aromatic (Pusa Basmati 1, Pant Sugandh Dhan 17, Taraori Basmati) and one non-aromatic (Narendra 359) indica rice (*Oryza sativa* L.) varieties were used for developing callus on Murashige and Skoog medium supplemented with 2, 4-dichlorophenoxy acetic acid (2, 4-D) (2.0 mg/L for Narendra 359 and 2.5 mg/L for Pusa Basmati 1, Taraori Basmati and Pant Sugandh Dhan17). Screening of calli was done by sub-culturing calli for15 days on Murashige and Skoog (MS) basal medium supplemented with different concentrations of polyethylene glycol (PEG- 6000) as chemical drought inducer. Narendra 359 showed best response in terms of callus growth at 70 g/L of PEG. The highest percentage somatic embryogenesis among selected calli was observed in Pusa Basmati 1 and the lowest in Pant Sugandh Dhan 17.

Cell cultures of sunflower (*Helianthus annus* L. cv. Myak) were established from callus tissues inoculated in MS liquid medium supplemented with 1.0 mg/L NAA (naphthalene acetic acid), 0.1 mg/L KIN (kinetin) (Hassan *et al*., 2004). PEG 6000 was added to the medium to induce water deficit. Water stress tolerant callus line was isolated by plating the cell suspension on agar-solidified medium containing the same solute potential of PEG (Polyethylene glycol). This selected line grew better than the non-selected one on various levels of water deficit induced by PEG. Moreover, the selected line accumulated more K+, Na+ and N but less Ca++ and P than the non-selected line. The proline level showed a positive correlation with the

degree of tolerance to water stress, which suggests that proline accumulation, accompanies survival and growth in drought environment. With the exception of polysaccharides, the sugar contents of both callus lines significantly increased with increasing PEG concentration. Protein profile of both selected and non-selected callus lines shows the presence of four major and three minor polypeptide bands. No qualitative differences have been obtained between both callus lines in presence or absence of water stress. No *de novo* proteins have been produced under PEG-induced water stress conditions. DNA banding pattern indicated the occurrence of four *de novo* DNA fragments in the non-selected callus line exposed to -0.8 and -1.0 MPa osmotic stresses. Exposing selected callus line to water stress was accompanied by the induction of a unique DNA fragment of an approximate size of 571 bp. The better performance of selected line under water stress may be attributed to its greater osmotic adjustment in relation to non-selected line.

2.11 Proline content and drought tolerance

Proline is one of the important osmolytes which accumulates during moisture stress condition. It helps to maintain turgor and promotes continued growth in low water potential soils (Mullet and Whitsitt, 1996). Singh and Singh (1983) observed that proline accumulation under drought condition is a good indicator of drought resistance capacity of plants. At cellular level water stress induces the production of reactive oxygen species (ROS), such as superoxide radical (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH), which ultimately cause membrane damage (Tambussi *et al.,* 2002). One of the applications of water stress tolerant species or cultivars is the dominance of the defense system of antioxidant enzymes. This enzyme includes superoxide dismustases (SOD), which scavenge superoxide radicals and convert them to O_2^- and H_2O_2. H_2O_2 is then detoxified by catalase (CAT) and ascorbate peroxidase (APOX). Catalase scavenges H_2O_2 by breaking down directly to form water and oxygen and an increase in its activity is

related with increase in stress tolerance (Kraus *et al.*, 1995). Catalase is indispensable for ROS detoxification during stress (Willekens *et al.*, 1997).

High proline content is a good index for moisture resistance in genotypes. Under moisture stress condition the protein degrades and consequently the proline content increases. An experiment was conducted with seven genotypes of rice where Monohar Sail recorded the highest increase in proline content (50%) over control at -0.4 MPa moisture stress imposed by PEG 6000 followed by Halodhar (46.1%) and Ranjit (37.50%). But at -0.8 MPa water stress Monohar Sail recorded the highest increase in leaf proline content (72.2%) followed by Halodhar (69.2%), George Sail (57.8%) and Kapili Dhan (57.1%). So, the rice genotypes Halodhar, George Sail and Kapili Dhan would be considered as potential genotypes for drought resistance (Roy *et al.,* 2009).

2.12 The genetics of drought tolerance using *in vitro* technique

The molecular and cellular processes underlying the acclimation of capsicum to abiotic stresses have attracted much interest because the response of this economically important crop to adverse environmental factors is not well understood as in other crop plants. Several genes are identified for tolerance to drought and other abiotic stresses. For example, *CaRZFP1* (*Capsicum annaum* RING Zinc Finger Protein 1), confers tolerance to drought, high temperature and cold stresses in tobacco (Zeba *et al.,* 2009). *CaBI1* (*Capsicum annaum*Bax Inhibitor 1), confers tolerance to drought in tobacco (Isbat *et al.,* 2009). *CaERFLP1 (Capsicum annaum*Ethylene Responsive Factor like Protein 1), confers tolerance to high salinity in tobacco (Lee *et al.,* 2004). *CaXTH3 (Capsicum annaum*Xyloglucanendotransglucosylase/hydrolase 3), improves drought and salt tolerance in *Arabidopsis* (Cho *et al.,* 2006). *CaPMEI1* (*Capsicum annaum*Pectin methylesterase inhibitor protein 1), confers drought and oxidative stress tolerance in *Arabidopsis* (An *et al.,* 2008). *CaCDPK3 (Capsicum*

*annaum*Calcium dependent Protein Kinase 3), implicated in various osmotic stresses and abscesic acid in pepper plant (Chung *et al.,* 2004). *CaSAR8.2* acts as a molecular marker for high salinity, drought or low temperature stresses in *Capsicum annaum*(Lee and Hwang, 2003).

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 of November, 2013 to December, 2014 to study the impact of polyethylene glycol induced water stress on calli growth and accumulation of proline in *Capsicum* spp. The experiment was performed in four different steps, viz. *in vitro* seed germination of different genotypes, *in vitro* callus initiation and sub-culturing of callus, *in vitro* drought treatment using polyethylene glycol (PEG) in different concentrations (0 g/L, 20 g/L, 40 g/L, 60 g/L and 80 g/L) and determination of proline content (µg/g) accumulated during stress. 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- 1207. The place is geographically located at about 23.77º North latitude and 90.37^0 East longitudes (Anonymous, 2014). The location of Sher-e-Bangla Agricultural University in Bangladesh map is shown in Appendix I.

3.2 Experimental material

3.2.1 Genotypes

Ten genotypes of *Capsicum* spp. were collected from Spices Research Centre, Bogra and Bangladesh Agricultural Research Institute, Joydevpur, Gazipur. Five of them were selected as explant source on the basis of their germination ability. The list of initial ten genotypes is given in Appendix II.

3.2.2 Genotypes for explant source

The seeds of ten genotypes were soaked in sterile water for two nights. The seeds of each genotype were placed on Whatman filter paper 3 in petridishes for ten days. The Whatman filter paper was kept in wet condition with sterile water. They were incubated in dark at 26±2^{o}C. The seedlings were counted after 10 days of incubation. The list of five selected *Capsicum* genotypes are presented in Table 1.

Table 1. List of the *Capsicum* genotypes used in the experiment

Sl. No.	Genotypes No.	Name/Accession No.	Collection area
01	G1	CO611	SRC, Bogra
02	G2	CO525	SRC, Bogra
03	G3	SRC02	SRC, Bogra
04	G4	SRC05	SRC, Bogra
05	G5	SRC14	SRC, Bogra

SRC= Spices Research Centre, Bogra

3.3 Laboratory materials

Laboratory preparation was started in early September, 2013 by collecting chemicals and instruments.

3.3.1 Chemicals

MS mixture (powder) and MS medium ingredients, seed treating chemicals (fungicide- 0.1% carbendazim), sterilizing chemicals (10% Sodium hypochlorite, NaOCl; 70% ethanol), distilled water (DW), sterilized distilled water, sucrose, agar, NaOH (10 N, 1 N), HCl (0.1 N), KCl (3 M), polyethylene glycol (PEG),

96% ethanol, absolute ethanol, methylated spirit, IAA (Indole-3-acetic acid), NAA (1-Naphthaleneacetic acid), 2,4-D (2,4-Dichlorophenoxyacetic acid), KIN (Kinetin), orthophosphoric acid (6 M), glacial acetic acid, ninhydrin, 3% sulfosalicylic acid and toluene.

3.3.2 Instruments, glassware and other accessories

Autoclave, hotplate with magnetic stirrer, automatic drying oven, microwave oven, freezers, shaking incubator, water bath, water purification system, pH meter, spectrophotometer, coarse and fine electric balances, pipettes, measuring cylinders, conical flasks, volumetric flasks, glass beakers, wash bottles, scalpel, forceps, scissors, hand gloves, parafilm, aluminium foil, autoclave tape, laminar air flow chamber, growth chamber, incubators, culture vials (petri dishes, test tubes 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 hormone supplements as culture medium for callus induction. The composition of MS medium has been presented in Appendix III. Different concentration of polyethylene glycol (PEG) was added to the basal MS medium supplemented with auxin and cytokinin for water stress treatment. Three types of culture media were used in this study, viz, hormone free basal MS medium for growing of seedlings for explants, MS medium supplemented with 2 mg/L of NAA, 0.5 mg/L of KIN and 5 mg/L of 2,4-D for callus induction and the same MS medium with hormones supplemented with different PEG concentrations (0 g/L, 20 g/L, 40 g/L, 60 g/L and 80 g/L) for stress treatment. Stock solutions were prepared at the beginning and stored in the refrigerator at 4±1ºC. Different steps of culture media preparation are presented in Plate 1.

3.4.1 Preparation of stock solutions

3.4.1.1 Preparation of NAA stock (10×)

A stock solution of NAA with 10× concentration was generally prepared. Firstly, 0.01 g NAA was dissolved by adding few drops of 1N NaOH in one liter beaker. This solution was dissolved in 100 mL of sterilized DW. Then the stock solution was labeled properly and poured into a clean sterilized glass container and stored in a refrigerator at 4°C for ready use. 20 mL stock provided 2 mg/L of NAA in callus induction medium.

3.4.1.2 Preparation of KIN stock (10×)

A stock solution of KIN with 10× concentration was generally prepared. Firstly, 0.01 g KIN was dissolved by adding few drops of 1 N NaOH in 100 mL beaker. Sterilized DW was added to make the final volume up to 100 mL. Then the stock solution was labeled properly and poured into a clean sterilized glass container and stored in a refrigerator at 4°C for ready use. 5 mL stock provided 0.5 mg/L of KIN in callus induction medium.

3.4.1.3 Preparation of 2,4-D (10×)

A stock solution of 2,4-D with 10× concentration was generally prepared. Firstly, 0.01 g 2,4-D was dissolved by adding few drops of 96% ethanol in 100 mL beaker. Sterilized distilled water was added to make the final volume up to 100 mL with slight heat using hotplate with magnetic stirrer. Then the stock solution was labeled properly and poured into a clean sterilized glass container and stored in a refrigerator at 4°C for ready use.

