THE EFFECT OF SALINITY ON GROWTH AND ACCUMULATION OF PROLINE IN CALLI OF *Capsicum* spp. GROWN *IN VITRO*

BY

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CERTIFICATE

This is to certify that thesis entitled, "The effect of salinity on growth and accumulation of proline in calli of Capsicum spp. grown in vitro" 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 Mr. Md. Rayhanul Islam, Registration No. 08-02733 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

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DEDICATED

TO

MY BELOVED PARENTS

Some commonly used abbreviations

Full Word	Abbreviation	Full Word	Abbreviation
2,4-Dichlorophenoxy acetic acid	2,4-D	Micro mole	µM
Abscisic Acid	ABA	Microgram per gram	µg/g
Agricultural	*Agril.*	Micromole per square meter per second	$\mu mol\text{-}m^{-2}\text{-}s^{-1}$
And others (at elli)	*et al.*	Mili mole	mM
As for example	e.g.	Molar	M
Benzyle Adenine	BA	Murashige and Skoog	MS
Benzyle Amino Purine	BAP	Nanometer	Nm
Biology	*Biol.*	Naphthalene Acetic Acid	NAA
Biotechnology	*Biotechnol.*	Negative logarithm of hydrogen ion concentration ($-\log[H^+]$)	pH
Botany	*Bot.*	Newsletter	*Newsl.*
Continued	Cont'd	Normal	N
Cultivar	cv.	Particular pages	pp.
Culture	*Cult.*	Parts per million	ppm
Days After Treatment	DAT	Percent	%
Degree (Latitude and longitude)	°	Physiology	*Physiol.*
Degree Celsius	°C	Plant growth regulator (s)	PGR(s)
Etcetera	etc.	Potassium chloride	KCl
Food and Agriculture Organization	FAO	Potassium ion	K^+
Gibbrelic Acid	GA_3	Pounds per square inch	Psi
Gram per Liter	g/L	Proceeding	*proc.*
Hectare	Ha	Publication	pub.
Horticulture	*Hort.*	Reports	Rep.
Hour	h	Research	Res.
Indole-3- Butyric Acid	IBA	Science	*Sci.*
Indole-3-Acetic Acid	IAA	Sodium chloride	NaCl
International	*Int.*	Species (plural)	spp.
Journal	*J.*	Spices Research Center	SRC
Kilometer	Km	Thidiazuron	TDZ
Kinetin	Kin or KIN	Ultra Violet	UV
Liter	L	Weight / Volume	w/v

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The Author

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THE EFFECT OF SALINITY ON GROWTH AND ACCUMULATION OF PROLINE IN CALLI OF *Capsicum* spp. GROWN *IN VITRO*

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ABSTRACT

The effect of NaCl salinity on growth and development of calli of *Capsicum* spp. was examined *in vitro*. Five genotypes of *Capsicum* were used in this study. The seeds were surface sterilized with absolute alcohol followed by 0.1% $HgCl_2$. The seeds were sown on the surface of hormone free MS (Murashige and Skoog) medium, solidified with 0.8% of agar and 3% of sucrose. The cultures of the seeds germinated initially at dark at 27°±1°C for ten days and then in light under 16 h photoperiod at 25°±2°C. Cotyledonary leaf and nodal segment explants from four weeks old seedlings were excised approximately 0.5 cm length. These explants were cultured on MS basal medium supplemented with 2 mg/L NAA, 0.5 mg/L KIN and 5 mg/L 2,4-D for callus formation. Addition of 50 mM, 100 mM, 150 mM, 200 mM and 200 mM of NaCl to the medium showed a negative effect on the growth and development of calli with some variation. Gradual increase of concentration of NaCl provoked a significant reduction of the calli size and weight to most of the genotypes, though there were some exceptions. Significant genotypic variation in tolerance was observed among the genotypes. The genotype G_3 showed better totipotency under salt stress and remained totipotent from lower to severe stresses. Genotype G_2 and G_4 showed better tolerance to salt stress at different duration as the biomass changing of calli in size and weight was comparatively better than those of other genotypes under stress condition. The proline accumulation in calli were evaluated and found that they correspond with the totipotency of genotype G_3 as well as size and growth of calli of genotype G_2 and G_4. These results suggest that genotype G_2, G_3 and G_4 have the potentiality to tolerate salt stress without their development being affected and could be selected for further regeneration.

Chapter I
Introduction

CHAPTER I

INTRODUCTION

World population is growing at an alarming rate and is anticipated to reach about between 8.3 and 10.9 billion by the end of year 2050 (Anonymous, 2013). On the other hand, agricultural productivity is not increasing at a required rate to keep up with the food demand. The reasons for these are water shortages, depleting soil fertility and mainly various abiotic and biotic stresses. Now, various abiotic and biotic stresses are the major area of concern for the fulfillment of the increasing food requirements (Shanker and Venkateswarlu, 2011).

Salinity is one of the abiotic stress factors, negatively affecting plant growth and development processes (Sengupta and Majumder, 2009; Zadeh and Naeini, 2007; Neumann, 1995), namely, physiological, morphological and biochemical processes, including seed germination, plant growth, water and nutrient uptake (Willenborg *et al.*, 2004) and thereby create a serious trouble in agricultural productivity, especially to the land located in sea costal and semi-arid zones. About 20-30 percent of the land losing arability due to salinity (FAO, 2002) and drastically reduce the agricultural productivity, worldwide every years (Gorai and Neffati, 2007). A considerable amount of land in the world is affected by salinity which is increasing day by day. More than 45 million hectares of irrigated land which account to 20 percent of total land have been damaged by salt worldwide and 1.5 million ha are taken out of production each year due to high salinity levels in the soil (Munns and Tester, 2008).

Bangladesh is a country with total area of 147570 km^2 of which major part (80%) consists of alluvial sediments deposited by the rivers Ganges, Brahmaputra, Tista, Jamuna, Meghna and their confluents. The coastal region covers almost 29000 km^2 which is about 20 percent of the total land and more than 30 percent of the cultivable lands of the country. Almost 98 percent of the coastal areas are coved by tidal and estuarine floodplains and small areas about 2

percent with river floodplains and peat basins are found in the northern part of the coastal area. Tidal floodplains alone covers 65 percent (1865000 ha) of the coastal area occur in Satkhira, Khulna, Bagerhat, Pirozpur, Jhalukhati, Barisal, Patuakhali, Chittagong and Cox's Bazar districts. The estuarine floodplains occur in Noakhali, Bhola and Patuakhali districts and in the north-western part of Chittagong district which cover about 33 percent (937000 ha) of the coastal area. About 53 percent of the coastal areas are affected by salinity problem, thereby agricultural land use efficiency of these areas become very low, which causes lowering countrys' average cropping intensity (Haque, 2006).

Plant has capability to regulate salt accumulation (Munns and Tester, 2008). Thus development of crops tolerant to salinity is a great challenge for plant breeder to increase cropping intensity as well as yields by taking marginal zones under cultivation (Turkan and Demiral, 2009; Queiros *et al.*, 2007; Davenport *et al.*, 2003).

Capsicum is one of the most important Solanaceous horticultural crops in the world. *Capsicum* is derived from the Greek word "Kapsimo" meaning 'to bite' (Basu and De, 2003). Capsium terminology is quite confusing; therefore, pepper, chili, chile, chilli, aji and paprika are synonymously used for '*Capsicum*' plants. *Capsicum* is a favorable food crop for *in vitro* 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. *Capsicum* originated in the New World, where at least 25-30 species have been reported among them five namely *C. annuum* L, *C. frutescens* Mill., *C. baccatum* L., *C. chinense* and *C. pubescens* Ruiz and Pavon have been domesticated and is widely cultivated for its fruits which have a recognized nutritional value (Csillery, 2006). In fact, they are an excellent source of various antioxidant compounds like flavonoids, carotenoids and vitamin C, A, B-complex and E along with different minerals like, manganese, potassium, molybdenum, folate, and thiamine (Kothari *et al.*, 2010; Chuah *et al.*, 2008). *Capsicum* contains seven times more vitamin-C than Orange

(Kothari *et al.*, 2010). Vitamins-C, A and Beta-carotenoids, present in chillies are powerful antioxidants that ruin free radicals and these later protects human body against oxidative damage and prevents various diseases such as cancer and cardiovascular diseases (Oboth and Rocha, 2007; Simonne *et al.*, 1997). The brilliant colors found in *Capsicum* are due to the mixture of esters of capsanthin, capsorubin, zeaxanthine, crytoxanthine and other carotenoids. These extractible colors of fruits are broadly used in the food processing industry to wide range of products such as sausages and meat products, as well as for cheeses, butters, salad dressings, condiment mixtures, gelatine desserts, coloring, flavoring of garnishes, pickles, barbecue sauces, ketchup, snack food, chili con came, curry powder tabasco sauce etc. (Govindarajan, 1986). The curative properties and pungency presented in *Capsicum* is assigned to a group of alkaloids known as 'Capsaicinoids' (Ochoa-Alejo and Ramirez-Malagon, 2001). The pepperiness of the chilli is mainly due to presence of Capsaicin ($C_{18}H_{27}O_3N$), (measure with Scoville scale) which is a produced by condensation of 3-hydroxy, 4-methoxy benzyl amino and decylenic acid. As a medicine it is used as a counter irritant in lumbago, neuralgia, rheumatic disorders and non-allergic rhinitis. It has also a tonic and carminative action (Kothari *et al.*, 2010). In combination with bark it is employed in sporadic and lethargic difficulties and also for gout, indigestion accompanied by flatulence, tympanitis and paralysis. Its most worthful application appears in cynanche maligna and scarlatina maligna. The plants have also been used remedies for hydrops, colic, diarrhoea, asthma, arthritis, muscle cramps and toothache (Ravishankar *et al.*, 2003). Red or hot peppers from *Capsicum annuum* L. and *Capsicum frutescens* L. are the most pungent peppers and are used extensively in Bangladeshi, Mexican and Italian foods.

Although *Capsicum* spp. have economic importance in the aspect of Bangladesh, but they faced problem with salt stress conditions in some parts of Bangladesh. By using conventional selection and breeding methods, improvements in the salinity tolerance of some agriculturally important plants have been attained (Ashraf and Harris, 2004), but conventional breeding methods have little success and some time failed to achieve desirable results (Purohit *et al.*, 1998). *In vitro*

culture has come forth as a useful technique for the study of salt stress as well as other stresses. In recent years *in vitro* technique has come forth as a faster, feasible and cost-effective alternative tool for developing stress-tolerant plants with limited space and time (Sakhanokho and Kelley, 2009; Woodward and Bennett, 2005; Yamaguchi and Blumwald, 2005; Borsani *et al.*, 2003; Naik and Widholm, 1993).

The aim of this study was to evaluate the behavior of callus of *Capsicum* spp. undergoing different NaCl concentrations during *in vitro* development. With conceiving the above scheme in mind, the present research work was undertaken in order to fulfill the following objectives:

- To determine the response of genotype, treatments and genotype-treatment interaction on calli size and weight as indicators of tolerance.
- To compare the accumulation of proline as an indicator of tolerance of genotypes under control and salt stress condition.
- To establish a protocol for *in vitro* callus induction of different genotypes of *Capsicum*.
- To assess the magnitude of genetic variation regarding callus induction under control and stressed condition.

Chapter II

Review of Literature

CHAPTER II

REVIEW OF LITERATURE

Salinity is one of the major abiotic stresses that adversely affect the growth and development process of plant (Roychoudury *et al.*, 2008). Yield potentiality of many crops has reported to be reduced due to saline growth conditions (Rani *et al.*, 2012). It has been estimated that due to the occurrence of high salinity levels in soils more than two million acres of agricultural land become inapplicable of production every year. In Bangladesh, the coastal region covers about 20 percent of the country and over 30 percent of the net cultivable area and extends inside up to 150 km from the sea coast. Agricultural productivity of these areas are very poor (Petersen and Shireen, 2001). In recent years, different *in vitro* techniques are being used as a useful tool to select salt tolerant cultivars and develop the mechanism involved in salt tolerance (Gu, 2004; Venkataiah *et al.*, 2003). On the other hand, *Capsicum* crops are cultivated for vegetables as well as condiments and also used around the world as sweet peppers, pungent chili peppers, or as a source of dried powders of various colors (Vema *et al.*, 2013; Ravishankar *et al.,* 2003). It is also importance as an ornamental plant due to its attractive, colorful and long-lasting fruit which stand erect on the plant (Shreya *et. al.*, 2014; Sanatombi and Sharma, 2007). The propagation through seeds is restricted by low germination rate and short span of viability of seeds (Sanatombi and Sharma, 2006). Cross pollination behavior of *Capsicum* plants is a constraint to propagation of agronomic traits and for commercial seed production for these reasons the traditional breeding for salt tolerant will be inefficient. So, an efficient *in vitro* selection protocol in case of *Capsicum* spp. would be a strong weapon to combat against salt stress.

2.1 Concept and background of plant tissue culture

Plant tissue culture can be defined as culture of plant seeds, organs, explants, tissues, cells, or protoplasts on nutrient media under sterile conditions. The science of plant tissue culture takes its roots from path breaking research in

botany like discovery of cell followed by propounding of cell theory. In 1839, Schleiden and Schwann proposed that cell is the basic unit of organisms (Rhoads, 2007). They visualized that cell is capable of autonomy and therefore it should be possible for each cell if given an environment to regenerate into whole plant. Based on this premise, in 1902, a German physiologist, Gottlieb Haberlandt developed the concept of *in vitro* cell culture (Haberlandt, 1902). He isolated single fully differentiated individual plant cells from different plant species like palisade cells from leaves of *Laminum purpureum*, glandular hair of *Pulmonaria* and pith cells from petioles of *Eicchornia crassiples* etc. and was first to culture them in Knop's salt solution enriched with glucose. In his cultures, cells increased in size, accumulated starch but failed to divide. Therefore, Haberlandt's prediction failed that the cultured plant cells could grow, divide and develop into embryo and then to whole plant. This potential of a cell is known as totipotency, a term coined by Steward in 1968. Despite lack of success, Haberlandt made several predictions about the requirements in media in experimental conditions which could possibly induce cell division, proliferation and embryo induction. Gottlieb Haberlandt is thus regarded as father of tissue culture. Taking cue from Haberlandt's failure, Hannig chose embryogenic tissue to culture. He excised nearly mature embryos from seeds of several species of crucifers and successfully grew them to maturity on mineral salts and sugar solution. In 1908, Simon regenerated callus, buds and roots from *Poplar* stem segments and established the basis for callus culture.

For about next 30 years (upto 1934), there was very little further progress in cell culture research. Within this period, an innovative approach to tissue culture using meristematic cells like root and stem tips was reported by Kotte (1922) and Robbins (1922) working independently. All these research attempts involving culture of isolated cells, root tips or stem tips ended in development of calli. There were two objectives to be achieved before putting Haberlandt's prediction to fruition. First, to make the callus obtained from the explants to proliferate endlessly and second to induce these regenerated calluses to undergo organogenesis and form whole plants. It was in 1929s, when progress in plant

tissue culture accelerated rapidly owing to an important discovery natural auxins were necessary for the growth of isolated tissues containing meristemsn by Went. This breakthrough came from Went who reported that not only could cultured tomato root tips grow but could be repeatedly sub cultured to fresh medium of inorganic salts supplemented with yeast extract. In 1926, Went discovered first plant growth regulator (PGR), indoleacetic acid (IAA) is a naturally occurring member of a class of PGRs termed 'Auxins' (Zhang and Duijn, 2014). Gautheret (1934) reported the successful culture of cambium cells of several tree species to produce callus and 2 addition of auxin enhanced the proliferation of his cambial cultures. Further research by Nobecourt (1937), who could successful grow continuous callus cultures of carrot slices and White (1939) who obtained similar results from tumour tissues of hybrid *Nicotiana glauca* x *N. langsdorffii*. Thus, the possibility of cultivating plant tissues for an unlimited period was independently endorsed by Gautheret, White and Nobecourt in 1939.

Adding to the ongoing improvements in the culture media, Van-Overbeek (1942) reported growth of seedlings from heart shaped embryos by enriching culture media with coconut milk besides the usual salts, vitamins and other nutrients. This provided tremendous impetus for further work in embryo culture. Stem tip cultures yielded success when Ball (1946) devised a method to identify the exact part of shoot meristem that gives rise to whole plant. After 1950, there was an immense advancement in knowledge of effect of PGRs on plant development. The fact that coconut milk (embryo sac fluid) is nutritional requirement for tobacco callus besides auxin indicated the non auxinic nature of milk. This prompted further research and so other classes of PGRs were recognized. Skoog and Tsui (1948) demonstrated induction of cell division and bud formation in tobacco by adenine. This led to further investigations by Skoog and Miller (1957) who isolated 'kinetin'- a derivative of adenine (6-furyl aminopurine). Kinetin and many such other compounds which show bud promoting activities are collectively called cytokinins, a cell division promoter in cells of highly mature and differentiated tissues. However, now the concept is

altered to multiple factors like source of plant tissue, environmental factors, composition of media, polarity, growth substances being responsible for determination of organogenesis. Besides PGRs, scientists tried to improve culture media by differing essentially in mineral content. In this direction, Murashige and Skoog (1962) prepared a medium by increasing the concentration of salts twenty-five times higher than Knops. This media enhanced the growth of tobacco tissues by five times. Even today MS medium has immense commercial application in tissue culture. Having achieved success and expertise in growth of callus cultures from explants under *in vitro* conditions, focus now shifted to preparation of single cell cultures. Muir *et al.* (1954) demonstrated that when callus tissues were transferred to liquid medium and subjected to shaking, callus tissues broke into single cells. Bergmann (1959) developed a technique for cloning of these single cells by filtering suspension cultures. This technique called Plating technique is widely used for cloning isolated single protoplasts. Next step for realization of Haberlandt's objectives was development of whole plant from the proliferated tissue of these cells.

Vasil and Hilderbrandt (1966), were first to regenerate plantlets from colonies of isolated cells of hybrid *Nicotiana glutinosa* x *N. tabacum*. The role of tissue culture in plant genetic engineering was first exemplified by Kanta *et al.* (1962). They developed a technique of test tube fertlization which involved growing of excised ovules and pollen grains in the same medium thus overcoming the incompatibility barriers at sexual level. In 1966, Guha and Maheshwari cultured anthers of Datura and raised embryos which developed into haploid plants initiating androgenesis. This discovery received significant attention since plants recovered from doubled haploid cells are homozygous and express all recessive genes thus making them ideal for pure breeding lines.

Next breakthrough in application of tissue culture came with isolation and regeneration of protoplasts first demonstrated by Cocking in 1960. Plant protoplasts are naked cells from which cell wall has been removed. Cocking produced large quantities of protoplasts by using cell wall degrading enzymes.