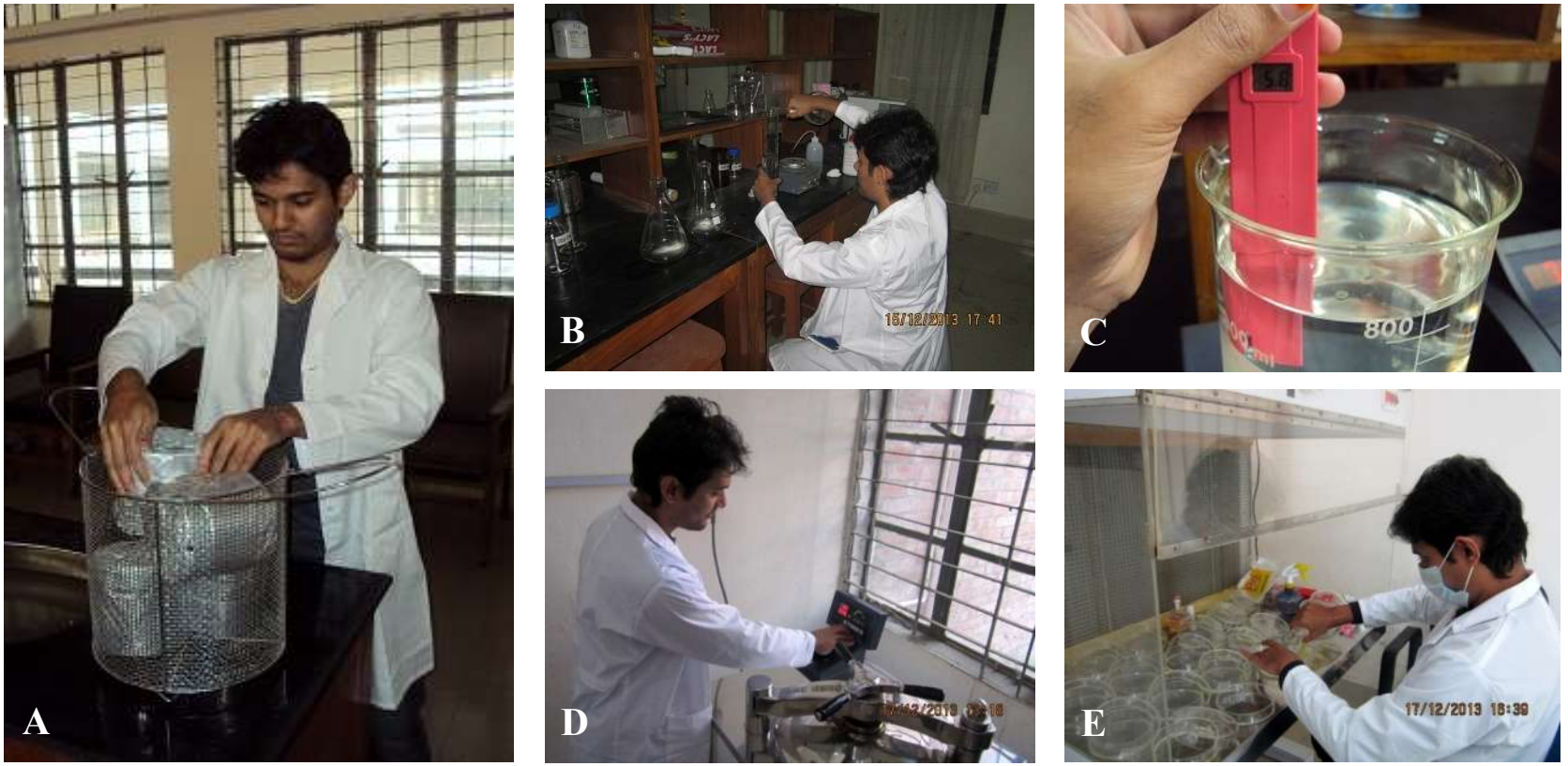

Plate 1. Steps of media preparation. A) Sterilization of glasswares, B) Preparation of medium solution, C) Adjustment of pH, D) Medium sterilization and E) Aliquot under laminar hood

3.4.2 MS media preparation

3.4.2.1 Steps of the MS media preparation

To prepare one liter of MS medium, 800 mL sterilized distilled water was taken into 1 liter beaker. 4.4 g of MS mixture was added to this sterilized distilled water. 30 g of sucrose was then dissolved in this solution with the help of magnetic stirrer. The whole mixture was then made up to 1 liter with further addition of sterilized distilled water. For germination medium, hormone was not added but for callus induction medium, 20 mL of NAA stock, 5 mL of KIN stock and 50 mL of 2,4-D was added to the medium. For treatment medium, 20 g/L, 40 g/L, 60 g/L and 80 g/L of PEG were added into the medium respectively. For control no PEG was added to the medium.

3.4.2.2 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 (Plate 1C).

3.4.2.3. Agar

The prepared media was equally divided into two 1000 mL conical flasks. 4 g of agar was added in each flask before autoclaving. The medium was allowed to cool and aliquote in petridishes under the laminar hood (Plate 1E). In case of test tubes, the medium containing agar was melted in microwave oven and aliquoted in the test tubes before autoclaving. The media was stored at 4 ± 1°C until use.

3.4.3 Preparation of other solutions

3.4.3.1 Preparation of 0.1% rekazim (carbendazim) solution

For preparing 0.1% rekazim (carbendazim) solution, 0.1 g of rekazim was taken in a 100 mL volumetric flask and the volume was made up to the mark.

3.4.3.2 Preparation of 0.1% $HgCl_2$ solution

For obtaining 0.1% concentration, 0.1 g of $HgCl_2$ was taken in 100 mL volumetric flask and the volume was made up to the mark.

3.4.3.3 Preparation of 1 N 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 1000 mL.

3.4.3.4 Preparation of 70% ethanol

In a 100 mL measuring cylinder 70 mL of 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.

3.4.3.5 Preparation of 6 M orthophosphoric acid

In a 500 mL measuring cylinder, 204 mL orthophosphoric acid was poured and filled with double distilled water up to the mark.

3.4.3.6 Preparation of ninhydrin reagent

At first 30 mL glacial acetic acid was poured in a 100 mL beaker then, 20 mL of 6 M orthophosphoric acid and 1.25 g ninhydrin solute was added and gently stirred with slight heat (not more than 70˚C) until completely dissolved.

3.5 Sterilization

3.5.1 Sterilization of culture media

The conical flasks and test tubes containing medium were capped with aluminium foil and were autoclaved at 15 psi pressure at 121˚C for 20 minutes (Plate 1D).

The medium was then transfer into the culture room and cooled at 24˚C temperature. Fixed volumes of medium were aliquot into petridishes. 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 stored at 4˚C until use. Marking was done for proper identification.

3.5.2 Sterilization of glassware and other accessories

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 15 psi pressure (Plate 1A). All types of glassware and accessories were washed properly by liquid detergent, cleaned with running tap water and finally washed with distilled water and dried in automatic drying oven prior to autoclaving.

3.5.3 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 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 with ultra violet light. The ultra violate ray kills the microbes inside the laminar air flow. 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 Pretreatment and sterilization of seed

Twelve to fourteen seeds per genotype were taken in a sieve and were washed in running tap water. The washed seeds were transferred into labeled beakers. Fungicide 0.1% rekazim (carbendazim) was poured in each beaker and shaken well. Then seeds were rinsed three times separately with distilled water and dipped in distilled water over night.

Seeds were treated with absolute alcohol for 4 minutes. After treatment, seeds were rinsed with sterilized distilled water for 2 times. Seeds were then surface sterilized with NaOCl (10%) for 6 minutes and rinsed 5 times with sterilized distilled water. The step of seed sterilization is presented in Plate 2A.

3.6 Seed inoculation and raising of seedlings

The sterilized seeds of selected genotypes (Table 1) were inoculated in hormone free MS (Murashige and Skoog, 1962) medium. The cultures were incubated in dark condition at 27±1˚C for 10 days to accelerate germination. After germination, they were shifted to a 16h/8h light/dark photoperiod with the illuminations of white fluorescent lights (40-50 µmol-m^{-2}-s^{-1}) at 25±2˚C. Twenty days old seedlings were used as explants source. Seed inoculation, germination and raising of seedlings are presented in Plate 2 (B-C).

3.7 Explant collection, inoculation and subculture

The explants (cotyledonary leaves and nodal segments) were collected from the 20 days old seedlings. For callus induction the protocol reported by Rakshit *et al*. (2010) was used. Briefly, cotyledonary leaves and nodal segments were excised at approximately 0.5 cm segments. The cultures were incubated at 16h/8h light/ dark photoperiod with the illuminations of white fluorescence lights (40-50 µmol-m^{-2}-s^{-1}) at 25± 2^{o}C for 4 weeks. Calli were cut into 5 mm pieces for subculturing onto freshly prepared medium after every four weeks. The explants collection,

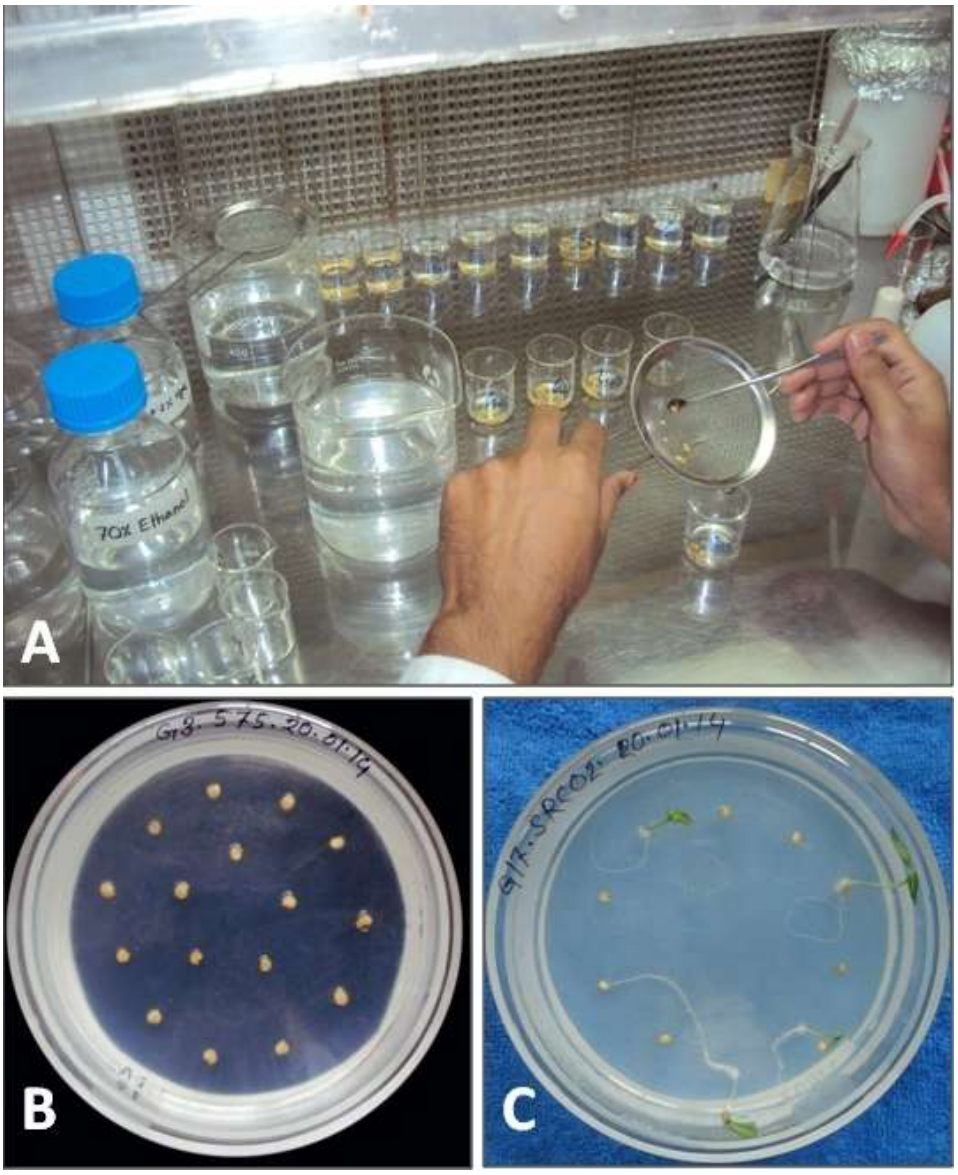

Plate 2. Steps of seed germination. A) Seed treatment with fungicide 0.1% rekazim (carbendazim), B) Seed inoculation in MS medium and C) Germination of seedling

Inoculation and subculture (Plate 3. A-C) was done for all five genotypes with three replications.

3.8 Drought tolerance assay

The drought tolerance assay was performed as Zeba *et al.* (2009). Briefly, the callus pieces of 5 mm (diameter) were inoculated in test tubes containing MS medium supplemented with 0 g/L, 20 g/L, 40 g/L, 60 g/L and 80 g/L of polyethylene glycol (PEG) (Plate 3D). The culture environment included 25^{o}C, 60% relative humidity, and a 16 h photoperiod from white fluorescent lamps (200 µmol photons-m^{-2}-s^{-1}). After 4 weeks, all the treatments were evaluated on the basis of their changing in size and weight from the day of inoculation to the 20 DAT (days after treatment) and 40 DAT.

3.9 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, sieves, brush, cotton 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 were sterilized in an autoclave following method of media sterilization. The necks of 50 mL conical flasks were flamed during aliquoting. Aseptic conditions were followed during each and every operation to avoid the contamination of cultures.

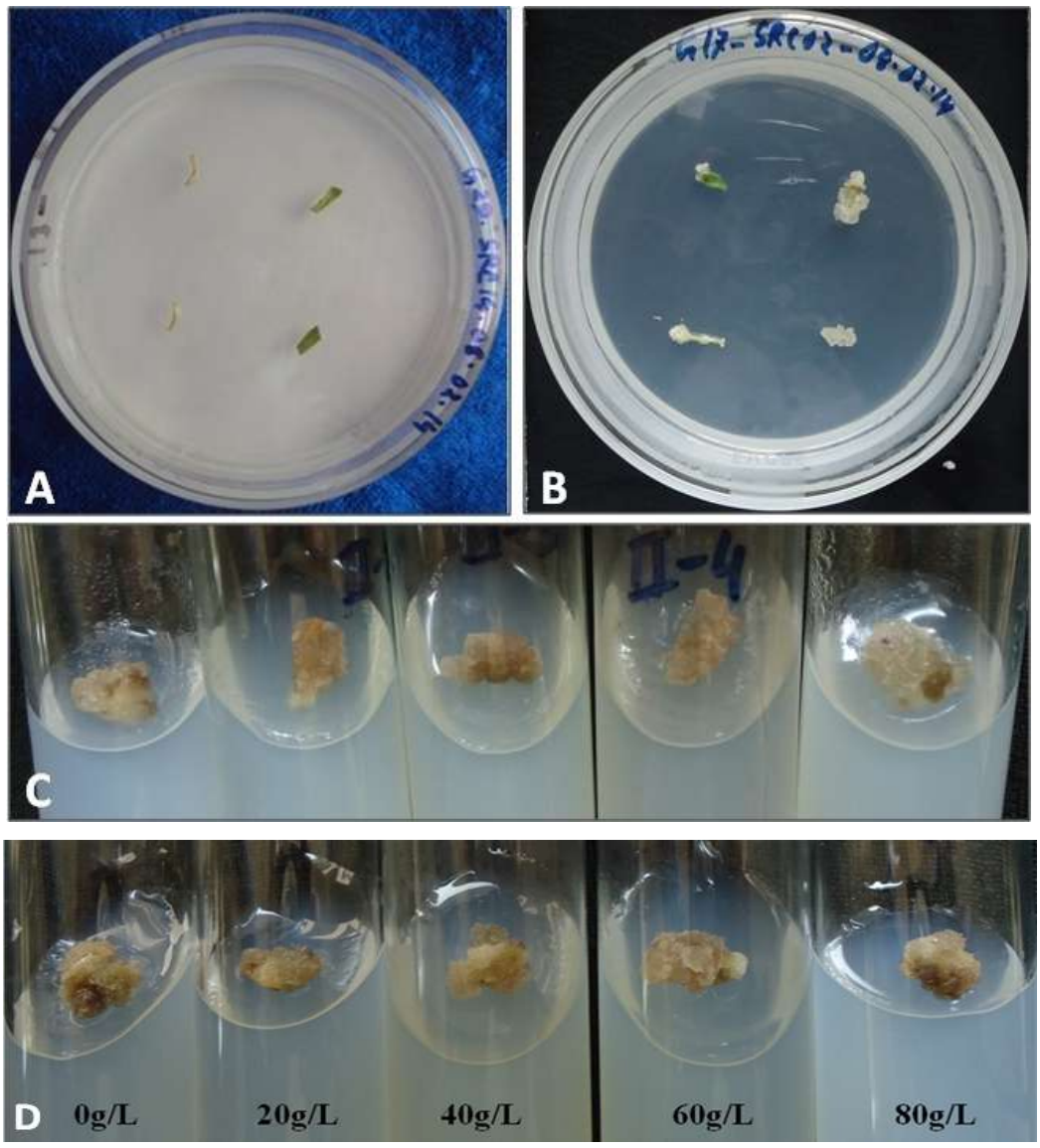

Plate 3. Callus initiation and subculture. A) Inoculation of cotyledon and nodal segment, B) Callus initiation, C) Subculturing of callus, D) PEG treatment to the calli at 0 g/L, 20 g/L, 40 g/L, 60 g/L and 80 g/L.

3.10 Determination of proline content

3.10.1 Proline extraction from calli

Proline accumulation was determined by the method as described by Sadasivam and Manickam (1996). Fresh calli (0.5 g) were grinded in mortar and pestle with 10 mL of 3% sulphosalicyclic acid and the homogenate was centrifuged at 18000× g. The homogenate was filtered and 2 mL of filtrate was added to the 2 mL of glacial acetic acid and 2 mL of acid ninhydrin and test tubes were kept for 1 h at 100°C in water bath, followed by ice bath. The reaction mixture was vortexed with 4 mL of toluene. Toluene layer was separated and absorbance was read at 520 nm. A standard curve of proline was used for calibration.

3.10.2 Preparation of proline standard curve

80 mg of pure proline was dissolved into 100 mL of distilled water to get 800 ppm proline stock solution for preparing proline standard curve. By diluting this solution, 50 ppm, 100 ppm, 200 ppm, 400 ppm and 800 ppm solution were prepared in 20 mL each. The absorbances were measured with the help of spectrophotometer at 520 nm. By plotting the concentration of proline (ppm) in 'X' axis and obtained absorbance reading in 'Y' axis a standard curve was prepared (Appendix V). From the absorbance reading obtained from samples, their respective proline content was estimated in ppm by using proline standard curve and converted into micro gram per gram (µg/g) unit using the following formula.

$$\text{Amount of proline } (\mu g/g) = \frac{x}{2} \times \frac{10}{500} \times 1000$$

3.11 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 digital verneer slide calipers at 0 DAT, 20 DAT, and 40 DAT (Plate 4A). Tubes were arranged on the shelves of

Plate 4. Data recording. A) Recording of callus weight with digital fine balance, B) Tubes were arranged on the shelves of the laboratory growth chamber according to a completely randomized design (CRD)

laboratory growth chamber according to a completely randomized design (CRD) (Plate 4B). Each tube had a single callus and was considered as an experimental unit. Callus response data were analyzed using the means and the genotype treatment interactions were analyzed using MSTATC program. Data were processed with analysis of variance (ANOVA) and the means were compared using DMRT (Duncan's Multiple Range Test) at 5% level of significance.

CHAPTER IV
RESULTS AND DISCUSSION

The experiment was carried out at the Genetics and Plant Breeding Laboratory, Sher-e-Bangla Agricultural University, Dhaka-1207 to study the performance of different concentrations of PEG for water stress on callus initiation and biomass changing of callus in terms of fresh weight and diameter of five genotypes. This study dealt with the *in vitro* selection of water stress tolerant callus lines in *Capsicum* to made the progress of regeneration and eventually gene expression analysis and thereby identify and isolate the genes involved in the process of water stress tolerance for future gene transformation events. As water stress in soil is variable and plant tolerance depends on the stage of plant development, in this study, calli were phenotyped at several water stress conditions and at the most sensitive stages (20 days, 40 days and 60 days old calli). The genotypes were denoted as G1, G2, G3, G4 and G5 and the PEG concentrations were denoted as T1 (0 g/L), T2 (20 g/L), T3 (40 g/L), T4 (60 g/L) and T5 (80 g/L).

4.1 Response of seedlings and callus induction

The seeds of five genotypes were surface sterilized and inoculated in hormone free basal MS medium. The seeds started to germinate within 10 days of incubation. Within three weeks of seed inoculation the length of seedlings were in appropriate size to serve as explants source for cotyledonary leaves and nodal segments. The explants were cultured on MS medium supplemented with auxin (NAA, 2,4-D) and cytokinine (KIN). The cotyledons and nodal segments were cut into about 0.5 cm sizes and inoculated in the petridishes. Within one week, the explants became enlarge and start swelling. After four weeks of culture the swelled explants gradually turned into whitish green callus. The gradual change of cotyledonary leaf and nodal segments to the calli is presented in ‘materials and methods’ section (Plate 2A and Plate 2B). For multiplication and maintenance, the calli of each

genotype, were cut into pieces of 5 mm (diameter) aseptically and subcultured in the fresh medium (Plate 2C). Auxin NAA and 2,4-D are important media supplements for callus initiation in culture. Previous most of the studies agreed with this study results in respect of the use of media for callus induction. It was 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; Gallo-Meagher *et al*., 2000). The use of auxin and cytokinin in the MS media for callus induction and proliferation also correspond with the result of Nadar *et al*. (1978), Bhansali and Singh (1982), Zang *et al.* (2004), Kulkarni (1989), Karim *et al*. (2002), Mamun *et al.*(2004), Nagai *et al*.(1991) and Islam *et al*.(1996). Snyman *et al*. (2000) also found that a good mass of callus could be obtained on MS medium containing 3.0 mg/L 2, 4-D after 3-4 weeks of inoculation. Niaz and Quraishi (2002) and Ramanand *et al*. (2006), also found that MS medium containing different concentrations of NAA and 2, 4-D improves the callus growth.