After success in regeneration of protoplasts, Carlson (1972) isolated protoplasts from *Nicotiana glauca* x *N. langsdorfii* and fused them to produce first somatic hybrid. Since then many divergent somatic hybrids have been produced. With the advent of restriction enzymes in early 1970s, tissue culture headed towards a new research area. The totipotent plant cells could now be altered by insertion of specific foreign genes giving rise to genetically modified crops. In Restriction enzymes are named according to a convention proposed by Smith and Nathans (Smith and Nathans, 1973). This led to construction of first recombinant organism by Cohen *et al.* in 1973.

2.2 Nutrient media

The type and composition of culture media vary with the growth and morphogenesis of explant tissues. The selection of tissue culture medium is usually depends on the basis of the species to be culture. For example some species require high salts or have different necessities for Plant growth regulators (PGRs). Some tissues show better response on solid medium while others response better on liquid medium. Therefore, considering specific requirements of a particular culture system development of culture medium formulations demands a systematic trial and experimentation.

Knop's solution (Knop, 1884) considered as the oldest medium and White's medium (White, 1963) is one of the earliest plant tissue culture media generally formulated for root culture. MS medium (Murashige and Skoog, 1962) is a high salt medium due to its content of potassium and nitrogen salts, which is most suitable medium for plant regeneration from tissues and callus and also commonly used. B5 medium (Gamborg *et al.*, 1968) is generally used for cell suspension or callus culture. It contains fewer amounts of nitrate and particularly ammonium salts than MS medium. NN medium (Nitsch and Nitsch, 1969) developed mainly for anther culture has a salt concentration intermediate to MS and White's medium. PCI (Protoplast culture isolation) medium (Frearson *et al.* 1973) and KM medium (Kao and Michayluk, 1975) used for protoplast culture.

2.2.1 Inorganic nutrients

In vitro growth of plants also requires combination of macro and micronutrients like *in vivo* growth. Macronutrients are those which are required in greater than 0.5 mM/L concentration, as for example nitrogen, potassium, phosphorus, calcium, magnesium and sulphur. They present in form of salts in media. Nitrogen is usually supplied in form of nitrate (NO_3^-) and ammonium (NH_4^+) ions. Nitrate is superior to ammonium as the exclusive Nitrogen source but use of NH_4^+ checks the increase of pH towards alkalinity. Culture media should contain at least 25 mM/L nitrogen and potassium. Other major elements are adequate in concentration range of 1-3 mM/L. Micronutrients are those elements which are required at a concentration less than 0.05 mM/L. These include iron, manganese, zinc, boron, copper and molybdenum. These inorganic elements although required in small quantity but essential for plant growth, most critical of them being iron which is not available at low pH. Therefore, it is provided as iron EDTA complex to make it available at wide range of pH (Rhitu and Shastri, 2007).

2.2.2 Carbon source

As source of energy Sugar is very important part of plant tissue culture nutrient medium. Because most of the plant cultures are unable to photosynthesize effectively due to lack of chlorophyll, inadequate cellular and tissue development, inability of gaseous exchange and fixation of carbon dioxide in tissue culture vessels etc. As they lost the autotrophic ability need external supply of carbon for energy. The most preferred carbon or energy source is sucrose at a concentration of 20-60 g/L. Sucrose is hydrolysed to glucose and fructoses which while the medium are autoclaved which are used for growth and development of plant cultures but, if Fructose is autoclaved it produces a toxic effect to the culture. Other carbon sources like glucose, sorbitol, raffinose etc. may be used depending upon plant species to be cultured. Carbohydrates also provide osmoticum for this reason in anther culture higher concentration of sucrose (6-12%) is used (Rhitu and Shastri, 2007). Galactose has been said to be

toxic to most plant tissues; it inhibits the growth of orchids and other plants in concentrations as low as 0.01% (0.9 mM) (Arditti and Ernst, 1984; Ernst *et al.*, 1971).

2.2.3 Organic supplements

There are many organic supplements as vitamins, amino acids, activated charcoal, complex organics etc. which are required for complotting different physiological and metabolic processes, stimulating or inhibiting *in vitro* cell growth and development.

Vitamins are organic substances works as cofactors or parts of enzymes for metabolic processes. So, achieving optimum growth and development, culture medium should be enriched with vitamins. Thiamine (B_1), nicotinic acid (B_3), pyridoxine (B_6), pantothenic acid (B_5) is some vitamins commonly used in tissue culture medium. The thiamine is essentially added to medium as it is essential for carbohydrate metabolism; other vitamins are promontory (Rhitu and Shastri, 2007). The advantage of adding thiamine was discovered almost at the same time by Bonner (1937, 1938), Robbins and Bartley (1937) and White (1937). Nicotinic acid and pyridoxine appear, in addition to thiamine, in media developed by Bonner (1940), Gautheret (1942) and White (1943).

Amino acids are another organic supplement mainly stimulates cell growth in protoplast cultures and also influence induction and development of somatic embryos. Lglutamine, L-asparagine, L-cystein, L-glycine are commonly used amino acids in culture medium in form of mixtures because individually they inhibit cell growth (Rhitu and Shastri, 2007).

Organic Complexes are group of supplements which are not clearly defined such as coconut milk, (Burnet and Ibrahim, 1973; Steward and Caplin, 1952; Caplin and Steward, 1948; Gautheret , 1942; Van-Overbeek *et al.*, 1942) , malt extracts (Carimi *et al.*, 1999, 1998; Das *et al.*, 1995; Jumin, 1995; De-Pasquale *et al.*, 1994; Rangan, 1984), yeast extract (Thom *et al.*, 1981; Bridson, 1978; Nickell and Maretzki, 1969; Morel and Muller, 1964; Skinner and Street, 1954; Straus

and La-Rue, 1954; Bonner and Addicott, 1937; Robbins and Bartley, 1937; White, 1934), Potato extract (McGregor and McHughen, 1990; Harvais, 1982; Sagawa and Kunisaki, 1982; Chuang *et al.*, 1978 ; Sopory *et al.*, 1978; Vacin and Went, 1949) tomato juice, orange juice etc. These compounds are frequently used when no other combination of defined components results the desired growth. Potato extract has been found useful for anther culture (Rhitu and Shastri, 2007).

Activated charcoal behaves both in promotion and inhibition of culture growth depending upon plant species being cultured. It is reported to promote growth and development in orchids, carrot, ivy, *Capsicum* and tomato whereas inhibits soybean, tobacco etc. It reduce toxicity by absorbing absorbs brown-black pigments and oxidized phenolics produced during culture. It also absorbs other organic compounds like PGRs, vitamins etc. which may cause the inhibition of growth. Another feature of activated charcoal is that it causes darkening of medium which helps in root formation and development (Rhitu and Shastri, 2007).

2.2.4 Plant Growth Regulators (PGRs)

Plant Growth Regulators stimulate cell division and thus regulate the proliferation and development of shoot and roots on explants and embryos in semi-solid or in liquid culture medium so that their addition is must. Auxins, cytokinin, gibberellins and abscissic acid are the four major PGRs used in the culture medium.

Auxins bring on cell division, elongation, apical dominance, adventitious root formation and somatic embryogenesis. In low concentration, auxins induce root initiation and callus formation in high. 1-Naphthaleneacetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D), Indole-3-Acetic Acid (IAA), Indolebutyric Acid (IBA) etc. are the most commonly used synthetic auxins . Due to photosensitivity the stock solutions of both IBA and IAA must be stored in the

dark. 2,4-D is mainly used for induction and regulation of somatic embryogenesis in *in vitro* studies.

Cytokinins stimulate *in vitro* cell division, initiation and growth of shoots. Commonly used cytokinins are Zeatin, 6-Benzylaminopurine (BAP), Kinetin and 2-iP (2-isopentenyl). They alter apical dominance by stimulate auxiliary shoot formation. Cytokinins when used in high concentration inhibit root formation and stimulate adventitious shoot formation (Rhitu and Shastri, 2007). The morphogenesis is decided by the ratio of auxin and cytokinin in the culture medium. A higher ratio of cytokinin to auxin promoting shoot formation, while a higher auxin to cytokinin ratio promotes rooting. A cytokinin: auxin ratio of about 50: 1 produces shoots with minimum callus formation. New shoots may be subcultured at an interval of about 4 weeks (George, 2007).

Gibbrellins and abscissic acid are lesser used PGRs. Gibbrellic acid (GA_3) is mostly used for internode elongation and meristem growth. Abscissic acid (ABA) is used only for somatic embryogenesis and for culturing woody species (Rhitu and Shastri, 2007).

2.2.5 Solidifying agents

Solidifying agents are utilized for preparing semisolid tissue culture media to enable explant to be placed in right contact with nutrient media on surface or slightly embedded for providing good aeration. Agar is high a polysaccharide with molecular weight and obtained from sea weeds which can bind water. It is added to the medium in concentration ranging from 0.5 percent to 1 percent (w/v). Agar is preferred over other gelling agents due to its inert nature, it does react with other media component and also not digested by plant enzymes. A purified extract of agar called 'Agarose' is used for protoplast culture. Gelrite is another gelling agent unlike agar which is translucent and therefore easier to detect contamination. Mechanical support to cell or tissue growth can also be supplied without using any gelling agent but with Filter Paper Bridge, perforated cellophane and polyurethane foam etc. (Rhitu and Shastri, 2007).

2.2.6 pH

The pH of a solution is the negative logarithm of hydrogen ions (H^+) concentration. Absorption of ions and also solidification of gelling agent is greatly affected by the pH of culture media. Optimum pH for culture media is 5.8 which must be adjusted before autoclaving. pH values of culture media lower than 4.5 or higher than 7.0 greatly limits the *in vitro* growth and development. The pH of culture media generally decreases by 0.3 to 0.5 units after autoclaving and due to oxidation, differential uptake and secretion of substances by growing tissue it keeps changing through the period of culture (Rhitu and Shastri, 2007).

2.3 Salt stress

Salt stress is one of the most important abiotic factors in limiting plant productivity (Munns, 2002a). It results from a number of detrimental processes including an ion imbalance and toxicity, the impairment of mineral nutrition, a reduction in the water status of the plant tissues and oxidative stress, linked to the production of reactive oxygen species, which cause damage to lipids, proteins and nucleic acids (Hernandez *et al.*, 2000). Salt stress is leading to negative impacts on almost every economic sector in Bangladesh, mainly in agriculture. The farmers of Bangladesh, especially in the coastal zone, are struggling with the adverse impacts of salt stress. About 53 percent of the coastal areas of Bangladesh are affected by salinity problem (Haque, 2006). Salinity intrusion in soil caused by climate-induced hazards, especially cyclones and sea level rise (Rabbani *et al.*, 2013) and also by man-induced hazard like unwise Shrimp culture, irrigation with salt water, the use of potassium as fertilizer, which can form sylvite, a naturally occurring salt etc. As soil salinity increases, salt effects can result in degradation of soils and vegetation.

Salt stress is a polymorphous stress that affects plant growth and reduces yield through three direct ways: First, the presence of salt reduces the ability of the plant to take up water which leads to reductions in the growth rate. This is referred to as the osmotic effect of salt stress, which starts immediately after the salt concentration

around the roots increases over a threshold level. There is a second and slower response due to the accumulation of ions in leaves. This ion-specific phase of plant response to salinity starts when accumulated salt reaches toxic concentrations in the leaves and the third one is nutritional stress (Montoliu *et al.*, 2009; Gomez-Cadenas *et al.*, 1998). Within many species, documented genetic variation exists in the rate of accumul ation of Na^+ and Cl^- in leaves, as well as in the degree to which these ions can be tolerated (Munns and Tester, 2008). For most species, Na^+ appears to reach a toxic concentration before Cl^- does. However for some Cl^- considered being the more toxic ion (Lopez-Climent *et al.*, 2008; Moya *et al.*, 2003).

2.3.1 Effects of salt stress on plant cell

Most crops are unable to grow well on salt containing soils. The main reasons behind that the salts prohibit the water uptake and reduce the amount of water up taken by plant for growth and development. Also, it is lethal for plant when some salts present in extravagant amount. The adverse effects due to salinity have been caused by mainly Na^+ and Cl^- ions and these ions create the critical conditions for plant survival through bugging different plant mechanisms. High salinity creates both hyperionic and hyperosmotic stresses and cause plant death (Hasegawa *et al.*, 2000). It is reported that plants growing under salt stress conditions are affected in three ways: reduced water potential in root zone causing water deficit for plant uptake, phytotoxicity caused by Sodium ions and Chloride ions and thereby nutrient imbalance, dejecting uptake and transport of nutrients (Hasanuzzaman *et al.*, 2013). K^+ is an essential plant nutrient ion but when in salt stress condition Na^+ (primary toxic ion) competes with K^+ for binding sites essential for cellular functions and may cause potassium deficiency (Munns, 2002b) as well as and disturbs stomatal regulation which ultimately causes water loss and necrosis. This is called salt-specific or ion-excess effect of salinity (Kumar *et al.*, 2008).

On the other hand, Cl^- which is the most dangerous than Na^+ (Tavakkoli *et al.*, 2010) induces chlorotic toxicity symptoms due to interfering with production of chlorophyll (Chl). Though in plant cells, Cl^- is required for the regulation of

some enzyme activities in the cytoplasm and also a co-factor in photosynthesis and is involved in maintaining turgor and pH but it is toxic to plants at high concentrations (White and Broadley, 2001; Xu *et al.*, 2000). Higher accumulation of Cl^- led to a significant reduction in growth and water use efficiency in plants. Nevertheless, the uptake of these ions depends on factors i.e. the plant growth stage, genetic characters, temperature, relative humidity, light intensity etc. The toxic ions enter into the plant with the water flow up taken by plant through root system. The ions move from soil to the vascular system of the plant root by apoplastic and symplastic pathways (Hasanuzzaman *et al.*, 2013).

2.3.2 Mechanism of salt tolerance

The salt tolerance of a plant is the degree to which the plant can withstand, without any significant adverse effects in growth and development. In practical, salt tolerance is a relative term. It varies plant to plant. Some are more tolerant to a high salt concentration than others. Though the mechanisms involved in salt tolerance are poorly understood but there are some points at which salt transport is regulated. For overcoming salt stress, plants have evolved protective mechanisms that allow them to acclimatize.

2.3.2.1 Physiological mechanisms

2.3.2.1.1 Accumulation of proline

The protective physiolocical mechanisms for salt tolerance, include osmotic adjustment that is usually accomplished by accumulation of compatible solutes such as proline, glycine betaine and polyols (Ghoulam *et al.*, 2001) but Proline (Pro) is probably the most common compound accumulated by plants as (Grigore *et al.*, 2011; Szabados and Savouré, 2010; Verbruggen and Hermans, 2008). Proline is an α-amino acid, one of the twenty main amino acids biosynthetically derived from the amino acid L-glutamate. Proline is known to accumulate under salt stress in many crops (Munns and Tester, 2008). Their main role is probably to protect plant cells against the ravages of salt by maintaining the osmotic balance, stabilizing subcellular structures, such as

membranes and proteins, and scavenging Reactive oxygen species (ROS) (Ashraf and Foolad, 2007).

There are many published reports showing that, under controlled laboratory conditions, the concentration of proline in plants increases in parallel with an increase of the external salinity level, and there are also some studies on proline contents in plants collected from the field (Parvaiz and Satyawati 2008; Munns, 2005; Tester and Davenport 2003; Marcum, 2002). Some of the earliest data regarding proline accumulation in halophytes are those recorded by Stewart and Lee (1974) who found that proline levels in *Triglochin maritima* were low in the absence of salt, but increased as the salinity was raised. More recently, Naidoo and Kift (2006) reported a significant increment of proline in plants of *Juncus kraussii* treated with NaCl. When comparing the levels of proline in plants with different degrees of salt sensitivity, they are often higher in the more resistant ones; however, there are also many examples in which there is no positive correlation between proline contents and tolerance (Ashraf and Foolad, 2007; Chen *et al.*, 2007; Guerrier, 1998; Lutts *et al.*, 1996).

2.3.2.1.2 Control of Na^+ infringement

There is physiological evidence that control Na^+ encroachment of the tissues is a key determinant of salt tolerance (Niu *et al.*, 1995; Yeo and Flowers, 1986). The mechanisms involved in this control are poorly understood. As for example selective uptake from the soil solution, loading of xylem, loading of the phloem, removal of salt from the xylem in the upper part of the plant and excretion through salt glands (Munns, 2002a,b). Based on osmotic potential, plant can control the toxic ions like Na^+ to enter into the cell through energy driven pathway (Garciadeblas *et al.*, 2003). In an experiment presenting the effects of the over expression of a Na^+/H^+ tonoplastic antiporter in *Arabidopsis* has noticed the first experimental evidence that control of Na^+ transport within tissues has a great effect on salt tolerance (Apse *et al.*, 1999).

2.3.2.2 Genetic mechanisms

Regulation of gene expression in salinity stress includes a wide range of mechanisms that are used by plants to up regulate or down regulate (increase or decrease) the production of specific gene products (protein or RNA). Various mechanisms of gene regulation have been identified during the central dogma, from transcriptional initiation, to RNA processing, and to the posttranslational modification of a protein.

Transcription factors are considered as most important regulators that control gene expressions. Among them, *bZIP, WRKY, AP2, NAC, C2H2* zinc finger gene, and *DREB* families comprise a large number of stress-responsive members. These transcription factor genes are capable of controlling the expression of a broad range of target genes by binding to the specific cis-acting element in the promoters of these genes (Gupta and Huang, 2014). Transcriptions factors such as OsNAC5 and ZFP179 showed an upregulation under salinity stress, which may regulate the synthesis and accumulation of proline, sugar, and LEA proteins that in turn play an integral role in stress tolerance (Song *et al.,* 2011).

A large number of genes and transcription factors are upregulated in response to salinity in different plant species, which serve diverse functions (Chakraborty *et al.,* 2012; Zou *et al.*, 2012; Lim *et al.*, 2010; Zou *et al.*, 2009 Roshandel *et al.,* 2009). Examples of salt-responsive are mainly classified into the following functional categories: ion transport or homeostasis (e.g., *SOS* genes, *AtNHX1*, and H^+-ATPase), senescence-associated genes (e.g., *SAG*), molecular chaperones (e.g., *HSP* genes), and dehydration-related transcription factors (e.g., DREB). Among stress-responsive genes, the *SOS* gene family is believed to play a very intriguing role in ion homeostasis, thereby conferring salt tolerance (Hasegawa *et al.*, 2000; Liu *et al.*, 2000; Shi *et al.*, 2000; Yen *et al.*, 2000). Some ROS-scavenging and osmotic-regulating genes are also upregulated by salinity in some plant species.