4.2 Performance of different genotypes under control and water stress condition based on biomass changing of callus size and weight

To illustrate the interaction effect of genotype and PEG treatment (water stress), 5 mm pieces of calli were inoculated in the MS medium supplemented with 0 g/L, 20 g/L, 40 g/L, 60 g/L and 80 g/L of PEG. Callus size and weight was the indicator of water stress tolerance. So, the callus diameter and weight were assayed three times after treating with different PEG concentration. Weight and diameter of each callus of each genotype were taken after 0 DAT (Days after treatment), 20 DAT, 40 DAT and 60 DAT with PEG. The callus growth was the highest in control condition (0 g of PEG) and at severe to more severe stresses gradually it decreases except with some genotypic variations as the water stress (PEG concentration) increases that is, in 20 g/L, 40 g/L, 60 g/L and 80 g/L. There

were also variations among the genotypes based on their stress period. Initially, the calli looked fresh and vigorous and it continues up to 20-40 DAT of stress period with some genotypic variation and almost all of them were whitish green (Plate 5 and Plate 6). At prolonged stress period (40 DAT to 60 DAT), they gradually became brown to dark brown and eventually died (Plate 7). Other researchers also evaluated the role of PEG on biomass growth of callus and found that, in the normal MS medium, biomass formation was very fast but when PEG used for changing the osmotic potential of the MS medium the biomass formation decreased (Aki, 2005). In this experiment callus diameter and fresh weight were measured up to 40 DAT under control and water stress condition 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 on genotypes

Significant genotypic variations were observed in the callus size and weight (Appendix IV). PEG treatment was given in every genotype and data were taken at 20 DAT and 40 DAT. The biggest size (diameter) of callus was found in the G3 (13.37 mm), G2 (12.27 mm) and G4 (12.15 mm) at 20 DAT and showed the same result up to 40 DAT (16.81 mm, 15.57 mm and 16.27 mm, respectively) (Table 2). The smallest size of callus was found in G1 (7.268 mm and 7.639 mm at 20 and 40 DAT, respectively). The heaviest callus was found in G2 and G4 for both at 20 DAT (1.24 g and 1.318 g, respectively) and at 40 DAT (1.895 g and 1.636 g, respectively). The lowest weight of callus was found in G1 and G5 (0.5124 g and 0.5146 g, respectively) at 20 DAT and only in G1 (0.5156 g) at 40 DAT (Table 2). Significant differences were also found among the genotypes regarding above traits by Rao and Jabeen (2013). Similar results were also found by Sakthivelu *et al*., 2008; Hassan *et al*., 2004. These genotypic

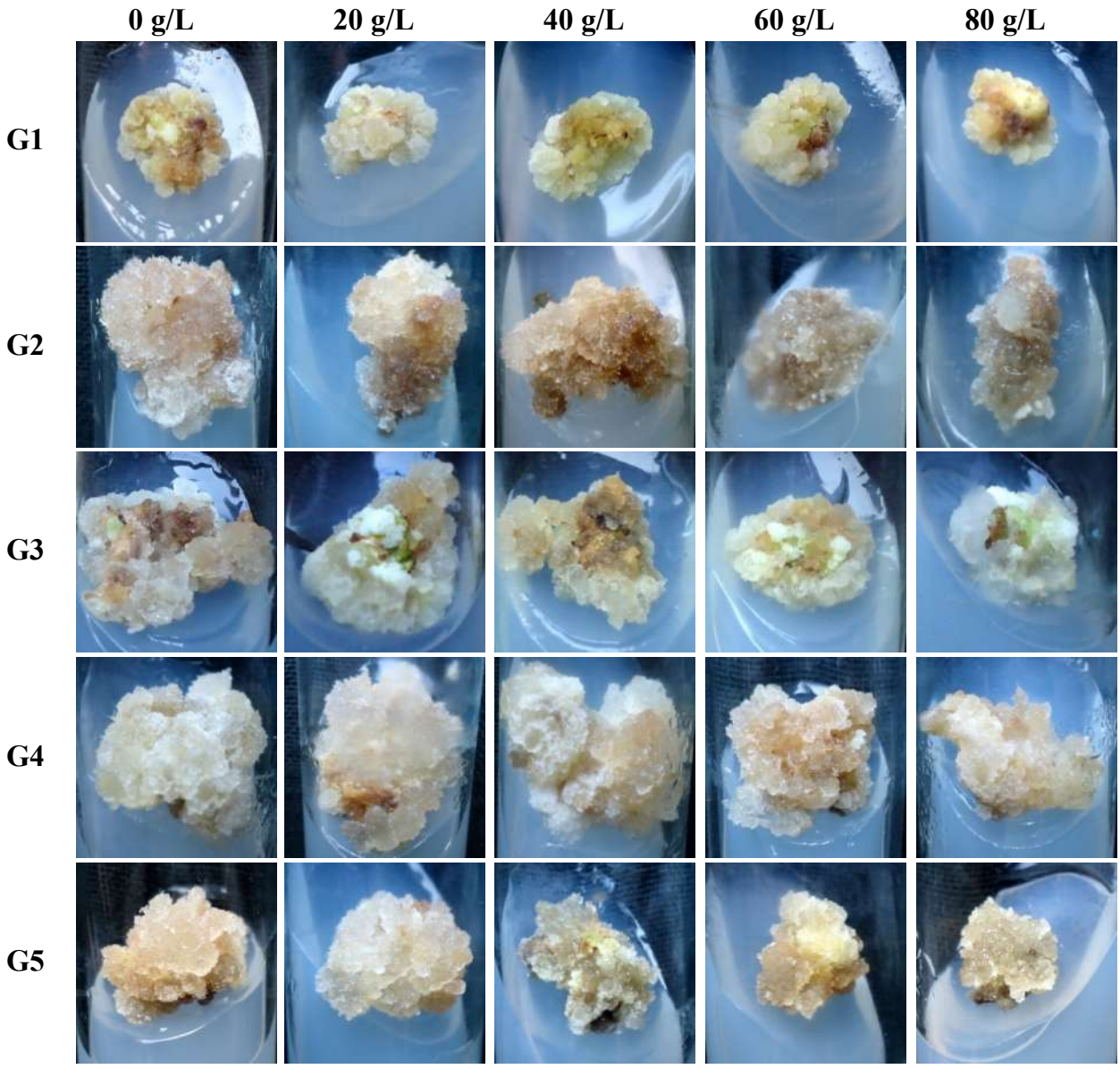

Plate 5. Biomass changing of callus at 20 DAT in different PEG concentrations (0 g/L, 20 g/L, 40 g/L, 60 g/L and 80 g/L)

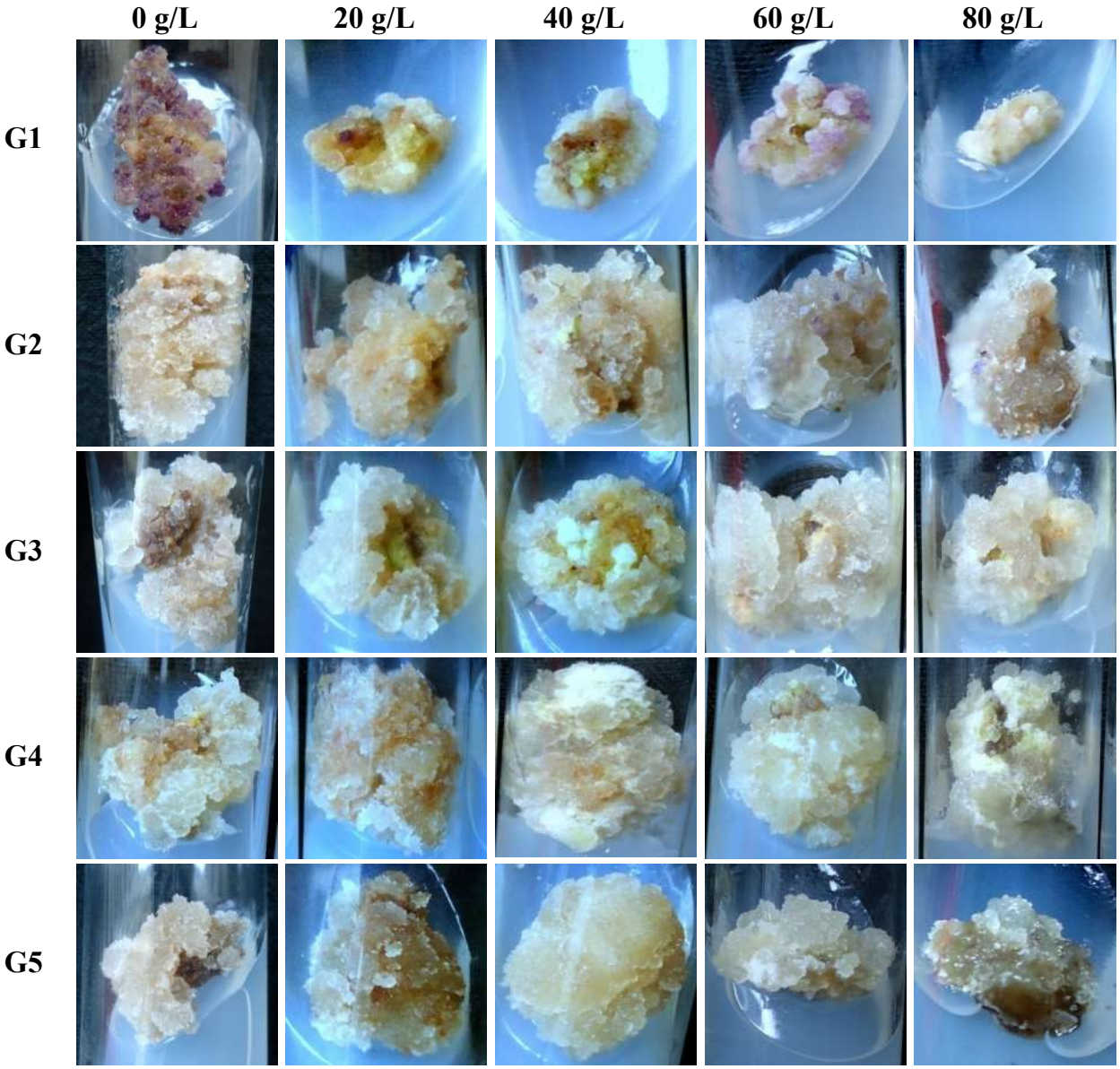

Plate 6. Biomass changing of callus at 40 DAT in different PEG concentrations (0 g/L, 20 g/L, 40 g/L, 60 g/L and 80 g/L)