Downregulated genes are emerging now as essential components of the response to salinity. For example downregulation of carotene hydroxylase increases carotene and total carotenoids enhancing salt stress tolerance in transgenic cultured cells of sweet potato (Kim *et al.*, 2012). It seems that mutual regulation mechanism exists between different genes and proteins and signals underlying different processes of plant adaptation to abiotic stress.

In addition to protein coding genes, recently discovered microRNAs (miRNAs) and endogenouks small interfering RNAs (siRNAs) have emerged as important players in plant stress responses. Initial clues suggesting that small RNAs are involved in plant stress responses stem from studies showing stress regulation of miRNAs and endogenous siRNAs, as well as from target predictions for some miRNAs (Sunkar *et al.*, 2007).

2.4 *Capsicum*

Capsicum is the most consumed spice in the world. It is used in the food, medical and chemical industries, in preparations as diverse as sauces, pain medication and agro-chemical insecticides. The genus *Capsicum* is a member of the Solanaceae family and consists of approximately 22 wild species and five domesticated species. The five domesticated species are *C. annuum* L., *C. baccatum* L., *C. chinense* Jacq., *C. frutescens* L. and *C. pubescens* R. and P.

The production of *Capsicum* is concentrated in a few countries, mostly developing nations. India is the dominant producer (44%), followed by China with a 9% market share in. From 1990 to 1999, Bangladesh was the world's fastest growing producer of *Capsicum* and by 2004, its third largest producer. Thailand and Peru are also significant exporters and producers. In 2004, the top three exporting countries of *Capsicum* were China, India and Spain, which managed to capture 27%, 16% and 11% of the market, respectively and the top three importers of *Capsicum* were the US, Malaysia and Germany, with a market share of 23%, 8% and 7% of the market, respectively (SADC, 2004). Malaysia is the largest importer of Indian *Capsicum* (~30%), followed by other traditional

importers like Bangladesh (~20%), Sri Lanka (15%), USA (9%), and UAE (8%) (Reddy *et al.*, 2014).

Capsicum is a valuable spice and one of the most important cash crops grown also in Bangladesh. It is available and used in the form of green, dried and powdered. It has become an essential ingredient in Bangladeshi meals. Most of our households always keep a stack of fresh hot green *Capsicum* at hand, and use them to flavor most curries and dry dishes. It is typically lightly fried with oil in the initial stages of preparation of the dish. Red *Capsicum*s contain large amounts of vitamin-C and small amounts of carotene (provitamin-A). Green *Capsicum*s (unripe fruit) contain a considerably lower amount of both substances. In addition, peppers are a good source of most vitamin-B and vitamin-B6 in particular. They are very high in potassium, magnesium and iron.

The *Capsicum* is a plant of tropical and sub-tropical region. It grows well in warm and humid climate. Deep, loamy, fertile soils rich in organic matter are preferred by the crop for satisfactory growth. Also need well drained soils with adequate soil moisture for the growth of the crop. *Capsicum* grows well in the dry and the intermediate part of the country. *Capsicum*s should not be in a position where the nightly temperature falls below 12°C. Growth will be inhibited if temperatures fall below 15°C. *Capsicum* pepper plants is a type of seasonal crops (annual plant) which only live for one season then died.

A total of 434757 acres of land are under *Capsicum* crop (BBS, 2014). Average gross margin and net return of green *Capsicum* were Tk.121315 and Tk.92250 respectively. The benefit cost ratio for green *Capsicum* was 1.64, which indicated that *Capsicum* cultivation is profitable in the areas (Hoq *et al.*, 2013).

2.4.1 *In vitro* investigations in *Capsicum*

In vitro is a Latin word meaning 'in glass'. Investigations or studies that are performed with cells or biological molecules outside of their normal biological circumstance, for example cells in artificial culture medium. *In vitro* investigations informally also called "test tube experiments", these studies

in biology and its sub-disciplines are traditionally done in test-tubes, flasks, Petridishes etc. *In vitro* studies include: the isolation, inoculation, growth, subcultures, cellular or sub cellular extracts; purification of molecules (often proteins, DNA, or RNA, either individually or in combination) and the commercial production of antibiotics and other pharmaceutical products etc. Several *in vitro* investigations have been conducted on *Capsicum* in different applications i.e., *in vitro* regeneration from *Capsicum* pepper (Berljak, 1999), production of virus free plants (Meena, *et al.,* 2014), genetic transformation (Arcos-Ortega *et al.*, 2010) and studies about the effect of variety and plant growth regulators on direct and indirect organogenesis and regeneration (Swamy *et al.,* 2014; Ashrafuzzaman *et al.,* 2009; Santana-Buzzy *et al.*, 2009; Kaparakis and Alderson, 2008; Rakshit *et al.*, 2007; Khan *et al.*, 2006; Stasolla and Yeung, 2003; Reddy *et al.,* 2002; Kintzios *et al.,* 2001; Nanakorn *et al.,* 1994; Harini and Lakshmi Sita, 1993).

In vitro plant regeneration from pepper (*Capsicum annuum* L. cv. 'Soroksari') explants were investigated by Berljak (1999). Shoot-tip, cotyledon and hypocotyl explants excised from 2 weeks old seedlings were cultured on Murashige and Skoog medium supplemented with B_5 or L_2 vitamins, and different content of growth regulators: 2,4-Dichlorophenoxyacetic acid, benzyladenine, indole-3-acetic acid, and zeatin.

Shoot formation from different explants as apical meristem, cotyledons, stems internodes, leaves, anthers and inflorescences has been reported in *Capsicum* (Verma *et al.*, 2013; Grozeva *et al.*, 2012; Otroshy *et al.* 2011a,b; Adhikery and Amin, 2011; Rakshit *et al.,* 2007; Kehie *et al.*, 2011; Aboshama, 2011; Kumar and Tata, 2010; Ashrafuzzaman *et al.,* 2009; Sanatombi *et al.*, 2007; Joshi and Kothari, 2006; Mathew, 2002; Shivegowda *et al.*, 2002; Kintzios *et al.,* 2001; Kim *et al.,* 2001; Zhu *et al.,*1996). Haploid technology in pepper includes induction and regeneration of haploid embryos from anthers or microspore culture. Genetic analysis is easy in case of plant regenerants containing a single set of chromosome, originating from *in vitro* anther culture due to its low

chromosome number (Pauk *et al.* 2010; Nervo *et al.* 2007; Andres *et al.*, 2004; Zagorska *et al.*, 2002; Steinitz *et al.*, 1999; Atanassov *et al.*, 1995). By doubling of the haploid genome a fully homozygotic line can be obtained within a short period of time which is important for creation of genetic variation with higher quality. Regeneration from haploid callus derived directly from anther or microspores and haploid embryos has been attempted successfully achieved by many scientist (Basay and Ellialtıoglu, 2013; Nowaczyk *et al.*, 2009; Lantos *et al.*, 2009; Kim *et al.*, 2008; Asakaviciute, 2008; Irikova, 2008; Bal and Abak, 2007; Rodeva *et al.* 2007; Nowaczyk and Kisiala, 2006; Supena, *et al.*, 2006; Ercan *et al.*, 2006; Touraev and Heberle-Bors, 2003; Comlekcioglu *et al.*, 2001; Dumas-de-Vaulx, 1990; Gresshoff and Doy 1972; Wang *et al.*, 1973). *In vitro* selection and screening of abiotic stress tolerant cell lines for genetic variability analysis have been also reported in *Capsicum* (Solis-Marroquin *et al.*, 2011; Mohammed and Mohammed, 2007).

2.4.2 Genotypic variation of *Capsicum* genotypes *in vitro*

As that of other factors like explants and media components, genotypes also play a significant role in *in vitro* response.

2.4.2.1 Variation due to different growth regulators

In an investigation conducted by Fari *et al.* (1990), two important breeding lines and seven cultivars of chilli were compared on the basis of their regeneration potential. Different excised organs of seedlings were used as explant. They found considerable shoot regeneration potential difference among chilli genotypes. The common phenomenon was that in all genotypes IAA stimulated shoot regeneration; however some moderate regeneration was observed even without IAA. In contrast to this, differential *in vitro* regeneration response was also observed among six cultivars and one Guatemalan wild accession. The wild accession showed the best regeneration response. Out of six cultivars, in *Yolo wonder* L. occasional elongation of shoots was attained by incubating cultures in the dark rather than light on a medium containing 10 mg/L BAP and 1 mg/L

IAA (Liu *et al.*, 1990) but Kumar *et al.* (2012) noticed that MS medium supplemented with 5 mg/L BAP and 1 mg/L IAA gave maximum shoots per explants. In another investigation, Christopher and Rajam (1994) detected maximum number of shoots on MS medium containing 88.8 µM BAP or 116.2 µM kinetin in *Capsicum annuum* and 66.6 µM BAP or 92.9 µM kinetin in *Capsicum praetermissum* after 4 weeks of culture, expected rooting was noticed on TIBA + BAP or kinetin media in both the species.

Szasz *et al.* (1995) studied the comparative shoot regeneration potential among 17 (10 Hungarian and 7 Italian) bell pepper genotypes on media supplemented with BAP + IAA in addition to a standard inbred lines (No. 40017, R-13). Only *cv.* Cuneo LS ISO and *cv.* Piknik regenerated shoots but formed pale leaf like structures on shoot elongation medium. In another study different responses to regeneration were also observed even within the *C. annuum* species. Better regeneration response was observed in Byadagi Dabbi compared to Arka Lohit (Mathew, 2002). In a study, Christopher and Rajam (1996) attained *in vitro* plant regeneration in *Capsicum annuum* cvs. *C. baccatum, C. praeteimissum*, Bhiwapuri sweet pepper, hybrid pepper and Cayenne pepper. Shoots were induced from hypocotyls, cotyledon and leaf explants on MS medium containing 5.7 µM, IAA + 13.3 µM BAP; 22 µM BAP; 44 µM BAP, respectively.

In counterpoint, Ge *et al.* (1991) could not find any considerable difference among the genotypes in terms of callus production when explants are excised from seedlings of *Capsicum annuum var. annuum* and *var. minimum, Capsicum baccatum var. baccatum* and *var. pendulum, Capsicum chinense, Capsicum frutescens* and *Capsicum pubescens.* Pundeva and Simeonova (1992a,b) also observe no significant difference among *Capsicum annuum* cultivars; Sivriya, Zlaten Medal, Pazardzishka Kapia, Kurtovska Kapiya and Safiyska Kapia to produce callus on MS medium containing IAA and NAA but differences among genotypes in terms of the type of callus.

2.4.2.2 Variation due to different explants to different medium composition

Cotyledonary leaf is most commonly utilized as explant for regenerating a new plant by tissue culture process. First effort to *in vitro* regenerate of *Capsicum* was made by Gunay and Rao (1978). They analyzed the effect of different cytokinins and noticed 6-benzyl amino purine (BAP) was more effectual in producing shoots from cotyledonary leaf. Verma *et al.* (2013); carried out an experiment to standardize a protocol for high efficiency *in vitro* plant regeneration system in *Capsicum annuum* L. cv. California Wonder using cotyledon explants. MS medium supplemented with 6.0 mg/L BA and 0.3 mg/L IAA gave highest percentage of shoot regeneration (80.95%). For elongation, regenerated shoots were transfer to MS medium supplemented with 2.25 mg/L BA and 2 mg/L GA_3. Half-strength MS medium containing 0.25 mg/L IAA used for obtaining highest percentage (50%) of *in vitro* root induction. The rate of plant regeneration was noticed to depend on genotype, explants type and culture medium. The cotyledons were more effective as explants for bud induction and subsequent plant elongation than hypocotyls. The highest regeneration frequency was obtained in cotyledons from Maritsa variety on culture medium containing 0.2 mg/L $AgNO_3$ but medium containing Humates containing 5.0 mL/L promoted plant regeneration in hypocotyls (Grozeva *et al.*, 2012).

The best performance for cotyledons was observed by Otroshy *et al.* (2011a) on MS medium containing 6 mg/L BAP and 1 mg/L IBA. From histochemical analysis he found that only the cotyledons brought out a direct induction of more embryological protuberances that grew around the cut end of the explants. In another study with nodal segment used as explants, Otroshy *et al.* (2011b) noticed that *in vitro* micro propagation among different concentrations of cytokinins BAP, KIN, Zea and TDZ the best buds per explant and percentage of rooting potentiality was observed on medium containing 2 mg/L BAP and 0.5 mg/L IBA. After hardening, the rooted plants were transferred to the greenhouse conditions where they grew, matured and flowered normally with a survival rate of 85 per cent (Otroshy *et al.*, 2011a). Adhikery and Amin (2011) conducted a

study with cotyledon of chilli as explants for regeneration on MS medium supplemented with different concentrations of auxins and cytokinins. He found that the highe percentage of callus (96.29%) was obtained from MS medium containing 1.0 mg/L kinetin and 3.0 mg/L 2,4-D within about 13 days. Among three genotypes he used, 'C1013' showed the height potential on callus induction. During working with two varieties Sujamukhi and Bally, Rakshit *et al.* (2007), found that callus derived from cotyledons was white and friable and showed excellent growth MS medium containing MS + 2.0 mg/L NAA or NAA + 0.5 mg/L Kn + 5.0 mg/L 2,4-D was the best for callus initiation. For shoot induction 0.5 mg/L IAA+ 1.0 mg/L BAP showed good response.

Kehie *et al.* (2011); found that maximum number of shoot was induced in *Capsicum chinense* Jacq. cv. Naga King Chili with bud-forming capacity (BFC) index of 10.8, by culturing nodal segments in MS medium enriched with 18.16 lM TDZ followed by 35.52 lM BAP and by culturing shoot tips as explants, multiple shoot was also induced with BFC 8.3 in MS medium supplemented with either 18.16 lM TDZ or 35.52 lM BAP. Best rooting of elongated shoot was noticed on MS medium containing 5.70 lM indole-3-acetic acids (IAA).

Kumar and Tata (2010) studied an experiment on shoot bud differentiation from hypocotyls explants *and* obtained best result from 15th day old hypocotyl explants and MS supplemented with BAP (4.0 mg/L) and IAA (0.5 mg/L) was found to be the best medium. From an investigation on regeneration potential determination of *Capsicum*, Ashrafuzzaman *et al.* (2009) reported that the callus induction as well as shoot initiation was higher in hypocotyls than cotyledon. The highest callus was brought on from hypocotyl in MS medium supplemented with BAP (5.0 mg/L) and NAA (0.1 mg/L) and cotyledon in a combination of BAP (5.0 mg/L) with IAA (1.0 mg/L). He also found that Shoot elongation was accelerated by using additional subjoining of GA_3 and $AgNO_3$ and on the MS medium supplemented with 0.1 mg/L NAA + 0.05 mg/L IBA regenerated shoots rooted best but Aboshama (2011) noticed that higher doses (1.5 and 2.0 mg/L) negatively affected the regenerative capacity .

In an investigation Sanatombi *et al.* (2007) used shoo-tip and axillary shoot-tip as explants in case of an ornamental chilli cultivar 'Morok Amuba'. Best performance he obtained from MS medium suplimented with 10 mg/L Zea and 5 mg/L BAP in combination with 1 mg/L IAA. Rooting and elongation of the shoot buds he used MS medium supplemented with 0.5 mg/L IAA or IBA. Auxiliary shoots were induced on the rooted plantlets by decapitation and the axillary shoot-tips explants were used for further induction of shoot buds by culturing them on a medium containing combinations of BAP with IAA. The shoot buds were rooted on a medium containing 0.5 mg/L IBA. The plantlets showed 80-90% survival during transplantation. In another experiment Sanatombi and Sharma (2006) used shoot tip and axillary shoot explants for *in vitro* regeneration and mass multiplication of two *Capsicum annuum* L. cultivars, Meiteimorok and Haomorok. They showed that shoot tip explants excised from *in vitro* raised seedlings can be used for multiple shoot induction in MS medium supplemented with BAP alone or in combination with IAA. Maximum number of shoots was obtained from MS medium supplemented with 22 μM BAP after four weeks of culture in the cultivar Haomorok.

Multiple shoots were induced from nodal segments explants excised from one month old seedlings of red pepper (*Capsicum annuum* L. *cv.* Pusa Jwala) on MS medium containing (0.1-10 μM) thidiazuron (TDZ). Best result obtained from MS medium supplemented with 1.0 μM TDZ (Siddique and Anis, 2006). Joshi and Kothari (2006) used cotyledonary explants of *Capsicum annuum* L. cv. X-235 to look into the effect of copper sulphate on differentiation and elongation of both shoot induction and elongation. Media were supplemented with different levels of $CuSO_4$ (0-5 μM). They noticed the levels of $CuSO_4$ in medium highly influenced the shoot formation and their subsequent elongation. Highest number of shoots per explant was received at the concentration of $CuSO_4$, 30 times increased to the normal MS level.

According to Mathew (2002) whole cotyledons cultured on MS with 2 mg/L BAP + 1 mg/L GA_3 resulted in maximum average number of buds. In an *in vitro*

regeneration study Shivegowda *et al.* (2002) used cotyledonary explants of chilli on MS medium supplemented with 9-18 µM zeatin in combination with GA_3 (2.89 µM). Venkataiah *et al.* (2004), observed that leaf explants are more conformable to adventitious shoot formation followed by cotyledon and hypocotyl on MS medium with 3 mg/L BAP + 1 mg/L IAA. By using stem segments and shoot tips Kintzios *et al.* (2001) developed an efficient protocol of direct somatic embryo genesis (without involving intermediate callus) of *Capsicum annuum* L. They cultured explants on MS medium supplemented with TDZ. Among the various concentrations of TDZ tested, best result obtained from 0.5 µM concentration. Induction, maturation and germination were attained on the same medium. Kim *et al.* (2001); used hypocotyls explants for obtaining plant regeneration on a MS medium supplemented with 3 mg/L zeatin (zea) and 0.3 mg/L IAA. Other explants that have been used for shoot induction include; embryo cultures, apical meristem, leaf discs. Zhu *et al.* (1996); used leaf disc as an explant and induced buds on 8 mg/L BAP and 2 mg/L IAA. This explant on medium with cytokinins (Kin, BAP, Zea, and Zip) dramatically increased both the percentage of explants forming buds and number of buds. By a comparative study on *in vitro* regeneration of leaf explants Fari *et al.* (1990) noticed that the best regeneration was obtained from cotyledonary explant. Using apical meristem as explants Madhuri and Rajam (1993) developed a protocol of *Capsicum* regeneration on medium with 2 mg/L BAP and observed it induced 5-7 percent multiple shoots.

Relatively high frequency of shoots was produced on MS medium with 0.5 mg/L TDZ from the cotyledonary leaves (Manoharan *et al.*, 1998). Rama (1997), showed that good morphogenetic response obtained from cotyledonary leaves than hypocotyls explant on MS medium containing 10 mg/L BAP and 2 mg/L GA_3 and callus was observed in the absence of GA_3 and presence of NAA. Agarwal *et al.* (1997); also reported that number of multiple shoots induced was more in cotyledon compared to hypocotyl explants on MS medium containing higher levels of BAP. About 95 percent shoot regeneration from cotyledonary explants was obtained on MS medium containing 5 mg/L BAP and 0.1 mg/L

IAA (Shen *et al.*, 1994; Gatz and Rogozinska, 1994). Ebida and Hu (1993), studied proliferation of buds from cut surfaces of cotyledon on 3.0 mg/L BAP and 0.1 mg/L NAA. TDZ was used to regenerate shoots directly from excised cotyledons of two genotypes of *Capsicum* by Szasz *et al.* (1995). However, increasing the concentration of BAP (5 mg/L) resulted in the increased induction of multiple buds directly in 80 percent of cotyledonary leaf (Agarwal *et al.*, 1989). Kniitel (1991), described the regeneration from cotyledons of mature zygotic embryos. Agarwal and Chandra (1983) noticed that shoot formation and increase in size of the cotyledon when embryos were cultured on 0.57 mM IAA and kin. But Arroya and Revilla (1991) observed that percent regeneration was better in case of hypocotyls then cotyledon explants.

Half seed, containing proximal part of the hypocotyls and the radical was used as explant by Binzel *et al.* (1996). Ezura *et al.* (1993); utilized proximal part of hypocotyl of *Capsicum* as explants for regeneration with Gellan gum (0.4%). A higher concentration of BAP (10 mg/L) was utilized by Ebida and Hu (1993) to proliferate multiple shoots from cut surfaces of hypocotyls. A novel protocol for regeneration of *Capsicum* was shown by Valera-Montero and Ochoa-Alejo (1992), from rooted hypocotyl on MS media supplemented with 5 mg/L BAP and 0.3 mg/L IAA, where shoot induction per cent ranged from 46.7-100. In an investigation of morphogenetic response of cultured hypocotyl segments of *Capsicum annuum* varied from apical to basal segments on MS medium supplemented with 0.5 mg/L IAA + 3 mg/L BAP where result shows that apical segments produced shoots and callus while basal segments produced callus and roots (Christopher *et al.*, 1991). Fari and Czako (1981), noticed shoots from apical segments of hypocotyl, while middle and basal segments produced roots and callus respectively. The best concentration for shoot differentiation from hypocotyls was 5 mg/L BAP (Agarwal *et al.*, 1989). Shoot tip and hypocotyls segments are also used for producing shoots. Shoot tip explants cultured on MS medium supplemented with 5 mg/L BAP produced vigorous shoots and a large number of shoots, intervening callus was observed with 0 to 5 mg/L BAP and

low levels of Indole Butyric acid (IBA) (Agarwal *et al.*, 1988; Agarwal *et al.*, 1989).

Elwan (2009) reported that leaf explants were significantly better than cotyledonary explants and also reported that the adaxial position of both explant types responded better comparing to abaxial one. Subhash and Christopher (1988), got multiple shoots from 10 percent of cultured leaf segments of *Capsicum* on medium with 1.0 mg/L kinetin (kin) and 1.0 mg/L naphthalene acetic acid (NAA). The regeneration of shoots from embryo culture was obtained by Christopher *et al.* (1986) on a medium fortified with 2,4-D (0.5 mg/L) and BAP (2.0 mg/L). Stem segments and cell suspensions established from hypocotyl explant showed very good, differentiation on MS medium supplemented with 1.0 mg/L IAA and 2.0 mg/L BAP (Garcia, 1990). Again in another study Liu *et al.* (1990) noticed that there is no significant difference among cotyledonary leaves, hypocotyls and leaf tissue in respect of shoots regeneration frequency.

2.4.3 Problems associated with *in vitro* studies of *Capsicum* spp.

Preliminary reports about various inherent problems related with *in vitro* studies of *Capsicum* as severe unmanageable morphogenic nature, formation of rosette shoots or poorly developed shoot buds, genotypic dependence have clearly noticed. These problems alone or together can affect the whole tissue culture attempts as well as crop improvement through genetic modification.

2.4.3.1 Recalcitrant morphogenic nature

Recalcitrance is the problem associated with *in vitro* culture, the inability of plant cells, tissues and organs responding to tissue culture. Ability of explants to response in tissue culture is greatly influenced by three main factors i.e. physiology of donor plant, *in vitro* manipulation and *in vitro* stress physiology (Benson, 2000). It is very hard to study *in vitro* with *Capsicum* as compared to other solanaceous crops like tobacco, tomato, potato which are frequently used as standard systems because of their great potentiality to regenerate new plants

(Rakshit *et al.*, 2007). Many researchers consider pepper a recalcitrant species and the reported number of androgenic embryos obtained per 100 cultured anthers often ranges from 0 up to approximately 10 embryos (Ercan and Ayar Şensoy, 2011; Segui-Simarro *et al.*, 2011).

Although, there are several relative success reports on shoot morphogenesis in *Capsicum* (Venkataiah *et al.*, 2003; Husain *et al.*, 1999; Ramirez-Malagon and Ochoa-Alejo, 1996; Valera-Montero and Ochoa-Alejo, 1992; Ochoa-Alejo and Ireta-Moreno, 1990; Agrawal *et al.*, 1989) but genetic engineering is still limited because of possessing low morphogenetic potential (Ochoa-Alejo and Ramirez-Malagon, 2001; Steinitz *et al.*, 1999). By Selecting proper explants at specific responsive stage, modifying different components in nutrient media and by adding other growth additives may helpful to overcome the recalcitrance problem.

2.4.3.2 Formation of abnormal shoots or rosette shoot buds

One of the most important factors, which limit the in *vitro* regeneration in *Capsicum,* is the formation of rosette shoots or abnormal leafy shoots which protest bud and shoot elongation. Several activities directed to overcome problems of shoot elongation have been noticed. Arroya and Revilla (1991) directly rooted the rosette of buds and elongation took place in the field itself. To overcome the problem of bud and shoot elongation different growth adjutants utilized like phenylacetic acid (Husain *et al.*, 1999) a lactone 24-epibrassinolide (Franck-Duchenne *et al.*, 1998) and $AgNO_3$ (Hyde and Phillips, 1996).

2.4.3.3 Genotypic dependence

Genotyp is another factor on which organogenesis of *Capsicum* culture depends. The strong potentiality of genotype in the regeneration capacity of different *Capsicum* cultivars demands a different regeneration protocol for each (Mathew, 2002; Christopher and Rajam, 1996; Szasz *et al.*, 1995; Fari *et al.*, 1990; Liu *et al.*, 1990). Alizah and Zamri (2013) studied the effect of genotype on regeneration of Malaysian chilli explants using two varieties MC11 and CB4

where, CB4 showed the best response and MC11 was the least responsive. In an *in vitro* study with three chili pepper cultivars (*Capsicum annuum* L., var. 'X-235', var. 'PC-1' and var. 'Pusa Jwala'), Kumar and Tata (2010) noticed that among three cultivar *Capsicum* L. var. 'X-235' gave better result than others. As because different genotypes shows different response to regeneration it is necessary to optimize *in vitro* propagation protocols for the specific *Capsicum* cultivar (Sanatombi and Sharma, 2006; Ezura *et al.*, 1993).

2.5 Callus

Callus refers to dividing unorganized mass of cells induced in artifitial culture. Generally, a higher auxin concentration in the growth medium induces callus formation (Skoog and Miller, 1957). The quantity and quality of callus produced depends on a wide variety of conditions like explants, genotypes, growth regulators and light/ dark incubation etc. (Patil, 2001). A Nobel callus experiment was conducted by Mangang (2014) with different explants the placental tissues of immature green pods of *Capsicum chinense* Jacq. cv. 'Umorok' and he found that MS media supplemented with 2 mg/L 2,4-D and 0.5 mg/L KIN produced a good amount of friable and proliferating callus.

2.5.1 Callus induction and proliferation in *Capsicum*

Callus Induction and Proliferation Callus production is an essential step in the use of tissue culture studies for various physiological phenomena including resistance against various abiotic stresses. Callus is an unorganized, proliferative mass of predominantly parenchyma cells. Studies have revealed that better response of callus was obtained when callus cultures were kept in dark. Chen *et al.* (1988); reported that morphogenic callus could form most readily from the leaf explants with most proliferating callus when kept in dark. Aftab *et al.* (1996); have also reported that embryogenic callus could be obtained from young leaves on modified MS medium under dark conditions. Studies have suggested that amongst all the media tested for callus induction and proliferation by different workers, the best medium was modified MS medium (Baksha *et al.*,

2002; Aftab *et al.*, 1996; Guiderdoni, 1986; Liu and Chen, 1974). Role of auxins have also been studied for callus induction and proliferation. Nadar *et al.* (1978); found that embryogenic callus forms when auxin is added to the medium. On the other hand, no embryogenesis was observed in callus cultures on auxin-free media. Callus proliferation in modified MS medium with various levels of auxins and cytokinins was also reported by Bhansali and Singh (1982). Studies have shown that amongst different auxins tested for callus induction, addition of 2,4-D in the medium always produced better callus growth than any other growth regulator.

In another *in vitro* propagation study, five varieties of *Capsicum* red, yellow, green, purple and white were cultured on MS medium, supplemented with hormones BAP, kinetin (Kin), the combination of BAP + Kin, BAP with NAA and BAP with IAA and 2.0 mg/L BAP with 0.1 mg/L NAA media was observed to be more suitable for callus formation and the number of regenerated shoot buds was highest in MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L IAA. IAA was observed to be more suitable for green variety of *C. annuum*. Plantlets were successfully acclimatized in greenhouse (Swamy *et al.*, 2014).

In an *in vitro* callus induction experiment of *Capsicum annuum* L. Rakshit *et al.* (2007); showed that callus developed from cotyledons was white and friable and showed excellent growth on MS media supplemented with 2.0 mg/L NAA or NAA, 0.5 mg/L KIN and 5.0 mg/L 2,4-D . Media containing 0.5 mg/L IAA and 1.0 mg/L BAP showed good response shoot induction.

Nanakorn *et al.* (1994); examined on effect of plant growth regulators on callus initiation and growth from leaf, petiole and internode segments of *Capsicum* spp. cultured on MS media supplemented with different concentration of NAA, 2,4-D, BAP and kinetin and the results showed that petiole had the highest percentage of callus initiation percent on media supplemented with 0.5 mg/L of NAA or 1.0 mg/L of 2,4-D and largest callus on media supplemented with 0.5 mg/L of 2,4-D. NAA facilitate the formation of compact callus while 2,4-D

promoted a formation of friable callus. The highest percentages of callus initiation were 100 percent and 96.67 percent from leaf and internode, respectively on the MS media supplemented with 1.0 mg/L of 2,4-D combined with 1.0 mg/L of BAP. The highest number of shoots was obtained from seed of *C. annuum* L. cultured on the media supplemented with 10.0 mg/L of BAP. He found that Kinetin had no effect on inducing multiple shoot in *C. frutescens* L. but node of *C. annuum* L. could form multiple shoot when supplied with 5.0 and 10.0 mg/L of kinetin.

Neither BAP nor kinetin (2, 4, 8 and 10 mg/L) along with NAA could regenerate plants from callus cultures on both solid and liquid media. On 2,4-D above 3 mg/L the highest proliferation was observed in callus from seeds compared to other explants such as shoot tips, internodes and leaf bases. Low level of 2,4-D (below 3 mg/L) showed inability to induce callus. On the other hand NAA induced long roots from cut ends of the explants (Mathew, 2002). When hypocotyl and root explants were cultured, Pundeva and Simeonova (1992a, 1992b) observed slight callus induction along with roots on medium with IAA and NAA and coarse friable callus on 2,4-D medium. But, cotyledons produced compact callus at the same level of 2,4-D (Agarwal *et al.*, 1997).

Kintzios *et al.* (2001); studied, the effect of different inorganic micronutrients and vitamins on callus induction, growth, and development of somatic embryos from young mature, fully expanded leaves of chilli pepper (*Capsicum annuum* L.). They utilized solid MS medium containing 8 percent (w/v) sucrose, 9 µM 2, 4-dich lorophenoxyacetic acid, 12.9 µM 6-benzyladenine, and 0.5 mg/L thiamin HCl in various concentration of 11 different vitamins. In lieu, explants were cultured onto a solid medium containing MS macro and micronutrients but the salts of Mn, Zn, Cu and Co were excluded, which were added at either the standard MS concentration or at a tenfold increased (Cu, Co) or decreased (Mn, Zn, I) concentration. At the end of the day they noticed that somatic embryogenesis from pepper leaves is preferred by the addition of nicotinic acid to the culture medium and the increase of copper concentration without reducing

embryo maturation and germination. Though protoplast can be obtained easily in chilli, but cell division and plant regeneration have been rarely found.

Saxena *et al.* (1981); got the first shoot regeneration from cultured protoplast by transferring the callus induced on MS medium with 4 mg/L IAA, 2.5 mg/L kinetin and 3 percent sucrose incubated in the dark for 15 days and then returned to the standard photoperiod. Prakash *et al.* (1997); reported that protoplast isolated from cotyledons was unable to divide and thereby degenerated finally, but protoplasts isolated from fully expanded leaves (21 days old) pure shoots when cultured on medium containing 1.0 mg/L NAA, 1.0 mg/L 2,4-D and 0.5 mg/L BAP succeed in division. In medium containing 2 mg/L NAA and 0.5 mg/L BAP micro calli and macro calli were formed, respectively. Regeneration of callus found on MS gelled medium suplemented with 10 mg/L BAP, 0.5 mg/L IAA and 2 mg/L GA_3 (Prakash *et al.*, 1997). Murphy and Kyle (1994) optimized procedure for isolation of protoplasts from leaf tissues of three cultivars of *Capsicum annuum* and two genotypes of *C. chinense* from sliced leaves. Protoplasts from these preparations were found to be excellent systems for viral RNA infection by electroporation. Power and Chapman (1985), isolated leaf protoplasts from pure shoot cultures formed callus on MS medium containing KMBP and zeatin respectively which regenerated plants on MS with 6 mg/L BAP.

By culturing mature embryos of *Capsicum annuum* variety MS medium enriched 0.5 mg/L, 2,4-D and 0.5 mg/L kinetin to induce callus from green cotyledons and complete plantlets were obtained from compact callus on MS medium supplemented with 0.1 mg/L IAA and 0.5 mg/L BAP (Christopher *et al.*, 1986). Petiole cultured on medium containing 0.4 mg/L 2,4-D and 0.03 mg/L Kinetins howed continuous callus growth. However, hypocotyls and cotyledon explants cultured on MS medium containing 2,4-D proved in vigorous, friable callus (Dix and Street, 1975). Shoot development of Pimento and *Capsicum fruitescens* cv. Bharat. was detected in California Wonder and Pimento genotypes only on medium supplemented with a commixture of 1 M and BAP

but, they failed to develop buds from callus or explant tissue itself. MS medium supplemented with 8.8 µM BAP and 5.7 µM IAA facilitate callus induction from hypocotyls (Fari and Czako, 1981). According to Nagata and Tabake (1971) recovery of callus was observed from protoplasts, cultured on MS containing 1 mg/L 2,4-D, 1.0 mg/L NAA and 1.0 mg/L BAP, 2 percent sucrose and 0.5 M mannitol, but no plant regeneration was reported.

2.5.2 Effect of NaCl on callus

NaCl decrease the growth of calli as the salinity is high (Vaziri *et al.*, 2004). This effect is more in calli grown in presence of light compared to those grown in the dark. Some parts of calli grown in the light show necrosis. The adverse effect of salt is more pronounced on total protein content of callus (Priya *et al.*, 2011). Previous results also suggest that the proline accumulation in callus is an index of salinity tolerance. NaCl also effects callus color in the medium. Gupta *et al.* (2014); studied the effect of NaCl on callus and obtained yellow-green and compact calli from *in vitro* raised Stevia leaves sub-cultured on MS medium supplemented with 2.0 mg/L NAA and different concentrations of NaCl (0.05-0.20%).

2.6 *In vitro* selection

In vitro selection is essential for desirable genotypes asceptically under control and treated environments. Reports on the use of this technology have dwindled over the years, after an explosion of reports following the awareness of its potential as a source of biological variation Using whole plants, organs or undifferentiated tissues (George, 2007). The production and productivity of several crops adversely affected by various biotic and abiotic stresses are responsible for tremendous economic losses worldwide. Traditional breeding technologies and proper management strategies continue to play a vital role in crop improvement. The conventional breeding programmers are being employed to integrate favorable genes of interest from inter crossing genera and species into the crops to induce stress tolerance. However, conventional breeding

methods have little success and have failed to provide desirable results (Purohit *et al.*, 1998) on the other hand *in vitro* techniques for developing stress tolerant plants, are known to be might demonstrate to be a faster way towards improving crop varieties (Borsani *et al.*, 2003; Yamaguchi and Blumwald, 2005). In recent years *in vitro* technique has come forth as a feasible and cost-effective alternative tool for selection of stress-tolerant plants with limited space and time (Sakhanokho and Kelley, 2009)

The selecting agents usually employed for *in vitro* selection include NaCl (for salt-tolerance), PEG or mannitol (for drought-tolerance), specific fungal culture filtrate (FCF) or phytotoxin such as fusaric acid or the pathogen itself (for disease-resistance). The explants are exposed to a broad range of these selective agents added to the culture medium. Only the explants capable of sustaining such environments survive in the long run and are selected (Purohit *et al.*, 1998). In spite of having many advantages, development of stress tolerant plants through *in vitro* selection has some limitations like loss of regeneration ability during selection, lack of correlation between the mechanisms of tolerance operating in cultured cell, tissue or organ and those of the whole plants, and phenomenon of epigenetic adaptation (Tal, 1994).

In vitro culture is a useful tool to evaluate the effect of salinity and to select salt-tolerant varieties in plant species (Queiros *et al.*, 2007; Davenport *et al.*, 2003). *In vitro* selection procedure and *Agrobacterium*-mediated transformation offer a meaningful tool for development of such tolerant lines (Hossain *et al.*, 2007). This is being done using a number of systems (callus, suspension cultures, somatic embryos, shoot cultures, etc.) which are screened for variation in their ability to tolerate relatively high levels of salt (NaCl) in media (Woodward and Bennett, 2005). In majority of salinity studies, the salt used is NaCl. Several researchers, however, have compared the response of other Cl^- and SO_2^- salts including KCl, Na_2SO_4, and $MgSO_4$ during *in vitro* screening. Different responses were found in *Nicotiana tabacum* when growth on seawater, synthetic seawater, manitol, NaCl and other Cl^- and SO_2^- salts was compared (Chen *et al.*,

1980). Use of multiple salts as a selection pressure will parallel the salinity under field conditions and may be a better choice. Most of works so far done in relation to *in vitro* selection are based on ion-homeostasis and compatible solutes mainly proline pool (He *et al.*, 2009; Hassan *et al.*, 2008; Lu *et al.*, 2007; Gandonou *et al.*, 2006; Singh *et al.*, 2003; Liu and Staden, 2000; Barakat and Abdel-Latif, 1996; Sabbah and Tal, 1990; Hassan and Wilkins, 1988).