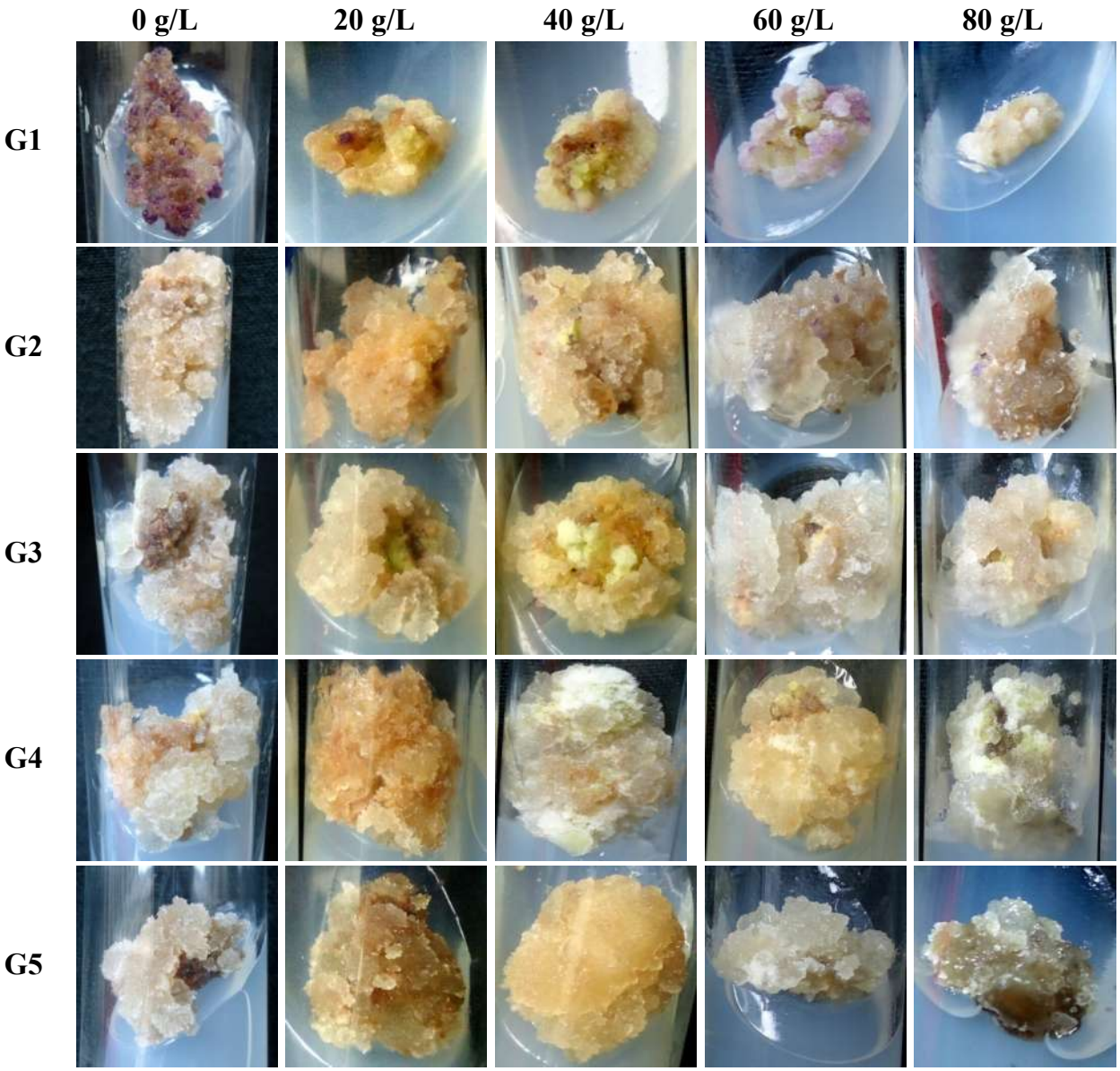

Plate 7. Biomass changing of callus at 60 DAT in different PEG concentrations (0 g/L, 20 g/L, 40 g/L, 60 g/L and 80 g/L)

Table 2. Biomass changing of callus in genotypes

Genotype	Biomass size (mm)			Biomass weight (g)		
	0 DAT	20 DAT	40 DAT	0 DAT	20 DAT	40 DAT
G1	5	7.268 c	7.639 c	0.1	0.5124 c	0.5156 c
G2	5	12.27 a	15.57 a	0.1	1.24 a	1.895 a
G3	5	13.37 a	16.81 a	0.1	0.9377 b	1.697 a
G4	5	12.15 a	16.27 a	0.1	1.318 a	1.636 a
G5	5	9.706 b	12.51 b	0.1	0.5146 c	0.8552 b
$LSD_{(0.5)}$	**NS**	**1.722**	**2.17**	**NS**	**0.2483**	**0.3206**

Table 3. Biomass changing of callus in treatments

Treatment	Biomass size (mm)			Biomass weight (g)		
	0 DAT	20 DAT	40 DAT	0 DAT	20 DAT	40 DAT
T1	5	11.04 b	14.39 a	0.1	0.9465 a	1.509 a
T2	5	13.26 a	15.38 a	0.1	1.066 a	1.451 a
T3	5	10.74 b	13.77 a	0.1	1.036 a	1.351 a
T4	5	10.86 b	13.85 a	0.1	1.03 a	1.333 a
T5	5	8.871 c	11.42 b	0.1	0.4439 b	0.9549 b
$LSD_{(0.5)}$	**NS**	**1.614**	**2.205**	**NS**	**0.2378**	**0.3584**

variation might be due to the induction or repression of many genes by water stress, involving a precise regulation of extensive stress-gene networks (Shinozaki and Yamaguchi, 2007; Delano *et al.*, 2011; Grativol *et al.*, 2012). Products of those genes might function in stress response and tolerance at the cellular level. Early plant response mechanisms prevent or alleviate cellular damage caused by the stress and re-establish homeostatic conditions and allow continuation of growth (Peleg *et al.*, 2011). Equilibrium recovery of the energetic, osmotic, and redox imbalances imposed by the stressor are the first targets of plant immediate responses.

4.2.2 Biomass changing of callus on PEG treatment

Involving the use of PEG in *in vitro* selection techniques is one of the reliable methods for screening desirable genotypes. Many researchers used PEG for selecting water stress tolerant genotypes (Kocheva *et al*., 2003; Sakthivelu *et al*., 2008). In this experiment different concentrations of PEG was used and the main effect of different concentrations of PEG showed significant variation on biomass changing of callus at different DAT. In all of the genotypes, 20 g (T2) PEG treatment showed the best callus size 13.26 mm, whereas 80 g (T5) PEG showed the smallest callus size at 20 DAT (8.871 mm) (Table 3). At 40 DAT, 80 g (T5) PEG showed the smallest callus (11.22 mm). The lowest biomass weight was observed by 80 g (T5) PEG treatment (0.4439 g and 0.9549 g at 20 DAT and at 40 DAT, respectively). Any increase in water stress levels in the media led to decrease of callus growth was also found by Rao and Jabeen (2013). Similar to this study, most of the researchers have used polyethylene glycol because of its high molecular weight and this feature of the molecules inhibits the entry to the plasmalemma and symplast with this way metabolic product does not affect directly (Cay and Yurekli, 1995). PEG induced water stress for callogenesis was also observed by Govindaraj *et al*. (2010); Corchete and Guerra (1986); Kaufman and Eckard (1971) and Ruf *et al.* (1967).

4.2.3 Biomass changing of callus on genotype × treatment interaction

The interaction effect of different chili genotypes and PEG concentrations showed significant variation on biomass changing of callus at different DAT (Table 4). 5 mm of calli were inoculated for all of the genotypes of chili under different PEG concentrations. At 20 DAT, the biggest callus was observed in G3T1 (17.50 mm) that is G3 treated with T1 (0 g PEG) at 20 DAT and the size continually increased (21.62 mm) up to 40 DAT. The smallest callus was found in G1T1 (5.15 mm) and G1T5 (6.14 mm) that is at control condition (0 g of PEG) the size was the lowest (5.15 mm) among all the genotypes. The callus size was gradually increased (8.88 mm) up to 60 g (T4) and then again decreased (6.14 mm) at severe stress (80 g PEG). It means G1 genotype is tolerant to water stress from low to severe water stress up to 20 DAT (Table 4). There was significant interaction effect between the genotypes and PEG concentrations on callus weight at 20 DAT and 40 DAT. 0.1 g was the initial weight for all the genotypes at all PEG concentrations. G3T1 also showed the highest weight (2.61 g) of callus at severe stress (80 g of PEG). G1 genotype was the most tolerant genotype as the callus weight was almost similar in control (0.43 g) as well as in stress conditions (0.43 g, 0.58 g, 0.72 g and 0.42 g at 20 g, 40 g, 60 g and 80 g of PEG, respectively) at 40 DAT. The addition of PEG to the culture media decreased the osmotic potential of the media inducing water stress that adversely affected the callus growth. Several authors reported the use of PEG for *in vitro* drought screening in different plants (Joshi *et al*., 2011; Zhao *et al*., 2007; Aki *et al*., 2005). In this study, the adaption capacity to different PEG level varies with degree of tolerance of the genotypes. The genotypic variation for water stress tolerance in this study agreed with the result of Joshi *et al.* (2011) and Nath *et al.* (2005). In this study callus grown with the increased PEG concentrations reduce the relative growth rate in all *Capsicum* genotypes as in the study of Khodarahmpour (2011), Govindaraj *et al*. (2010) and Sakthivelu *et al*. (2008). *In vitro* plant tissue culture is useful and quick tool to evaluate plant

Table 4. Interaction effect of genotype and treatment

Interaction	Biomass size (mm)			Biomass weight (g)		
	0 DAT	20 DAT	40 DAT	0 DAT	20 DAT	40 DAT
G1T1	5.00	5.15 l	7.32 no	0.10	0.22 j	0.43 k
G1T2	5.00	8.43 ij	6.45 o	0.10	0.60 hi	0.43 k
G1T3	5.00	7.74 j	8.15 mno	0.10	0.75 gh	0.58 jk
G1T4	5.00	8.88 ij	9.32 lm	0.10	0.74 gh	0.72 jk
G1T5	5.00	6.14 l	6.97 no	0.10	0.25 j	0.42 k
G2T1	5.00	12.83 de	16.92 cde	0.10	1.68 b	2.31 b
G2T2	5.00	13.99 cd	16.98 cde	0.10	1.27 cd	2.01 cd
G2T3	5.00	12.37 ef	16.07 def	0.10	1.23 cd	1.85 de
G2T4	5.00	10.90 g	13.99 hi	0.10	0.89 fg	1.49 fg
G2T5	5.00	11.27 fg	13.90 hi	0.10	1.13 de	1.82 de
G3T1	5.00	17.50 a	21.62 a	0.10	1.83 b	2.61 a
G3T2	5.00	15.41 b	17.59 bcd	0.10	1.02 ef	1.62 ef
G3T3	5.00	11.38 fg	14.67 fgh	0.10	0.78 gh	1.29 gh
G3T4	5.00	13.75 cd	18.33 bc	0.10	0.93 fg	1.73 def
G3T5	5.00	8.82 ij	11.86 jk	0.10	0.14 j	1.24 gh
G4T1	5.00	13.44 cde	17.38 bcd	0.10	0.78 gh	1.51 fg
G4T2	5.00	14.37 bc	18.96 b	0.10	1.80 b	2.18 bc
G4T3	5.00	10.99 fg	15.81 defg	0.10	1.41 c	1.81 de
G4T4	5.00	11.36 fg	15.20 efgh	0.10	2.11 a	1.88 de
G4T5	5.00	10.61 gh	14.02 hi	0.10	0.48 i	0.80 ij
G5T1	5.00	6.26 kl	8.68 lmn	0.10	0.22 j	0.69 jk
G5T2	5.00	14.12 bcd	16.93 cde	0.10	0.64 hi	1.01 hi
G5T3	5.00	11.20 fg	14.18 ghi	0.10	1.01 ef	1.23 gh
G5T4	5.00	9.44 hi	12.41 ij	0.10	0.49 i	0.85 ij
G5T5	5.00	7.51 jk	10.34 kl	0.10	0.22 j	0.50 k
Lsd $_{(0.5)}$	**NS**	**1.25**	**1.60**	**NS**	**0.18**	**0.26**

G1= Genotype 1, G2= Genotype 2, G3= Genotype 3, G4= Genotype 4, G5= Genotype 5, T1= 0g, T2= 20g, T3= 40g, T4= 60g, T5= 80g of PEG

tolerance to water stress. In this study, we found decreased callus induction potential with increasing PEG levels. A similar observation was found by Rao and Jabeen (2013) and Hassan *et al.* (2004) using tissue culture techniques for *in vitro* selection for drought tolerance. Results of this study indicate that biomass changing in five genotypes have shown differences in *in vitro* conditions might also be due to the differences in their endogenous hormone levels. Aki *et al*. (2005) also gave similar observation in his study.

4.2.4 Biomass changing of callus size under water stress

Size of callus of five genotypes was recorded for 20 days and 40 days after treatment (DAT) in different PEG concentration and significant differences were recorded (Plate 5, Plate 6 and Plate 7 and Figure 1). The genotype G3 showed the highest biomass size in control condition (0 g PEG) from 20 DAT to 40 Days and remains constant up to 60 days (Plate 6 and 7). Genotype G5 showed increased size of callus up to 60 g PEG until 40 DAT and remains constant up to 60 DAT (Plate 7). Under stress condition G3 gradually decreased in size up to 40 g of PEG but at severe stress at 60 g of PEG it recovered slightly. It might be due to the adaptation capacity of the genotype. Similar result was observed by Bray *et al*. (2000) in *Mesembryanthemum crystallinum* of MIP-related genes where turgor was lost at early stage of water stress but later it recovered. Genotype G1 showed constant tolerance up to 60 g of PEG until 40 DAT. The Genotype G2 and G4 showed tolerance at low water stress (20 g PEG) and then gradually lost their tolerance at severe stress. Genotypic variation for biomass size is evident in control (0 g PEG) and in stressed condition (20 g, 40 g, 60 g and 80 g) (Figure 1). Up to 20 DAT (days after treatment) callus size was not reduced at all at low water stress (20 g PEG) but increased in case of G1, G2, G4 and G5 (-3.28 mm, -1.16 mm, -0.93 mm and -7.86 mm, respectively) (Table 5). At moderate stress that is at 20-40 g of PEG, all of the genotypes reduced in their callus size but G1 showed the lowest reduction in size of callus (0.69 mm). But later, that is at moderate to

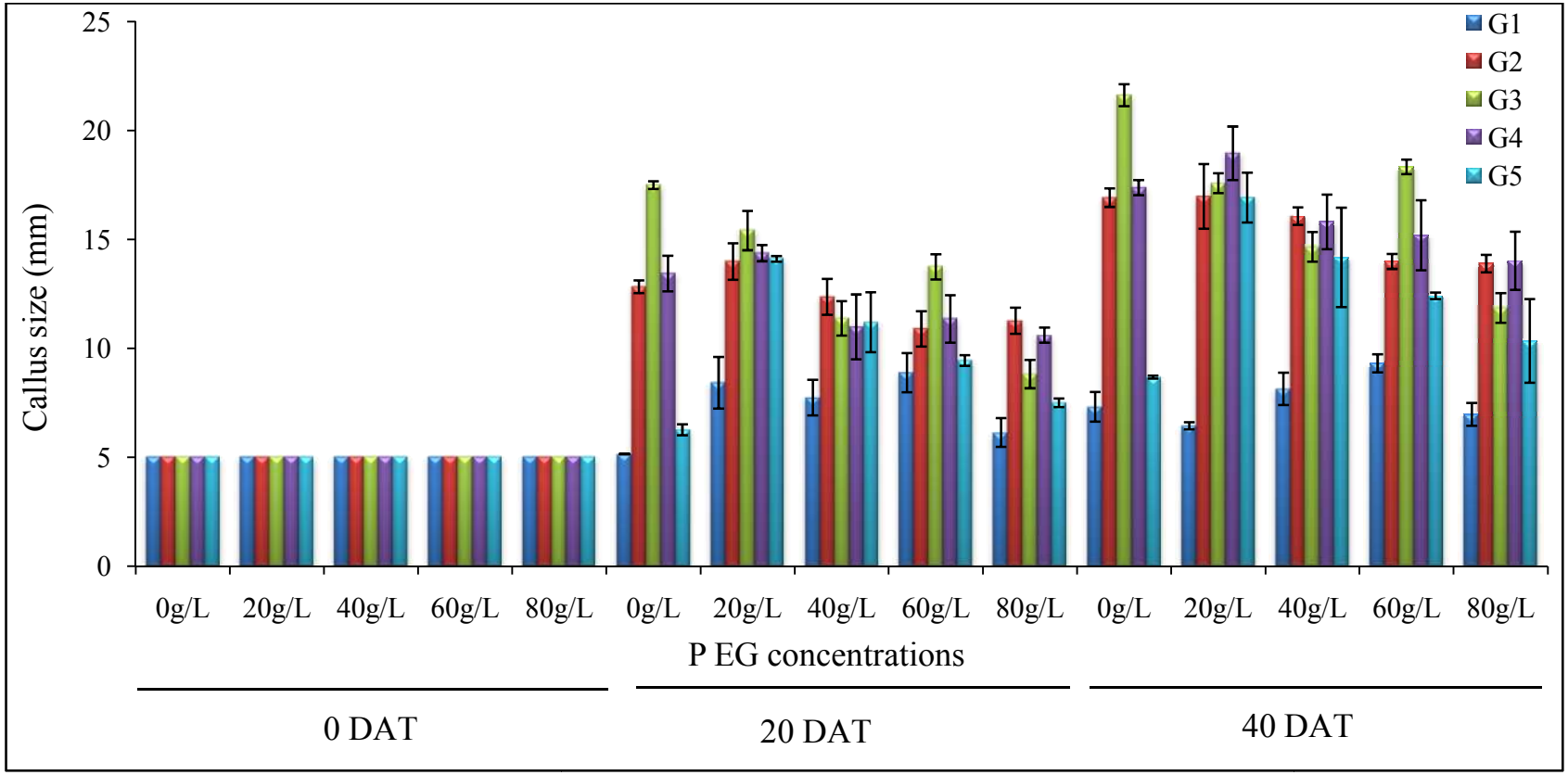

Figure 1. Size (diameter) of callus of five genotypes at 0 DAT, 20 DAT and 40 DAT in different PEG concentrations (0 g/L, 20 g/L, 40 g/L, 60 g/L and 80 g/L)

high water stress (40-60 g PEG), some of the genotypes recovered such as G1, G3 and G4 showed negative reduction in callus size (-1.14 mm, -2.37 mm and -0.37 mm). At prolonged stress period that is at 40 DAT, G1 and G3 showed the lowest reduction (negative) in callus size i.e., -1.17 mm and -3.66 mm at moderate to high PEG level (40-60 g PEG) and thus showing tolerance.

4.2.5 Biomass changing of callus weight under water stress

Biomass weight of calli was recorded in control (0 g PEG) and in water stress condition (20 g, 40 g, 60 g and 80 g PEG) at 0 DAT, 20 DAT and 40 DAT. Significant genotypic variation for biomass weight was recorded (Plate 5, Plate 6 and Plate 7 and Figure 2). The callus of genotype G3 showed the highest weight at 20 DAT under control condition (0 g PEG) and it gradually increased up to 40 DAT and remains fresh. The weight of G3 remain constant up to 60 DAT but gradually loss their totypotency and turned brown to dark brown and gradually died. Genotype G1 showed constant tolerance (increasing weight) up to 60 g of PEG until 40 DAT (Figure 2) and without losing weight gradually turned brown to dark brown (plate 6). G4 also showed better performance from control (0 g PEG) to 60 g PEG up to 40 DAT. G5 also increased weight up to 40 g of PEG and then decreased (Figure 2). This situation remained constant up to 40 DAT to 20 DAT (days after treatment). Callus weight was not reduced at all at low water stress (20 g PEG) but increased in case of G1, G4 and G5 (-0.37 g, -1.02 g and -0.42 g respectively) (Table 6). At moderate stress that is at 20-40 g of PEG, G1 and G5 did not reduced at all but increased in weight as the callus weight reduction is negative (-0.15 g and -0.37 g). Later, that is at moderate to high water stress (40-60 g PEG), some of the genotypes recovered such as G3 and G4 showed negative reduction in callus weight (-0.15 g and -0.70 g) that is they recovered and increased in weight. At prolonged stress period that is at 40 DAT, G1, G3 and G4 showed the tolerance at severe stress (40 g to 60 g PEG) and showed the negative reduction in callus weight i.e., - 0.44 g and -0.07 g .

Table 5. Reduction of callus size from low to severe water stress

Callus size reduction (mm)								
Genotype	20 DAT				40 DAT			
	0g-20g	20g-40g	40g-60g	60g-80g	0g-20g	20g-40g	40g-60g	60g-80g
G1	-3.28	0.69	-1.14	2.74	0.87	-1.7	-1.17	2.35
G2	-1.16	1.62	1.47	-0.37	-0.06	0.91	2.08	0.09
G3	2.09	4.03	-2.37	4.93	4.03	2.92	-3.66	6.47
G4	-0.93	3.38	-0.37	0.75	-1.58	3.15	0.61	1.18
G5	-7.86	2.92	1.76	1.93	-8.25	2.75	1.77	2.07

Table 6. Reduction of callus weight from low to severe water stress

Callus weight reduction (g)								
Genotype	20 DAT				40 DAT			
	0g-20g	20g-40g	40g-60g	60g-80g	0g-20g	20g-40g	40g-60g	60g-80g
G1	-0.37	-0.15	0.02	0.49	-0.01	-0.14	-0.14	0.29
G2	0.41	0.04	0.34	-0.24	0.30	0.16	0.36	-0.33
G3	0.81	0.24	-0.15	0.79	0.99	0.33	-0.44	0.49
G4	-1.02	0.39	-0.70	1.63	-0.67	0.37	-0.07	1.08
G5	-0.42	-0.37	0.52	0.27	-0.32	-0.22	0.38	0.35