2.6.1 *In vitro* selection of salt tolerance in other crops

To cope with abiotic barriers for crop production the mechanism of resistance/tolerance to these stresses is must (Dita *et al.*, 2006). The development of salt-tolerant crops that can tolerate high levels of salinity in the soils would be a practical solution of such problem (Yamaguchi and Blumwald, 2005). Since first report in *Nicotiana sylvestris* (Zenk, 1974) many attempts have been made to produce salt tolerant plants using *in vitro* techniques (Hossain *et al.*, 2007; Gandonou *et al.*, 2006; Zair *et al.*, 2003; Singh *et al.*, 2003; Patnaik and Debata, 1997a,b; Winicov, 1996; Barakat and Abdel-Latif, 1996; Tal, 1994).

In vitro selection and screening for salinity tolerance have been reported in tomato by (Mercado *et al.*, 2000; Yusuf *et al.*, 1994). Aazami *et al.* (2010); conducted an experiment that the response of calli of six tomato cultivars (*Lycopersicon esculentum* Mill.) to salt stress was investigated under *in vitro* conditions. Callus relative growth rate (RGR), dry matter percentage (DM), osmotic potential and proline content were evaluated. Significant differences were found among cultivars regarding above traits. Any increase in salinity levels in the media led to decrease of RGR and in contrast increased DM and osmotic potential in all treatments compared to control. In all cultivars, proline levels increased in response to salinity stress. High callus formation was correlated with low proline content. Differences were also found from callus of 0.2 g in callus relative growth rate (RGR), fresh and dry weights, proline, Na^+ and K^+ contents from 0.2 g callus of hypocotyls in tomato grown under previous salt levels for four weeks. Rooting parameters are the most useful traits for rapid

evaluation and screening of tomato species and segregating populations through *in vitro* shoot apex culture (Cano *et al.*, 1998).

Using *in vitro* selection development of abiotic stress tolerant plants especially for salt reported in a wide range of plant species including cereals, vegetables, fruits and other plant species commercially important (Raia *et al.*, 2011) as for example Cucumber (*Cucumis sativus* L.) by Abu-Romman (2010), Malik *et al.*(2010); Potato (*Solanum tuberosum*) by Sajid and Aftab, (2014), Amin *et al.* (2013), Queiros *et al.* (2007), Ochatt *et al.*(1999), Sabbah and Tal (1990); Sour orange (*Citrus aurantium*) by Koc *et al.* (2009); Sweet potato (*Ipomoea batatas*) by He *et al.* (2009); Tobacco (*Nicotiana tabacum*) by Rout *et al.* (2008); Mungbean (*Vigna radiata*) by Hassan *et al.* (2008); Bermuda grass (*Cynodon transvaalensis* × *C. dactylon*) by Lu *et al.* (2007); Chrysanthemum (*Chrysanthemum morifolium*) by Hossain *et al.* (2007); Sugarcane (*Saccharum* sp.) by Gandonou *et al.* (2006); Mulberry (*Morus* sp.) by Vijayan *et al.* (2003); Wheat (*Triticum aestivum*) by Zair *et al.* (2003); Barakat and Abdel-Latif, (1996), Karadimova and Djambova, (1993), Vajrabhaya *et al.* (1989), Galiba and Yamada, (1988), Bamboo (*Dendrocalamus strictus*) by Singh *et al.* (2003); Kaller grass (*Diplachne fusca*) by Nanakorn *et al.* (2003); Cauliflower (*Brassica oleracea*) by Elavumoottil *et al.* (2003); Strawberry (*Fragaria* × *ananassa*) by Dziadczyk *et al.* (2003); Sunflower (*Helianthus annus*) by Davenport *et al.* (2003); Rice (*Oryza sativa*) by Zinnah *et al.* (2013), Lee *et al.* (2003), Shankhdhar *et al.* (2000), Basu *et al.* (1997), Binh and Heszky (1990); Tomato (*Solanum esculentum*) by Kripkyy *et al.* (2001); Soya bean (*Glycine max*) by Liu and Staden (2000); Palma rosa (*Cymbopogon martinii*) by Patnaik and Debata (1997a); Lemon (*Citrus limon*) by Piqueras *et al.* (1996); Alfalfa (*Medicago sativa*) by McCoy (1987), Safarnejad *et al.* (1996); Rapeseed (*Brassica napus*) by Rahman *et al.* (1995); Indian mustard (*Brassica juncea*) by Jain *et al.* (1990), Kirti *et al.* (1991); Shamouti orange (*Citrus sinensis*) by Ben-Hayyim and Goffer (1989); Wild tomato (*Solanum peruvianum*) by Hassan and Wilkins (1988); Barley (*Hordeum vulgare*) by Ye *et al.* (1987) and flax (*Linum usitatissimum*) by

McHughen (1987). *Sapindus trifoliatus* can tolerate high concentrations of NaCl without affecting growth.

2.6.2 *In vitro* selection for salt tolerant calli in *Capsicum* spp.

Though *in vitro* method is a vary rich method for selecting stress toletent genotype and *Capsicum* also a very important crop, but a few works has been conducted on *in vitro* selection of salt tolerant *Capsicum* spp. Solis-Marroquin *et al.* (2011); conducted an experiment to understand the effect of NaCI salinity on growth and development of somatic embryos of Habanero pepper. They notiched that the addition of 75 and 100 mM NaCI into the medium greatly increased the growth and development of somatic embryos and favored the proliferation but supplementation of 200 and 300 mM NaCI to the medium showed a negative effect. Concentration increases of NaCl caused a significant reduction of the embryos survival rate with the average lethal dose (46%) being registered in the treatment of 100 mM. The results they obtained suggest that somatic embryos of *C. chinense* can tolerate concentrations of NaCl up to 100 mm without their development being affected.

In an experiment Mohammed and Mohammed (2007), reported that leaf explants are higher tolerated to salinity levels than cotyledon explants and the leaf explants produced more shoots exposed to 50 mM NaCl compared with the control condition. During the regeneration process he also found that, enhanced shoot fresh weight as well as shoot survival rate obtained under higher NaCl concentration (200 mM) from leaf explants than with lower salt level (50 mM) but no rooting occurred when the regenerated small shoots were exposed to higher salinity level (200 mM).

2.6.3 *In vitro* selection on the basis of proline accumulation as an indicator of salt tolerance

Proline is the most common compatible osmolyte in plants and has therefore been extensively studied. The accumulation of this amino acid is an important regulatory mechanism under osmotic stress (Bojórquez-Quintal *et al.*, 2014;

Huang *et al.*, 2013). Proline is a multifunctional amino acid (Szabados and Savouré, 2010). In many plant species, the accumulation of proline has been associated with tolerance to salt stress and has even been used as a marker to select tolerant genotypes (Ashraf and Harris, 2004). However, a negative correlation between the accumulation of proline and salt tolerance has also been reported, indicating discrepancies in its function (Lutts *et al.*, 1999; Chen *et al.*, 2007). Proline accumulation is made possible by the increase in the expression and activity of the synthesis enzymes (pyrroline-5-carboxylate synthetase, P5CS; pyrroline-5-carboxylate reductase, P5CR) or by the decrease in the degradation enzymes, proline dehydrogenase or proline oxidase (PDH or POX), and P5C dehydrogenase (P5CDH) (Huang *et al.*, 2013). Under salt stress, the *P5CS1* and *PDH* genes are positively and negatively regulated, respectively (Jaarsma *et al.*, 2013; Verslues and Sharma, 2010; Kishor *et al.*, 2005). Similarly, the overexpression of the *P5CS* gene increases proline synthesis under salt stress and improves tolerance to salt (Hmida-Sayari *et al.*, 2005; Kishore *et al.*, 1995).

Chapter III

Materials and Methods

CHAPTER III

MATERIALS AND METHODS

The experiment was conducted in three different steps, viz. selection of plant material for explants using ten genotypes of *Capsicum* spp., *in vitro* callus induction using the selected five genotypes and *in vitro* salt tolerance assay at different concentration of NaCl (0 mM, 50 mM, 100 mM, 150 mM and 200 mM). The materials and methods of this experiment are presented in this chapter under the following headings –

3.1 Experimental Site

The experiment was conducted at the Genetic Engineering Laboratory, Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University, Dhaka. The geographical location of the place is about 23.77° North latitude and 90.37° East longitude (Anonymous, 2014). The location of Sher-e-Bangla Agricultural University in Bangladesh map is shown in Appendix I.

3.2 Experimental materials

Genotypes of *Capsicum* spp. were collected from Spices Research Centre, Bogra and Bangladesh Agricultural Research Institute, Joydebpur, Gazipur. Five genotypes from them were selected as explant source on the basis of their germination ability. The list of initial five genotypes is given in Table 1.

Table 1. List of the selected genotypes of *Capsicum* spp. for getting explants *in vitro*

Sl. No.	Genotype No.	Accession No.	Collected From
01	G_1	CO 611	SRC, Bogra
02	G_2	CO 525	SRC, Bogra
03	G_3	SRC 02	SRC, Bogra
04	G_4	SRC 05	SRC, Bogra
05	G_5	SRC 14	SRC, Bogra

3.3 Laboratory materials

The instruments, chemicals, glasswares and other accessories used in this study are stated below:

3.3.1. Instruments

Aautoclave, hotplate with magnetic stirrer, automatic drying oven, freezers, microwave oven, dark incubator, laminar air flow cabinet, pipette, pipette pump, plant growth chamber, water distillation system, pH meter, electric oven, fine and course electric balance, spectrophotometer and digital camera .

3.3.2 Glass wares

Petri dishes, test tubes, conical flasks, beakers, volumetric flasks, measuring cylinders and glass pipette.

3.3.3 Other accessories

Autoclave tape, hand sprayer, stainless steel scalpel, forceps, scissors, wash bottle, mortar and pestle, marker pen, pencil, Whatman no.1 and 3 filter paper and sieve.

3.3.4 Chemicals

3.3.4.1 Chemicals for culture media

MS medium with vitamins (powder) (Duchefa, Netherlands) was used for seed germination, callus induction and salt tolerance assay. The MS powder includes macro nutrients, micro nutrients, iron source and vitamins (Appendix II) Sucrose was used for carbon source and agar was used for solidification of culture media. Growth hormones such as, Napthalene acetic acid (NAA), 2,4- Dichlorophenoxy acetic acid (2,4-D) and Kinetin (KIN) were used in the media for callus induction and growth, NaCl (laboratory grade) was used in the medium for giving stress to the calli, NaOH (10 N, 1 N) and HCl were used for adjusting pH, KCl (3 M) to keep pH at neutral level, methilated spirit was used in lamp .

3.3.4.2 Chemicals for sterilization

Absolute ethanol, 70% ethanol, fungicide (carbendazim), mercuric chloride (0.1% $HgCl_2$), Tween-20, and detergents were used for sterilization of seeds and other accessories such as scalpel, forcep, scissor and spatula.

3.3.4.3 Chemicals for proline extraction

Ninhydrin, glacial acetic, sulphosalicylic acid and toluene were used for extraction of proline from control and stressed calli.

3.4 Culture media

Three types of media were used for this experiment. Basic Murashige and Skoog (1962) medium (powder) was used as *in vitro* aseptic seed germination and raising of seedlings. MS media supplemented with auxin (NAA and 2,4-D) and cytokinin (KIN) were used as callus induction media and this medium containing different concentrations of NaCl (0 mM, 50 mM, 100 mM, 150 mM and 200 mM) were used for salt stress treatments. The composition of basal MS medium has been presented in Appendix II. The methods of preparation of these three types of media are stated below and some of the steps of media preparation are presented in Plate 1.

3.4.1 Preparation of media for germination

A glass beaker (1 L) filled with 800 mL double distilled water was taken on the hot plate with magnetic starrier. Stirring speed was adjusted at optimum level. In stirring condition 4.4 g of MS powder was added.Then 30 g of sucrose was added. After proper mixing the pH of the solution was adjusted to 5.8 with the addition of 0.1N NaOH and/or 0.1 N HCl whichever was necessary. The volume was made to the 1000 mL by adding double distilled water. The prepared media were equally divided into two 1000 mL conical flasks (500 mL each). 4 g agar was added in each flask before autoclaving. The medium was allowed to cool and aliquote under the laminar hood. The media was stored at 4±1°C until use.

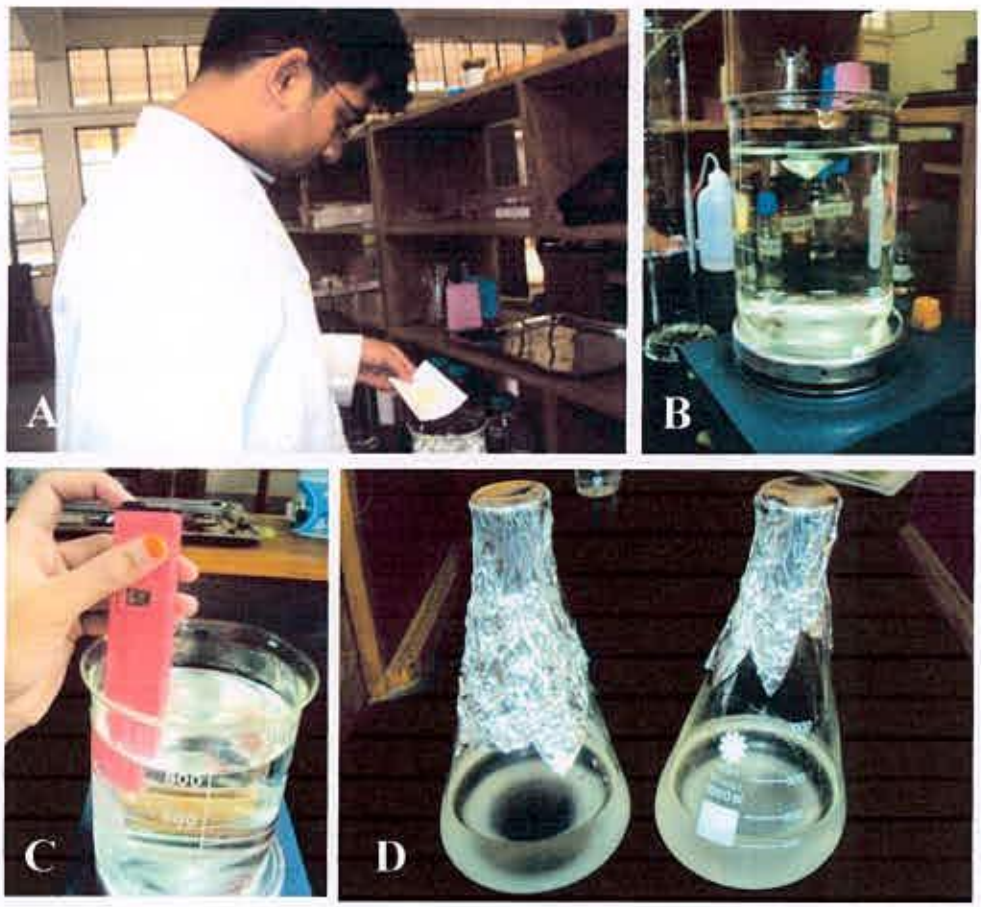


Plate 1. Media preparation: (A) Adding of ingredients of MS medium, (B) Stirring of medium ingredients (C) pH adjustment (D) Media with agar before sterilization

3.4.2 Preparation of media for callus induction

MS medium supplemented with different hormones viz., NAA, KIN and 2,4-D. was used as callus induction media. The hormones were added in the medium after adding sucrose. Stock solutions for NAA, KIN and 2,4-D were prepared at 100× concentrations. 1 mL of stock solution will provide 0.1 mg NAA, KIN and 2,4-D per litre of MS medium. The preparations of stock of these hormones are stated below.

3.4.2.1 Preparation of NAA stock

2.0 mg/L of NAA was used for callus induction and treatment media. For preparing 0.1 mg/L NAA stock 10 mg NAA was taken in a 100 mL beaker and then dissolved with few drops of 1 N NaOH solution. The volume was made up to the 100 mL with sterilized distilled water and stirred well until mixed properly. 20 mL/L stock provided 2 mg/L NAA.

3.4.2.2 Preparation of 2,4-D stock

5 mg/L of 2,4-D was used for callus induction and treatment media. For the preparation of 0.1 mg/L 2,4-D stock 10 mg 2,4-D was taken in a 100 mL beaker and then dissolved with few drops of 1N NaOH solution. The volume was made up to the 100 mL and stirred well until fully mixed. 50 mL/L stock provided 5 mg/L of 2,4-D.

3.4.2.3 Preparation of KIN stock

0.5 mg/L of KIN was used for callus induction and treatment media. For the preparation of 0.1 mg/L KIN stock 10 mg KIN was taken in a 100 mL beaker and dissolved with few drops of 1N NaOH solution. The volume was made up to the 100 mL and stirred well until fully mixed. 5 mL/L stock provided 0.5 mg/L of KIN.

3.4.3 Preparation of media for salt tolerance assay

Callus induction media supplemented with different concentrations of NaCl (0 mM, 50 mM, 100 mM, 150 mM and 200 mM) were used for salt tolerance assay. The molecular weight of NaCl is 58.44 g. For preparing 1 M NaCl solution 58.44 g NaCl was added in 1 L of distilled water. So, for preparing 50 mM concentration, 2.92 g of NaCl was added to 1 L of MS medium. 5.84 g of NaCl was added to 1 L of MS medium for getting 100 mM concentration. 8.77 g of NaCl was added to the 1 L of MS medium to get 150 mM concentration and to obtain 200 mM concentration; 11.68 g of NaCl was added to the 1 L of MS medium.

3.5 Preparation of other required solution

3.5.1 Preparation of 0.1% Carbendazim solution

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

3.5.2 Preparation of 0.1% $HgCl_2$ solution

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

3.5.3 Preparation of 1 N NaOH

40 g of NaOH pellets were weighed and dissolved in 900 mL of sterilized distilled water under stirring condition. The flask was kept in a thermo regulator at 20°C for an hour and made the volume with sterilized distilled water up to 1 L.

3.5.4 Preparation of 70% ethanol

In a 100 mL measuring cylinder, 70 mL of 99.9 percent pure ethanol (absolute ethanol) was poured. Then measuring cylinder was filled with double distilled water up to the mark. The solution was stored in a sterilized glass bottle. This solution was made fresh each time before use.

3.5.5 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.

3.5.6 Preparation of ninhydrin reagent

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

3.6 Sterilization

3.6.1 Sterilization of culture media

The mouth of conical flasks containing prepared culture media were wrapped carefully with aluminum foil and marked with different codes with the help of a water proof permanent glass marker to indicate specific hormonal supplements and different concentration of NaCl. The medium was autoclaved at 15 psi pressure at 121°C for 20 minutes. The medium was then transfer into the culture room and aliquot to petridishes and cooled at 24°C temperature before used. This process was for petridishes but in case of test tube, the culture media were melted perfectly with microwave oven and media was aliquot to test tube and mouths of each test tube were wrapped with aluminum foil and marked with different codes with the help of a water proof permanent glass marker to indicate supplements, then autoclaved at 15 psi pressure at 121°C for 20 minutes.

3.6.2 Sterilization of glassware and instruments

Glass wares like test tubes, beakers, petridishes, pipettes, slides, plastic caps and other instruments such as forceps, needles, scissors, spatula, surgical blades, brush, cotton, instrument stand and aluminum foil were sterilized in an autoclave at a temperature of 121°C for 20 minutes at 15 psi pressure.

3.6.3. 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 thoroughly by

gentle wash of the floors, walls and rakes with detergent. This was followed by careful wipe 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 UV (Ultra Violet) ray for 30 minutes which kills the microbes inside the cabinet. The working surface was wiped 30 minutes before starting the transfer work and frequently during working with 70% ethanol.

3.6.4 Seed sterilization

At first about 20 seeds of each selected *Capsicum* lines were taken and soaked in small beakers for 48 h separately. Then they were taken under the laminar hood and washed three times with sterilized distilled water. After that they were surface sterilized with 70% ethanol for five minutes, rinsed three times in sterile distilled water, soaked in a solution of 0.1% fungicide (Carbendazim) for 4 minutes, then again rinsed three times in sterile distilled water and soaked in a solution of 0.1% $HgCl_2$ plus 2 drops of Tween-20 for five minutes and washed three times with sterilized distilled water. Seed sterilization is shown in Plate 2A.

3.7 *In vitro* raising of seedling as source of explants

The sterilized seeds of selected genotypes (Table 1) were inoculated in hormone free MS (Murashige and Skoog, 1962) basal medium containing 30 g sucrose and 0.8% agar adjusted with pH 5.8. All the steps of inoculation were performed under laminar hood to prevent contamination. 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 $\mu mol\text{-}m^{-2}\text{-}s^{-1}$) 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-D).

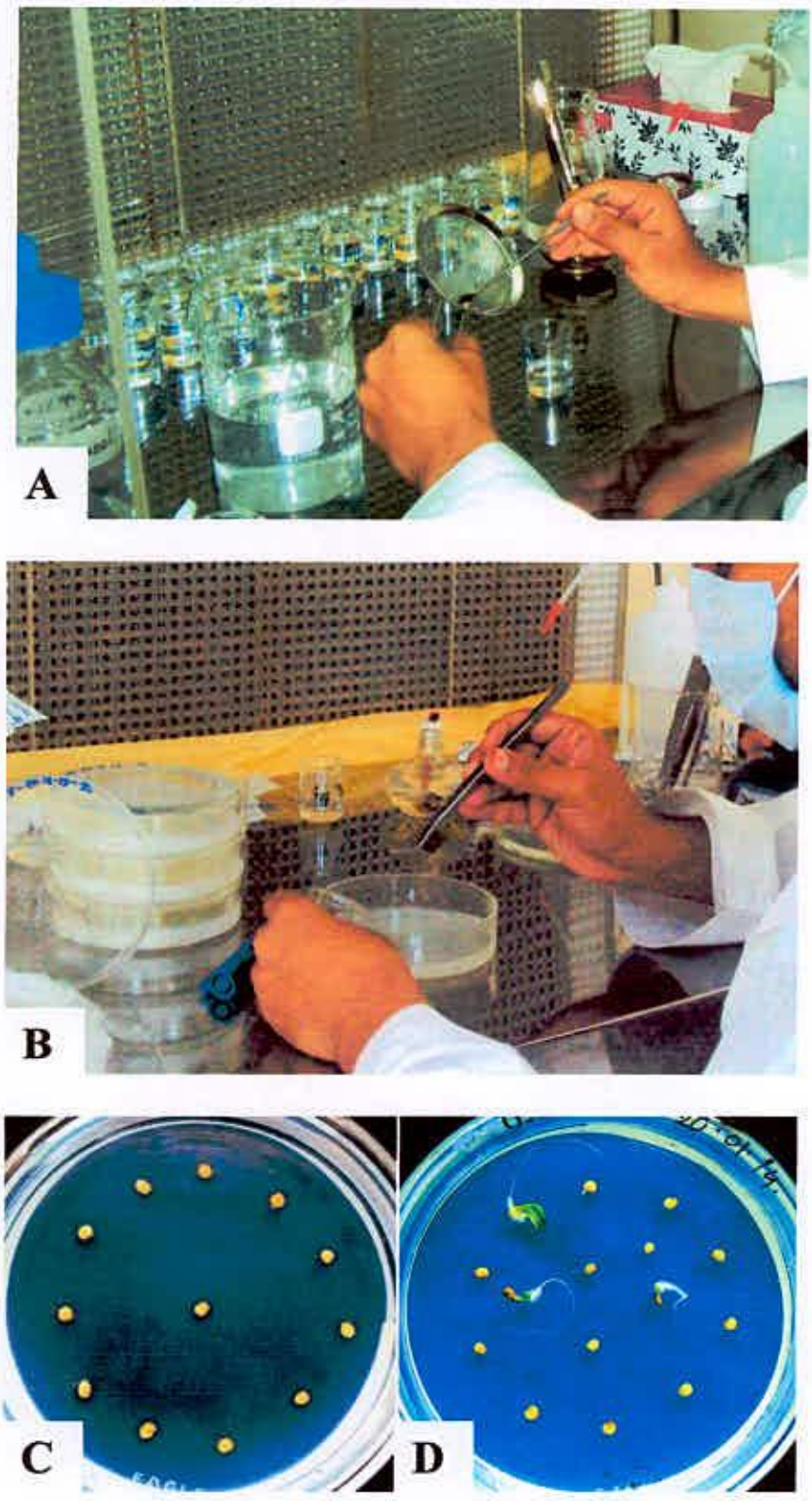


Plate 2. Seed sterilization, inoculation and rising of seedling: (A) Seed sterilization (B) Seed inoculation in hormone free basal MS medium (C) Inoculated seeds (D) Germinated seedling for collection of explants

3.8 Explant collection, inoculation and subculture

The explants (cotyledons leaves and nodal segments) were collected from the 20 days old seedlings. Cotyledone and nodal segments were excised at approximately 0.5 cm segments. For callus induction the protocol reported by Rakshit *et al.* (2007) was used. Briefly, the explants were cultured on MS basal medium supplemented with 2 mg/L NAA, 0.5 mg/L KIN and 5 mg/L 2,4-D for callus formation. The media were solidified with 0.8% Agar and the pH was adjusted to 5.8 before sterilization (121°C for 15 min). The cultures were incubated at 16 h/8 h light/ dark photoperiod with the illuminations of white fluorescence lights (40-50 $\mu mol\text{-}m^{-2}\text{-}s^{-1}$) at 25±2 °C for 4 weeks. Calli were cut into 5 mm pieces for subculturing onto freshly prepared medium after every four weeks. The explants collection, inoculation and subculture (Plate 3) were done for all five genotypes with three replications.

3.9 Precautions to ensure aseptic condition

All inoculation and aseptic manipulations were carried out under laminar air flow cabinet. The cabinet was usually switched on with Ultra Violet light (UV) half an hour before use and wiped with 70% ethanol to reduce the chances of contamination. The instruments like scalpels, forceps, needles, surgical blades, scissor, pipettes, slides, plastic caps, spatula, brush, cotton etc. were 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. It is also necessary to wear apron and mask to avoid contamination rate. Other required materials like distilled water, culture vessels, beakers, glass plates, petridishes etc. were sterilized by autoclaving. The neck of test tubes was flamed after open and before closing. Aseptic conditions were followed during each and every operation to avoid the contamination of cultures.

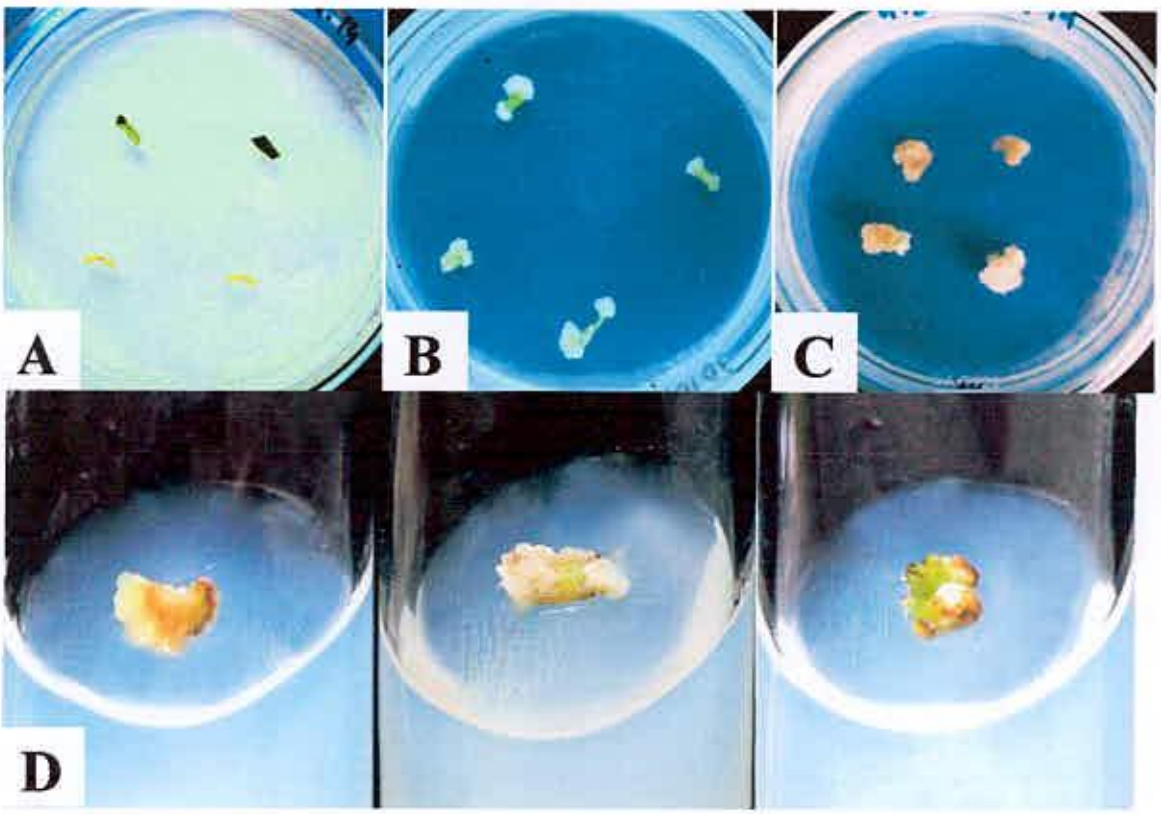


Plate 3. Explant inoculation, callus initiation and subculture: (A) Inoculation of cotyledonary leaves and nodal segments (B) Callus induction (C) Fully developed calli (D) Subculture of calli

3.10 Salt tolerance assay

The salt tolerance assay was performed as described by Zeba (2009). To evaluate the effect of NaCl on biomass changing of callus size and weight, calli pieces of 5 mm size were transferred on to the MS medium supplemented with different NaCl concentrations (0 mM, 50 mM, 100 mM, 150 mM, and 200 mM). Culture condition was same as describe in 3.8. After 4 weeks, all treatments were evaluated on the basis of their change in size and weight from the day of inoculation to the 18^{th} and 36^{th} day.

3.11 Determination of Proline content

3.11.1 Proline extraction from calli

Free proline content was estimated according to Bates *et al.* (1973) method. At first 0.5 g of fresh callus sample from each treatment was taken separately. Then they were homogenized in 3 percent aquous sulphosalicylic acid with the help of mortar and pestle. Then the solutions were filtered by What man No. 1 filter paper. 2 mL supernatant was mixed with 2 mL ninhydrin and 2 mL glacial acetic acid in a test tube and covered with aluminum foil. They were then boiled at 100°C for 1 h in water bath. By cooling the tubes in an ice bath the reaction was stopped. Then 4 mL of toluene per test tube were added and shaken vigorously for 15-20 seconds and were allowed to separate in layers for 30 minutes. The chromophore formed was extracted and the absorbance of the resulting organic layer was measured with the help Spectrophotometer at 520 nm.

3.11.2 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 IV). 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:

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

Here, x= proline content in ppm

3.12 Data recorded and statistical analysis

Data were collected and evaluated in terms of the biomass callus weight with digital fine balance and size (diameter) using verneer slide calipers, at 0 DAT (Days after treatment), 18 DAT and 36 DAT. Tubes were arranged on the shelves of laboratory growth chamber according to a Completely Randomized Design (CRD). 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 software. Data were processed with analysis of variance (ANOVA) and the means were compared using DMRT (Duncan's New Multiple Range Test) at 5% level of significance.

Chapter IV

Results and Discussion

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 five *Capsicum* genotypes under different NaCl concentrations for salt stress on callus initiation and biomass changing of callus in terms of fresh weight and diameter. This study dealt with the *in vitro* selection of salt 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 salt stress tolerance for future gene transfer program. As salt stress in soil is variable and plant tolerance depends on the stage of plant development, in this study, calli were phenotyped at several salt stress conditions and at the most sensitive stages (18 Days and 36 Days old calli). The genotypes were denoted as G_1, G_2, G_3, G_4 and G_5. The NaCl concentrations were denoted as T_1 (0 mM), T_2 (50 mM), T_3 (100 mM), T_4 (150 mM) and T_5 (200 mM).

4.1 *In vitro* seed germination and response of explants to callus induction medium

The seeds of five *Capsicum* genotypes were surface sterilized and placed on hormone free basal MS medium in petridishes. The seeds started to germinate within twelve days of incubation and took about 20 days for maximum germination. Within one month of seed inoculation the length of seedlings were in suitable size to serve as explants source for cotyledonary leaves and nodal segment. Berljak (1999) agreed with this regeneration process. He also regenerated pepper plants *in vitro.* Some other previous studies about the seed germination and regeneration were also in agreement with this germination and regeneration processes (Swamy *et al.,* 2014; Ashrafuzzaman *et al.,* 2009; Santana-Buzzy *et al.*, 2009; Kaparakis and Alderson, 2008; Rakshit *et al.*, 2007).

In this study, the cotyledonary leaves and nodal segments from five *Capsicum* genotypes were segmented into about 0.5 cm sizes and inoculated on MS media

supplemented with auxin (NAA and 2,4-D) and cytokinin (KIN) and incubated in light. Within one week the explants became enlarge and start swelling. Within four weeks of culture the swelled explants gradually turned into whitish green coloured callus. For amplification and maintainance, the callus of each genotype cut into pieces aseptically and subcultured in the fresh callus induction medium. The gradual change of cotyledonary leaves and nodal segment to the callus is presented in Plate 3. Different *Capsicum* genotypes showed significant variation for days to callus initiation, growth and callus type. The genotype G_1 and G_3 was produced compact type and genotype G_2, G_4 and G_5 produced friable type of callus. The induction and proliferation of callus largely depends on the explants, growth regulators, genotypes and light/ dark incubation etc. Similar to this experiment, cotyledonary leaves and nodal segments were also used by several researchers for callogenesis (Kehie *et al.*, 2011; Kumar and Tata, 2010; Ashrafuzzaman *et al.*, 2009; Siddique and Anis, 2006). However, unlike this experiment some researchers used other explants for callogenesis like, shoot-tip (Berljak, 1999), axillary shoot tip (Sanatombi *et al.*, 2007), leaf explants (Venkataiah *et al.*, 2004; Fari *et al.*, 1990), leaf disc (Zhu *et al.*,1996), petiole (Nanakorn *et al.*, 1994) and embryo (Christopher *et al.*,1986). Like in this study, NAA, 2,4-D and KIN hormones were used by different researchers (Mangang, 2014; Otroshy *et al.*, 2011b; Rakshit *et al.*, 2007; Nanakon *et al.*, 1994). However in disagreement with this result, other hormones like, IAA, BAP and GA_3 were also used by some other researchers (Swamy *et al.*, 2014; Verma *et al.*, 2013; Prakash *et al.*, 1997). Studies of some of the previous researchers have revealed that better response of callus was obtained when callus cultures were kept only in dark (Aftab *et al.*, 1996; Chen *et al.*, 1988) which was not in agreement with the incubation condition of this research where the calli were incubated in light.

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

To illustrate the interaction effect of genotype and NaCl treatment (salt stress), 5 mm pieces of calli containing 0.1 g weight, were inoculated in the MS medium

supplemented with 0 mM, 50 mM, 100 mM, 150 mM and 200 mM of NaCl. Callus size and weight was the indicator for salt stress tolerance. So, the callus diameter and weight were assayed two times after treating with different NaCl concentrations. Initial weight and diameter of each callus of every genotype were also measured to compare with those data which were taken after 18 and 36 days after treating with NaCl. The callus growth was the highest in control condition (0 mM of NaCl) and at severe to more severe stresses gradually it decreases except with some genotypic variations as the salt stress (NaCl concentration) increases that is, in 50 mM, 100 mM, 150 mM and 200 mM respectively. There were also variations among the genotypes based on their stress period (Appendix III). Initially the calli looked fresh and vigorous and it continues up to 18 days of stress period with some genotypic variation and almost all of them were whitish green (Plate 4 and Plate 5). At prolonged stress period that is, from 18 days to 36 days they gradually became brown to dark brown and eventually died (Plate 6).