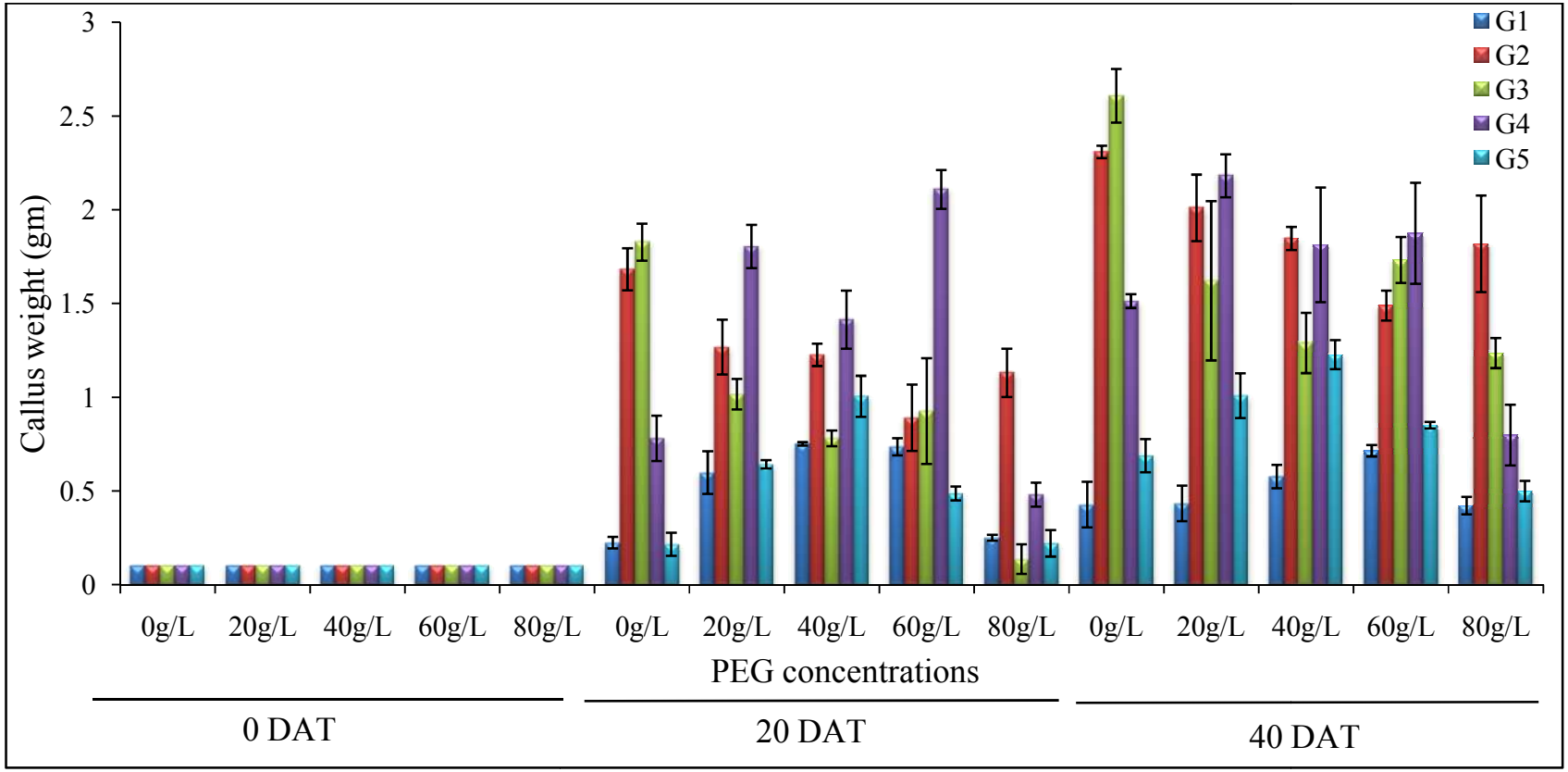

Figure 2. Weight of callus of five genotypes at 0 DAT, 20 DAT and 40 DAT in different PEG concentrations (0 g/L, 20 g/L, 40 g/L, 60 g/L and 80 g/L)

4.3 Performance of different genotypes under control and water stress condition based on proline accumulation

Proline is one of the well-known osmoprotectants and its accumulation is widely observed in various organisms under abiotic stress including salt stress. Proline accumulation is regulated by multiple factors, such as its synthesis, catabolism, utilization for protein synthesis and transport from other tissues. Improved accumulation of proline conferred salt and drought tolerance and this result supported that proline functions to regulate cellular osmotic balance (Kishore *et al*., 1995). Additionally, proline is also utilized for protein synthesis. Proline serves to regulate osmotic adjustment. In this experiment proline was extracted from control and water stressed calli and significant variations were observed in different genotypes (Plate 8, Figure 3). The proline content in the calli of different genotypes under control and stressed condition are presented in (Figure 3). Proline content gradually increased as the increase of stress from 0 g/L to 80 g/L of PEG. G1 showed the highest proline content as compared to the other genotypes under control as well as under stress condition (Figure 3). As the proline content is not equal in all genotypes under control condition, a comparative increase of proline from control to different stress condition was estimated (Table 7). The proline content increased in case of G3 gradually from low to moderate to severe stress (Table 7).

Considering that proline accumulation is associated with stress tolerance, that core enzymes regulate proline biosynthesis, and that these core enzymes are likely rate-limiting steps for its accumulation, logic dictates that overexpression of biosynthetic proline enzymes might increase the levels of the compatible solute and thus improve the tolerance in plants against abiotic stress. Several studies have tested this by overexpressing genes for P5CS or P5C enzymes in different plant species, reporting the expected rise in proline levels and the associated resistance

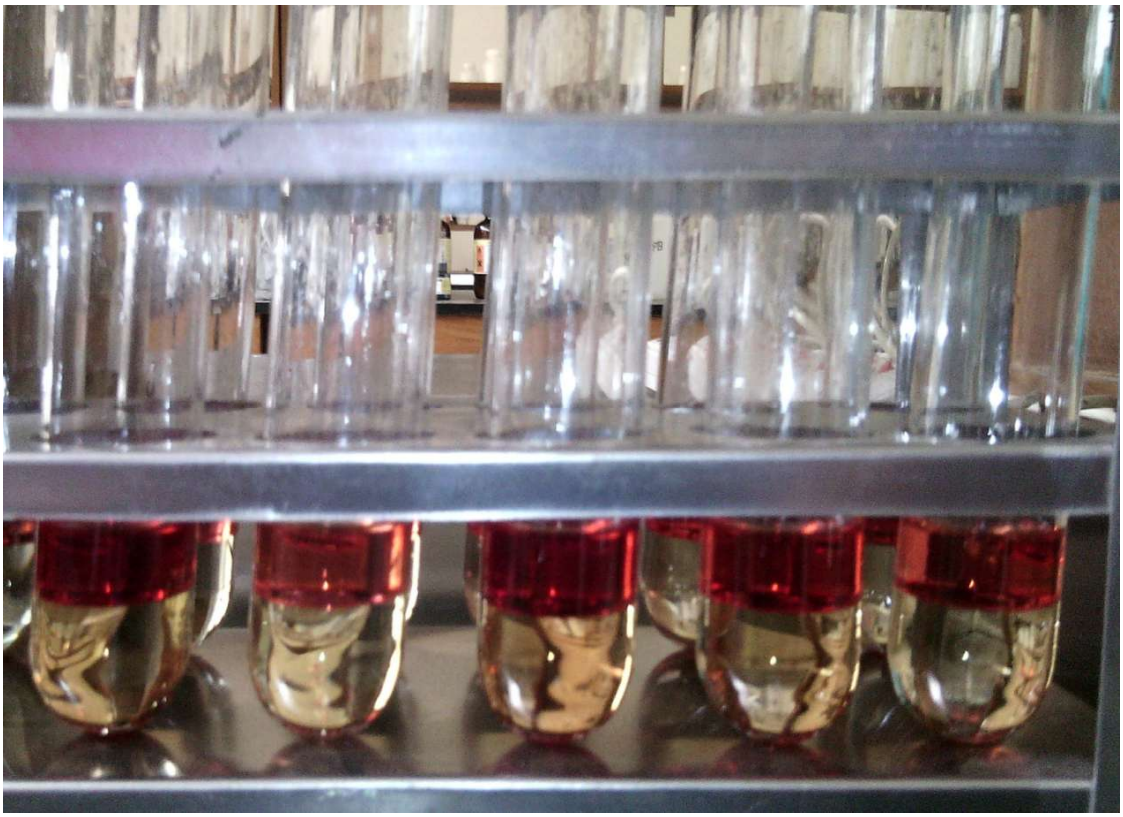

Plate 8. Variation in proline content in different genotypes. Light red colour indicates less proline and deep red indicates high proline content in the respective callus

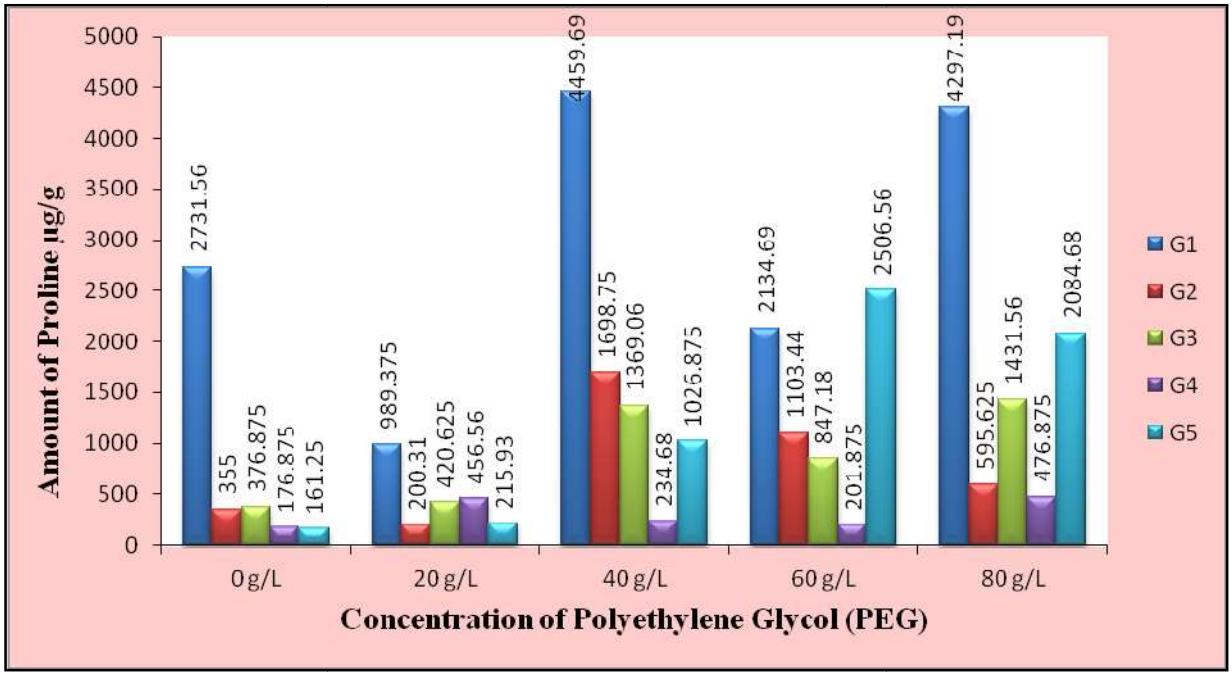

Figure 3. Proline content in control and stressed condition

Table 7. Increase of proline content from low to severe water stress

Proline increase (µg/g)				
Genotype	**20g-0g**	**40g-20g**	**60g-40g**	**80g-60g**
G1	-1742	3470.3	-2325	2162.5
G2	-154.7	1498.4	-595.3	-507.82
G3	43.75	948.44	-521.9	584.38
G4	279.69	-221.9	-32.81	275
G5	54.68	810.95	1479.7	-421.88

to dehydration, salinity, or freezing (Kishor *et al.*, 1995; Zhu *et al.*, 1998; Sawahel and Hassan, 2002; Su and Wu, 2004; Parvanova, 2004; Gleeson *et al.*, 2005; Yamada *et al.*, 2005; Vendruscolo *et al.*, 2007).