For controling as well as providing salt stress, MS media was used and similar to this study, some researchers' studies have suggested that among all the media tested for callus induction and proliferation, the best medium was MS medium (Baksha *et al.*, 2002; Aftab *et al.*, 1996; Guiderdoni, 1986; Liu and Chen, 1974) but almost all of them modified their media according to their experiment in the concentrations and type of carbon source, growth regulators, macronutrients, micronutrients, vitamins etc. The callus diameter and fresh weight were measured up to 36 days 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 are discussed below:

4.2.1 Performance of different genotype on the basis of their callus biomass change

Calli sizes were significantly varied among different *Capsicum* genotypes (Appendix III). NaCl treatment was given in every genotype and data were taken

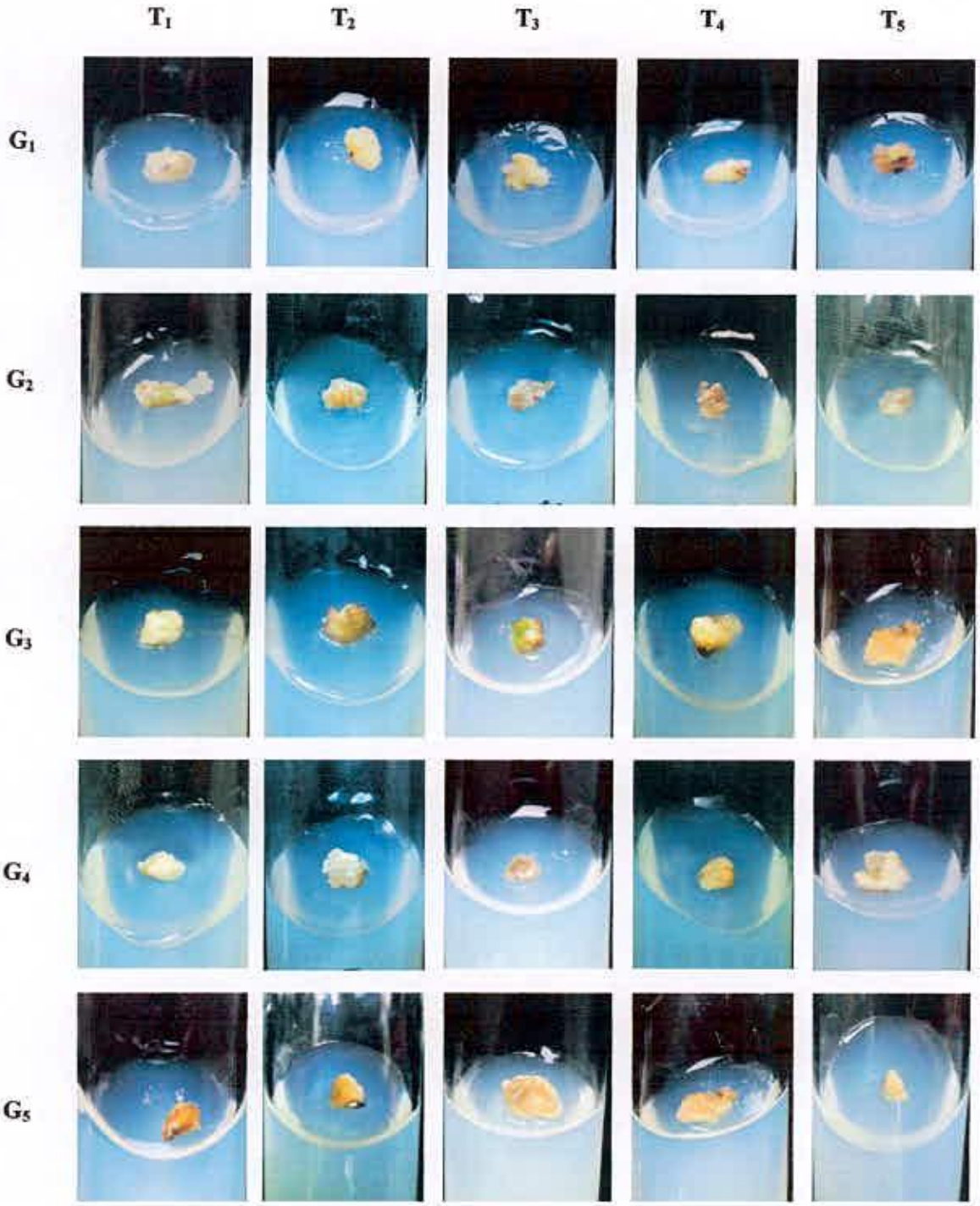


Plate 4. Biomass changing of callus at 0 DAT (days after treatment) under different NaCl treatments (T_1 = 0 mM, T_2 = 50 mM, T_3 = 100 mM, T_4 = 150 mM, T_5 = 200 mM (NaCl); G_1 = CO 611, G_2 = CO 525, G_3 = SRC 02, G_4 = SRC 05, G_5 = SRC 14)

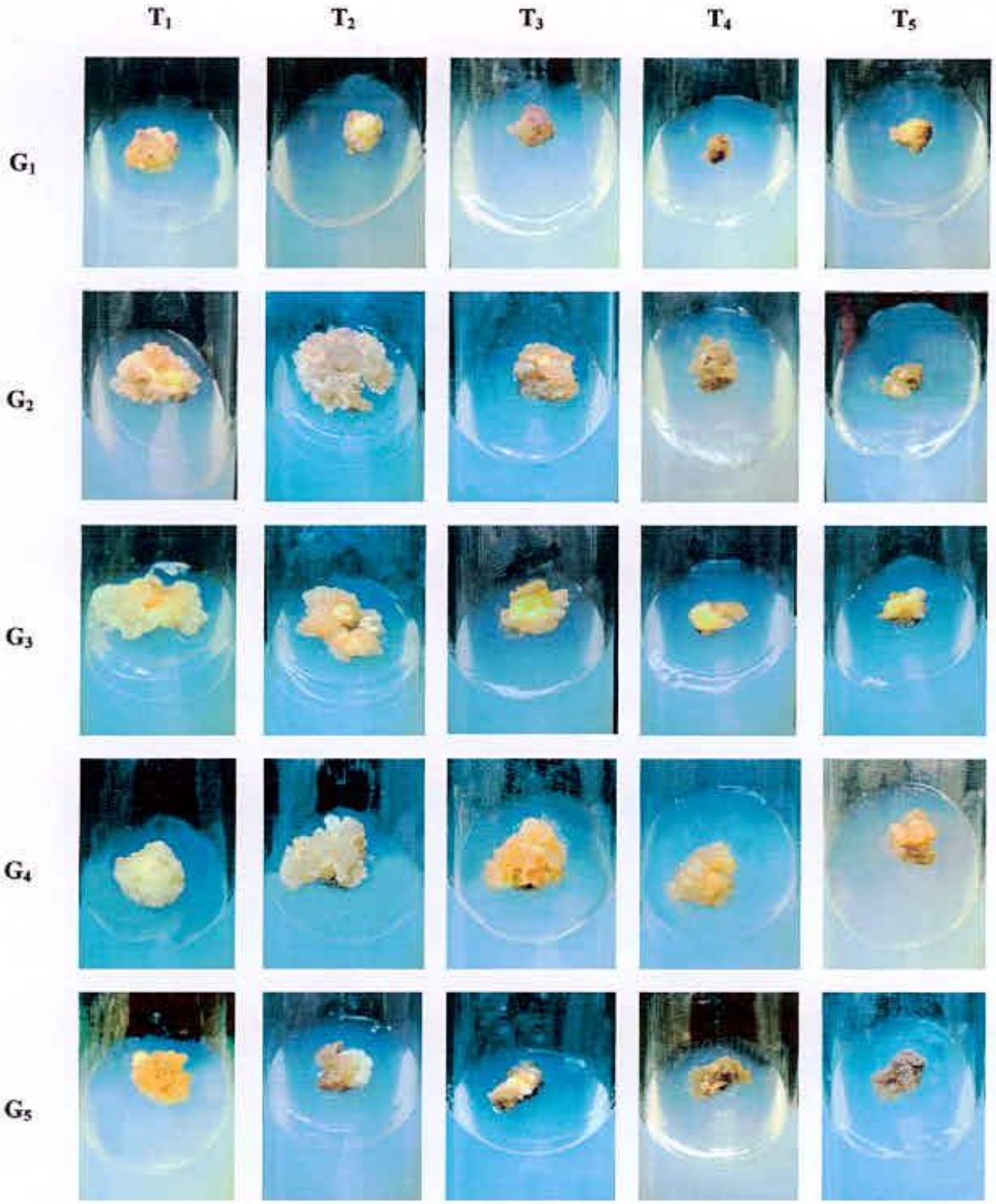


Plate 5. Biomass changing of callus at 18 DAT (days after treatment) under different NaCl treatments (T_1 = 0 mM, T_2 = 50 mM, T_3 = 100 mM, T_4 = 150 mM, T_5 = 200 mM (NaCl); G_1 = CO 611, G_2 = CO 525, G_3 = SRC 02, G_4 = SRC 05, G_5 = SRC 14)

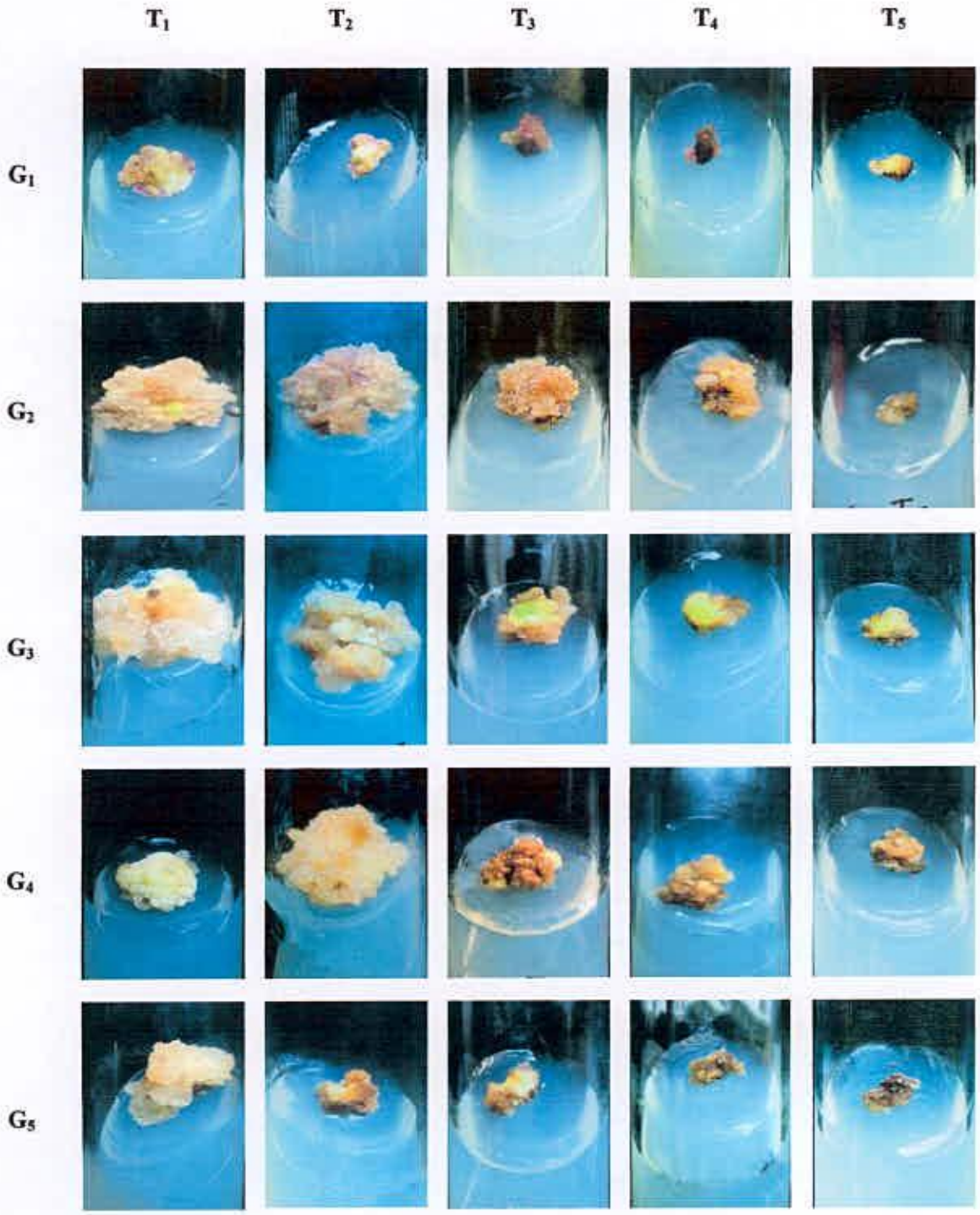


Plate 6. Biomass changing of callus at 36 DAT (days after treatment) under different NaCl treatments (T_1 = 0 mM, T_2 = 50 mM, T_3 = 100 mM, T_4 = 150 mM, T_5 = 200 mM (NaCl); G_1 = CO 611, G_2 = CO 525, G_3 = SRC 02, G_4 = SRC 05, G_5 = SRC 14)

at 18 DAT and 36 DAT. The biggest size (diameter) of callus was found in the genotype G_3 (13.37 mm), which was statistically similar with genotype G_2 (12.27 mm) and G_4 (12.15 mm) at 18 DAT and gradually increased upto 36 DAT (16.81 mm, 15.57 mm and 16.27 mm, respectively) (Table 2). The smallest size of callus was found in genotype G_1 (7.268 mm and 7.639 mm, respectively at 18 and 36 DAT). At 18 DAT the heaviest callus was found in genotype G_2 and G_4 (1.24 g and 1.318 g, respectively) and at 36 DAT in genotype G_2, G_3 and G_4 (1.895 g, 1.697g and 1.636 g, respectively). The lowest weight of callus was found in genotype G_1 and G_5 (0.5124 g and 0.5146 g, respectively) at 18 DAT and only in genotype G_1 (0.5156 g) at 36 DAT (Table 2). Different genotypes showed different response to callus induction and growth, and it was necessary to optimize *in vitro* callus induction protocols for the specific *Capsicum* genotypes as were done by other researchers (Sanatombi and Sharma, 2006; Ezura *et al.*, 1993).

Similar significant genotypic variation was found by Swamy *et al.* (2014) while working with five varieties of *Capsicum*. Significant differences were also found among cultivars of different plants regarding above traits by Aazami *et al.* (2010), Vijayan *et al.* (2003), Tewary *et al.* (2000) and Ben-Hayyim (1989).

4.2.2 Effect of different NaCl treatments on callus biomass change

The main effect of different concentrations of NaCl showed significant variation on biomass changing of callus at different DAT. In all of the genotypes, 0 mM (T_1), 50 mM (T_2) and 100 mM (T_3) treatments showed the best callus size at 18 DAT (8.053 mm, 8.266 mm and 7.24 mm, respectively) and at 36 DAT only 0 mM (T_1) and 50 mM (T_2) showed the best callus size (10.45 mm and 10.60 mm, respectively) whereas 200 mM (T_5) NaCl showed the smallest callus size for both 18 DAT and 36 DAT (4.615 mm and 4.371 mm, respectively) (Table 3).

Table 2. Performance of different genotype on the basis of their callus biomass change

Genotypes	Biomass size (mm)			Biomass weight (g)		
	0 DAT	18 DAT	36 DAT	0 DAT	18 DAT	36 DAT
G_1	5	7.268 c	7.639 c	0.1	0.5124 c	0.5156 c
G_2	5	12.27 a	15.57 a	0.1	1.24 a	1.895 a
G_3	5	13.37 a	16.81 a	0.1	0.9377 b	1.697 a
G_4	5	12.15 a	16.27 a	0.1	1.318 a	1.636 a
G_5	5	9.706 b	12.51 b	0.1	0.5146 c	0.8552 b
$LSD_{(0.05)}$	NS	1.722	2.17	NS	0.2483	0.3206

NS Non significant

Means having similar letter(s) is/are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05 level of probability

Table 3. Effect of different NaCl treatments on callus biomass change

Treatments	Biomass size (mm)			Biomass weight (g)		
	0 DAT	18 DAT	36 DAT	0 DAT	18 DAT	36 DAT
T_1	5	8.053 a	10.6 a	0.1	0.2974 b	0.6044 a
T_2	5	8.266 a	10.45 a	0.1	0.3557 a	0.5421 b
T_3	5	7.24 a	7.639 b	0.1	0.2595 c	0.1931 c
T_4	5	5.673 b	6.302 b	0.1	0.1595 d	0.1876 c
T_5	5	4.615 b	4.371 c	0.1	0.1439 d	0.1471 d
$LSD_{(0.05)}$	NS	1.343	1.601	NS	0.03206	0.03926

NS Non significant

Means having similar letter(s) is/are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05 level of probability

Any increase in salt levels in the media led to decrease of callus growth was also found by Aazami *et al.* (2010). The heaviest callus was found in 50 mM (T_2) at18 DAT (0.3557 g) and in 0 mM (T_1) at 36 DAT. The lowest biomass weight was observed by 200 mM (T_5) NaCl treatment for both 18 DAT and 36 DAT (0.1471 g and 0.1439 g, respectively) (Table 3). The results of other researchers also agreed with this experiment in regard to the supplementation of NaCl at different concentrations in modified MS media including hormones (Solis-Marroquin *et al.* 2011; Zeba, 2009; Mohammed and Mohammed, 2007).

4.2.3 Interaction effect of genotype and treatment on the basis of callus biomass change

The interaction effect of different *Capsicum* genotypes and NaCl concentrations showed significant variation on biomass changing of callus at different DAT (Table 4). 5 mm sizes of calli were inoculated for all of the genotypes of *Capsicum* under different NaCl concentrations and evaluated at 18 DAT and 36 DAT. At 18 DAT the biggest callus was observed in G_3T_1 (13.66 mm) that is genotype G_3 treated with T_1 (0 mM NaCl) and the size continually increased (18.44 mm) up to 36 DAT. At 18 DAT the smallest callus was found in G_5T_5 (3.301 mm) which was statistically identical with G_2T_4 (3.63 mm), G_2T_5 (3.91 mm), G_4T_5 and G_5T_3 (4.26 mm) (Table 4) whereas in G_5T_5 interaction the lowest callus size (3.12 mm) was also observed at 36 DAT which was statistically identical with G_5T_4 (3.78 mm) and G_4T_5 (3.22 mm) among all the genotypes. Interaction of *Capsicum* genotypes and salinity treatments significantly affects the callus weight at 18 DAT. 0.1 g was the initial weight for all the genotypes at all NaCl concentrations. Maximum callus weight was found in G_3T_1 interection (0.6729 g) and whereas minimum callus weight was found in G_5T_4 (0.0638 g) (Table 4). At 36 maximum callus weight was found in G_3T_1 (0.9977 g) whereas minimum callus weight was found in G_5T_4 (0.0122 g) which was statistically identical with G_5T_3 (0.0185 g) (Table 4). Several authors reported the use of NaCl for *in vitro* salt tolerant screening in different plants (Vijayan *et al.*, 2003; Zhao *et al.*, 2007). In this study, the adaption capacity to different NaCl level varied with the genotype's degree of tolerance.