SUMMARY AND CONCLUSION

Drought is one of the major stress factors among the abiotic stresses. Water stress affects almost every aspect of the physiology, biochemistry and genetics of plants and significantly reduces yield. As drought is common around the world, great effort has been devoted to understanding physiological aspects of tolerance to drought in plants, as a basis for plant breeders to develop drought-tolerant genotypes. In spite of this great effort, only a small number of cultivars, partially tolerant to drought, have been developed. Further effort is necessary if the exploitation of drought stressed soils and the waters that are not currently usable e.g, saline water are to be achieved. Drought affects yield and quality, so that yield characters must be taken into account when breeding for drought tolerance. But not only yield-related characters are important. As drought affects almost every aspect of the physiology and biochemistry of the plant, the enhancement of crop drought tolerance will require the combination of several to many physiological traits not simply those directly influencing yield. As drought in soils is variable and plant tolerance depends on the stage of plant development, plants should be phenotyped at several water stress concentrations and at the most sensitive plant stage(s). A screening of *Capsicum* genotypes for *in vitro* explants source was performed in this study based on their germination. Most of them were collected from Bangladesh Agricultural Research Institute (BARI). Germination test eliminated five genotype of *Capsicum* and the best five germinated genotypes of *Capsicum* were selected for explants source of this experiment. The cotyledonary leaves and nodal segment of three weeks old seedlings were taken as explants. Calli were developed from these explants using modified MS medium supplemented with different concentrations of hormones. The calli were then cut into pieces and inoculated into callus induction medium supplemented with 0g/L, 20g/L, 40g/L, 60g/L and 80g/L of PEG. PEG prevent cell from taking water from

media. Evaluation of response of these calli in low to severe stresses has been carried out in different duration. The environment was controlled with 25^{o}C, 60% relative humidity, and a 16 h photoperiod from white fluorescent lamps (50 µmol photons-m^{-2}-s^{-1}). Drought tolerance assay was performed by estimation of callus size and weight as indicators of tolerance response of these genotypes. Under stress condition G3 gradually decreased in size and weight up to 40 g of PEG at 20 DAT but at severe stress at 60 g of PEG it recovered. It might be due to the adaptation capacity of the genotype which indicates tolerance to water stress. This result correlates to the proline accumulation i.e., at low stress (20 g/L PEG) the proline accumulation decreased but later it increased at high stress indicated the function of proline in tolerance to drought. At prolonged stress period, G1 and G4 showed better performance at severe stress period which correlates with the accumulation of proline.

In this experiment disease free drought tolerant healthy calli were produced. The hypocotyls and cotyledon calli grew well in the basal MS medium supplemented with 2 mg/L NAA, 0.5 mg/L KIN and 5 mg/L 2,4-D for callus formation. The genotypes G1, G3 and G4 showed tolerance to water stress condition at different duration as the biomass changing of calli in *Capsicum* was comparatively better than those of other genotypes under stress condition. G3 could be selected for drought tolerance for short duration stress period (20 days) and G1 and G4 could be selected for prolonged drought stress (40 days). It indicates the expression of functional gene occurs at high water stress in case of G1 and G4. Proline content gradually increased as the PEG concentration increased except some variation. Proline accumulation continuously increased in G3 up to 60 g/L of proline which supports the increased callus size and weight in G3.

The regeneration of water stress tolerant calli and field trial is quiet necessary for development of drought tolerant lines. Gene expression pattern of previously reported genes such as PIP, LTPs, APETALA2 GRP, Arabinogalactan protein,

PRP, Glyceraldehyde 3-phosphate dehydrogenase etc. is necessary for confirming that the tolerance is due to the action of gene rather than environment which will give stability to the genotype. Genes who will express could play a protective role under stress condition. So, further experiment by RT-PCR or Northern hybridization is required. Hybridization and genetic transformation are the powerful tools in the breeding of complex characters, such as tolerance to drought, if progress is made on the identification of genes involved in the process of drought tolerance.

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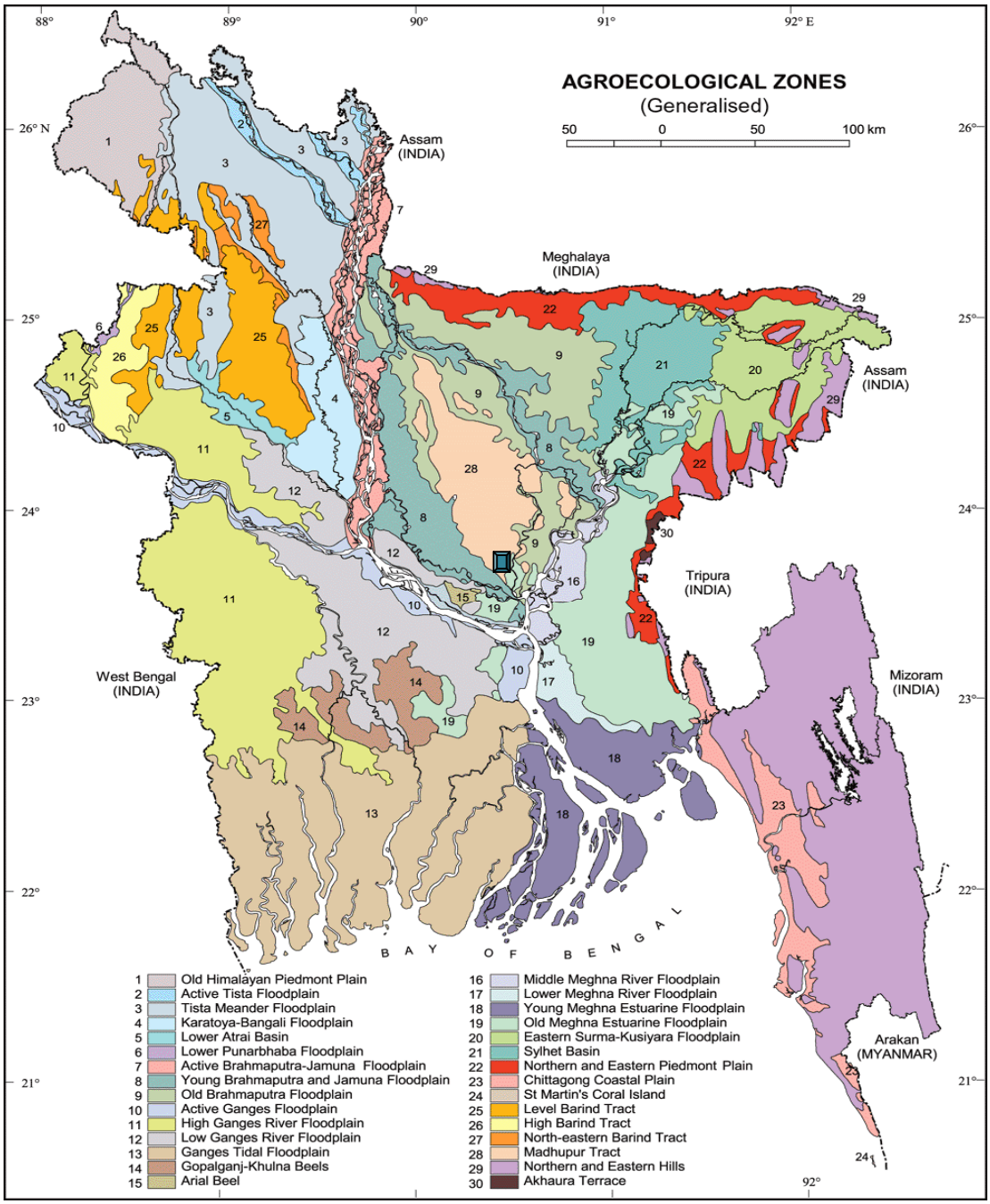
APPENDICES

Appendix I. Map showing the experimental site under the study



The experimental site under study

Appendix II. List of ten *Capsicum* genotypes with their accession number, source of collection and germination percentage

Sl. No.	Accession No.	Source of collection	Germination percentage (%)
1.	AC 520	SRC, Bogra	05
2.	AC 612	SRC, Bogra	35
3.	BARI-1	BARI, Dhaka	0
4.	C0 525	SRC, Bogra	90
5.	CO 611	SRC, Bogra	85
6.	HP 1001	SRC, Bogra	0
7.	SRC 02	SRC, Bogra	100
8.	SRC 05	SRC, Bogra	95
9.	SRC 07	SRC, Bogra	45
10.	SRC 14	SRC, Bogra	100

Appendix III. **Murashige and Skoog (1962) medium salts**

Components		Concentrations (mg/L)
Macronutrients or Major salts	Potassium nitrate (KNO_3)	1900
	Ammonium nitrate (NH_4NO_3)	1650
	Magnesium sulfateheptahydrate ($MgSO_4.7H_2O$)	370
	Calcium chloride (anhydrous) ($CaCl_2$)	332.20
	Potassium phosphate (KH_2PO_4)	170
Micronutrients or Minor salts	Boric acid (H_3BO_3)	6.2
	Manganese sulphate tetrahydrate ($MnSO_4.4H_2O$)	22.3
	Zinc sulfate tetrahydrate ($ZnSO_4.4H_2O$)	8.6
	Potassium iodide (KI)	0.83
	Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)	0.25
	Cobalt chloride hexahydrate ($CoCl_2.6H_2O$)	0.025
	Cupric sulfate pentahydrate ($CuSO_4.5H_2O$)	0.025
Iron Sources	Ferrous sulfate heptahydrate ($FeSO_4.7H_2O$)	27.80
	Disodium EDTA dihydrate ($Na_2EDTA.2H_2O$)	37.260
Vitamins	Thiamine-HCl	0.1
	Nicotinic acid	0.5
	Pyrodoxine-HCl	0.5
Sucrose		3000.00
Agar		8000.00

Appendix IV: Analysis of variance of the data on callus size and callus weight.

Source of variation	Degrees of freedom (df)	Mean Sum of Square					
		Size (mm)			Weight (g)		
		0 DAT	20 DAT	40 DAT	0 DAT	20 DAT	40 DAT
Factor A (genotype)	4	0.000^{NS}	90.629*	217.346*	0.000^{NS}	2.212*	5.384*
Factor B (PEG treatment)	4	0.000^{NS}	36.484*	31.911*	0.000^{NS}	1.023*	0.702*
A×B	16	0.000^{NS}	11.779*	15.678*	0.000^{NS}	0.561*	0.398*
Error	50	1.000	0.577	0.946	1.000	0.012	0.025

* Significant at 0.01 level of probability, ^{NS} Non significant

Appendix V. Proline standard curve