Table 4. Interaction effect of genotype and treatment on the basis of callus biomass change

Interaction	Biomass size (mm)			Biomass weight (g)		
	0 DAT	18 DAT	36 DAT	0 DAT	18 DAT	36 DAT
G_1T_1	5	6.974 fgh	8.522 g	0.1	0.346 e	0.463 f
G_1T_2	5	6.416 ghij	6.227 ijk	0.1	0.274 f	0.275 h
G_1T_3	5	5.567 ijkl	4.727 lmn	0.1	0.254 g	0.175 jk
G_1T_4	5	4.952 lmn	4.557 lmn	0.1	0.127 l	0.163 k
G_1T_5	5	6.245 ghijk	5.258 klm	0.1	0.173 j	0.123 l
G_2T_1	5	6.571 ghi	8.669 g	0.1	0.114 l	0.579 e
G_2T_2	5	8.256 de	9.924 ef	0.1	0.460 c	0.673 c
G_2T_3	5	7.701 ef	7.298 hi	0.1	0.427 d	0.281 h
G_2T_4	5	3.633 o	6.714 hij	0.1	0.228 i	0.455 f
G_2T_5	5	3.910 no	4.433 mno	0.1	0.250 gh	0.280 h
G_3T_1	5	13.660 a	18.44 a	0.1	0.672 a	0.997 a
G_3T_2	5	12.510 b	15.54 b	0.1	0.541 b	0.827 b
G_3T_3	5	9.692 c	12.38 c	0.1	0.218 i	0.263 h
G_3T_4	5	7.383 efg	8.777 fg	0.1	0.144 k	0.193 j
G_3T_5	5	5.348 jklm	5.815 jkl	0.1	0.117 l	0.157 k
G_4T_1	5	6.498 ghi	6.373 ijk	0.1	0.234 hi	0.372 g
G_4T_2	5	5.994 hijkl	11.95 cd	0.1	0.343 e	0.671 c
G_4T_3	5	8.969 cd	8.566 g	0.1	0.284 f	0.227 i
G_4T_4	5	7.257 efg	7.684 gh	0.1	0.233 i	0.114 l
G_4T_5	5	4.269 mno	3.224 op	0.1	0.096 m	0.125 l
G_5T_1	5	6.559 ghi	11 de	0.1	0.118 l	0.609 d
G_5T_2	5	8.150 de	8.616 g	0.1	0.158 jk	0.262 h
G_5T_3	5	4.268 mno	5.219 klm	0.1	0.114 l	0.018 n
G_5T_4	5	5.142 klm	3.780 nop	0.1	0.063 n	0.012 n
G_5T_5	5	3.301 o	3.124 p	0.1	0.080 m	0.048 m
LSD (0.05)	**NS**	**0.998**	**1.158**	**NS**	**0.016**	**0.023**
CV (%)	0.0	9.00	8.97	0.0	8.57	6.22

NS Non significant; G_1 = CO 611, G_2 = CO 525, G_3 = SRC 02, G_4 = SRC 05, G_5 = SRC 14; T_1 = 0 mM, T_2 = 50 mM, T_3 = 100 mM, T_4 = 150 mM, T_5 = 200 mM (NaCl).

Means having similar letter(s) is/are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05 level of probability

The genotypic variation for salt stress tolerance in this study agreed with the result of Rus *et al.* (2001) and Perez-Alfocea *et al.* (1994). Callus growing in this study under the increasing NaCl concentrations reduced relative growth rate. A similar observation was found by Yusuf *et al.* (1994); Cano *et al.* (1998) while Mercado *et al.* (2000) using tissue culture techniques for *in vitro* selection of salt tolerance. The reduced relative growth rate in all *Capsicum* genotypes, except variation in some of the genotypes in this study was also in agreement with Ben-Hayyim (1989); Zhang *et al.* (2004) and Amini and Ehsanpour (2006).

As in this study, they also concluded that *in vitro* plant tissue culture is useful and quick tool to evaluate plant tolerance to salt stress. The addition of NaCl to the culture media decreased the osmotic potential of the media inducing water stress that adversely affected the callus growth. Several The genotypic variations observed in this experiment might be due to the function of different genes controlling the traits. Some of the transcription factor genes regulate stress response such as, *bZIP, WRKY, AP2, NAC, C2H2* zinc finger gene, and *DREB*. These transcription factor genes are capable of controlling the expression of a broad range of target genes by binding to the specific cis-acting element in the promoters of these genes (Gupta and Huang, 2014) and thereby might regulate the synthesis and accumulation of proline, sugar, and LEA proteins that in turn play an integral role in stress tolerance (Song *et al.*, 2011).

4.2.4 Biomass changing of callus size under salt stress

Size of callus of five genotypes was recorded for 18 days and 36 days after treatment (DAT) in different NaCl concentration and significant differences were recorded (Appendix III, Plate 5, Plate 6 and Figure 1). The genotype G_3 showed the highest biomass size in control condition (0 mM NaCl) at 18 DAT and upto 36 Days (13.66 mm and 18.44 mm, respectively) (Plate 5, Plate 6 and Figure 1). Genotype G_2 showed better performance at low to moderately high salt stress condition that is from 50 mM to 100 mM at short duration salt stress period (18 DAT) (Figure 1).

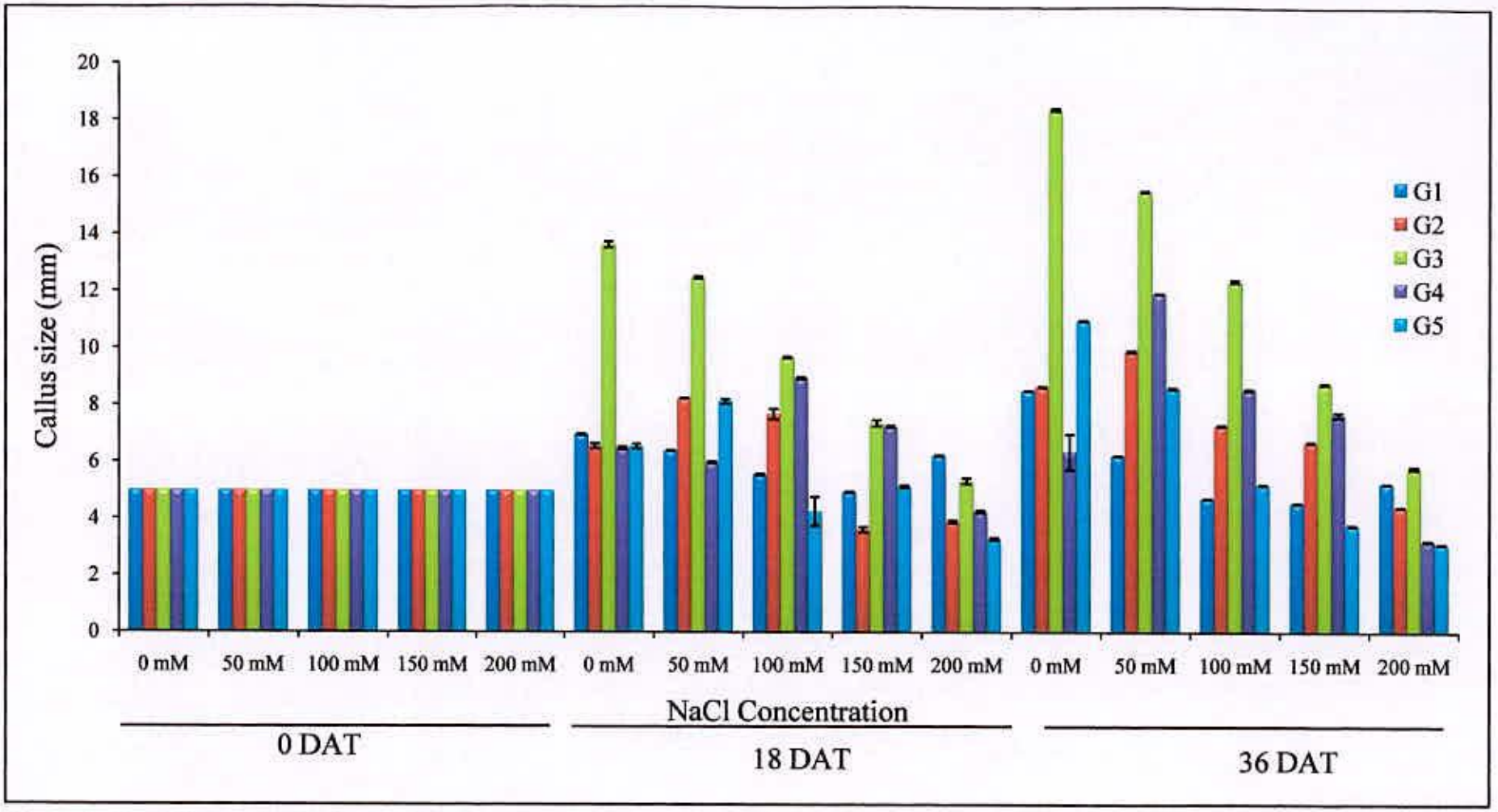


Figure 1. Size (diameter) of callus of five genotypes at 0 DAT, 18 DAT and 36 DAT in different NaCl concentrations (0 mM, 50 mM, 100 mM, 150 mM and 200 mM)

At prolonged stress period that is at 36 DAT G_2 also showed better performance at low salt stress condition (50 mM). Whereas, regarding totypotency, G_3 showed better performance at moderate to severe stress and from short duration (18 DAT) to long duration (36 DAT) stress period. They remained whitish green, fresh and live for long duration (Plate 5 and Plate 6). Under stress condition G_4 initially decreased in size at low salt stress (50 mM) but later as the stress increased it recovered in size at moderate stress (100 mM). It might be due to the adaptation capacity of the genotype. Similar result was observed by Bray *et al.* (2000) in *Mesembryanthemum crystallinum* due to the function of MIP-related genes where turgor was lost at early stage of NaCl stress but later it recovered. Genotypic variation for biomass size is evident in control (0 mM) and in stressed condition (50 mM, 100 mM, 150 mM and 200 mM) (Figure 1). Up to 18 DAT (days after treatment) callus size was not reduced at all at low salt stress (50 mM NaCl) but increased in case of genotype G_2 and G_5 (-1.69 mm and -1.59 mm, respectively) followed by better performance in genotype G_4 (0.505 mm) (Table 5). At moderate stress (50-100 mM NaCl) genotype G_4 increased in callus size (-2.97 mm) and genotype G_2 showed lowest reduction of callus size (0.56 mm) among other genotypes. Later due to stress shock genotype G_2 reduced in size (4.07 mm) at 150 mM of NaCl but later recovered at 200 mM of NaCl (-0.277 mm) (Table 5) due to acclimatization. Similar result was observed by Bray (2000) in *Mesembryanthemum crystallinum* due to the function of MIP-related genes where turgor was lost at early stage of salt stress but later it recovered. It suggests that for short duration salt stress (18 days) G_4 was the best performer at low to moderate salt stress whereas G_2 was the best performer under severe stress. At prolonged stress period i.e., at 36 DAT, also G_4 and G_2 showed better tolerance under low salt stress, i.e., their callus increased in size under low salt stress (-5.57 mm and -1.25 mm, respectively) (Table 5).

4.2.5 Biomass changing of callus weight under salt stress

Biomass weight of calli was recorded in control (0 mM) and in salt stress condition (50 mM, 100 mM, 150 mM and 200 mM) at 0 DAT, 18 DAT and

Table 5. Reduction of callus size from low to severe salt stres

Genotypes	Size reduction (mm)							
	18 DAT				36 DAT			
	0-50 mM	50-100 mM	100-150 mM	150-200 mM	0-50 mM	50-100 mM	100-150 mM	150-200 mM
G1	0.558333	0.848333	0.615333	1.042	2.294533	1.500333	0.169667	-0.701
G2	-1.68567	0.555333	4.068	-0.277	-1.25467	2.625667	0.584333	2.281
G3	1.151667	2.819333	2.309333	2.034667	2.898667	3.157	3.607667	2.961667
G4	0.504	-2.97467	1.711667	2.987533	-5.572	3.379667	0.881667	4.46
G5	-1.59133	3.881667	-0.87333	1.840667	2.383	3.396667	1.439333	0.655667

G_1 = CO 611, G_2 = CO 525, G_3 = SRC 02, G_4 = SRC 05, G_5 = SRC 14

36 DAT. Significant genotypic variation for biomass weight was recorded (Plate 4, Plate 5 and Figure 2). The genotype G_2 showed better performance at low stress and more or less remained constant up to moderate salt stress (100 mM) and then gradually decreased as the salt stress increased at short stress period (18 DAT) (Figure 2). At prolonged stress period G_2 showed the same performance up to 50 mM of NaCl concentration. G_4 increased in their weight up to 50 mM of NaCl concentration (low stress) and then gradually decreased but still up to the severe stress condition (150 mM) it showed the same weight as it was in control condition (0 mM) (Figure 2). At prolonged stress period (36 DAT), G_4 increased in its weight up to 50 mM of NaCl concentration and then gradually descreased as the salt stress increased. Upto 18 DAT (days after treatment) callus weight was not reduced at all at low salt stress (50 mM NaCl) but increased in case of G_2, G_4 and G_5 (-0.35 g, -0.11 g and -0.04 g, respectively) (Table 6). At moderate stress that is at 50-100 mM of NaCl, G_2 continued showing tolerance but G_1 also recovered at this level. At moderate to severe stress, G5 recovered well and followed by G_4. So, G_2, G_4 and G_5 continued showing tolerance from low to moderate severe stage. At prolonged stress period (36 DAT), G_4 followed by G_2 were the best performer under low stress (-0.30 g and -0.09 g, respectively) (Table 6).

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

Proline is one of the well-known osmoprotectants and its accumulation is widely observed in various organisms under salt stress. Significant genotypic variation was found in this experiment regarding proline accumulation under control as well as under salt stress condition (Plate 7). In Plate 7, the amount of proline content (µg/g) acuumulated in calli is presented below each respective test tube. Under control condition (0 mM), G_1 calli showed the highest accumulation of proline (1846.67 µg/g) followed by G_3 (1055 µg/g). Under low stress (50 mM), G_3 calli followed by G_1 calli showed better proline accumulation (1503.33 µg/g and 1046.67 µg/g). Under treatment T_3 (100 mM) G_4 and G_3 increasingly accumulated proline (2968.33 µg/g and 1610 µg/g, respectively). Under

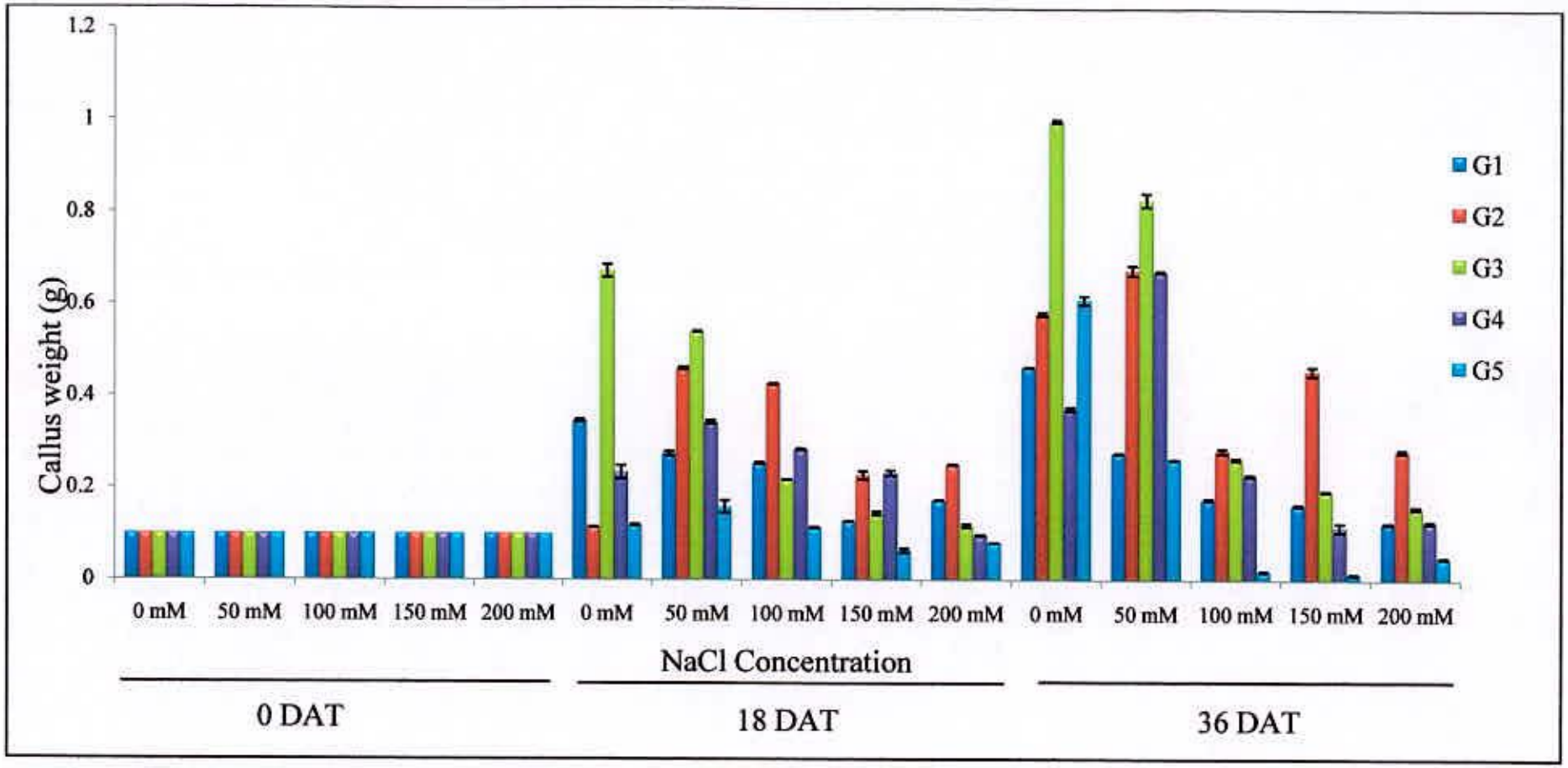


Figure 2. Weight of callus of five genotypes at 0 DAT, 18 DAT and 36 DAT in different NaCl concentrations (0 mM, 50 mM, 100 mM, 150 mM and 200 mM)

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

Genotypes	Weight reduction (g)							
	18 DAT				36 DAT			
	0-50 mM	50-100 mM	100-150 mM	150-200 mM	0-50 mM	50-100 mM	100-150 Mm	150-200 mM
G1	0.0715	0.020567	0.1266	-0.04567	0.1875	0.1004	0.012167	0.039633
G2	-0.346	0.0335	0.198733	-0.02253	-0.09437	0.392467	-0.17383	0.174533
G3	0.131633	0.3232	0.073367	0.0271	0.1705	0.5642	0.069833	0.0358
G4	-0.1087	0.0588	0.051367	0.136067	-0.2995	0.444	0.113233	-0.01147
G5	-0.04003	0.044933	0.0502	-0.0169	0.347333	0.243967	0.0063	-0.03603

G_1 = CO 611, G_2 = CO 525, G_3 = SRC 02, G_4 = SRC 05, G_5 = SRC 14

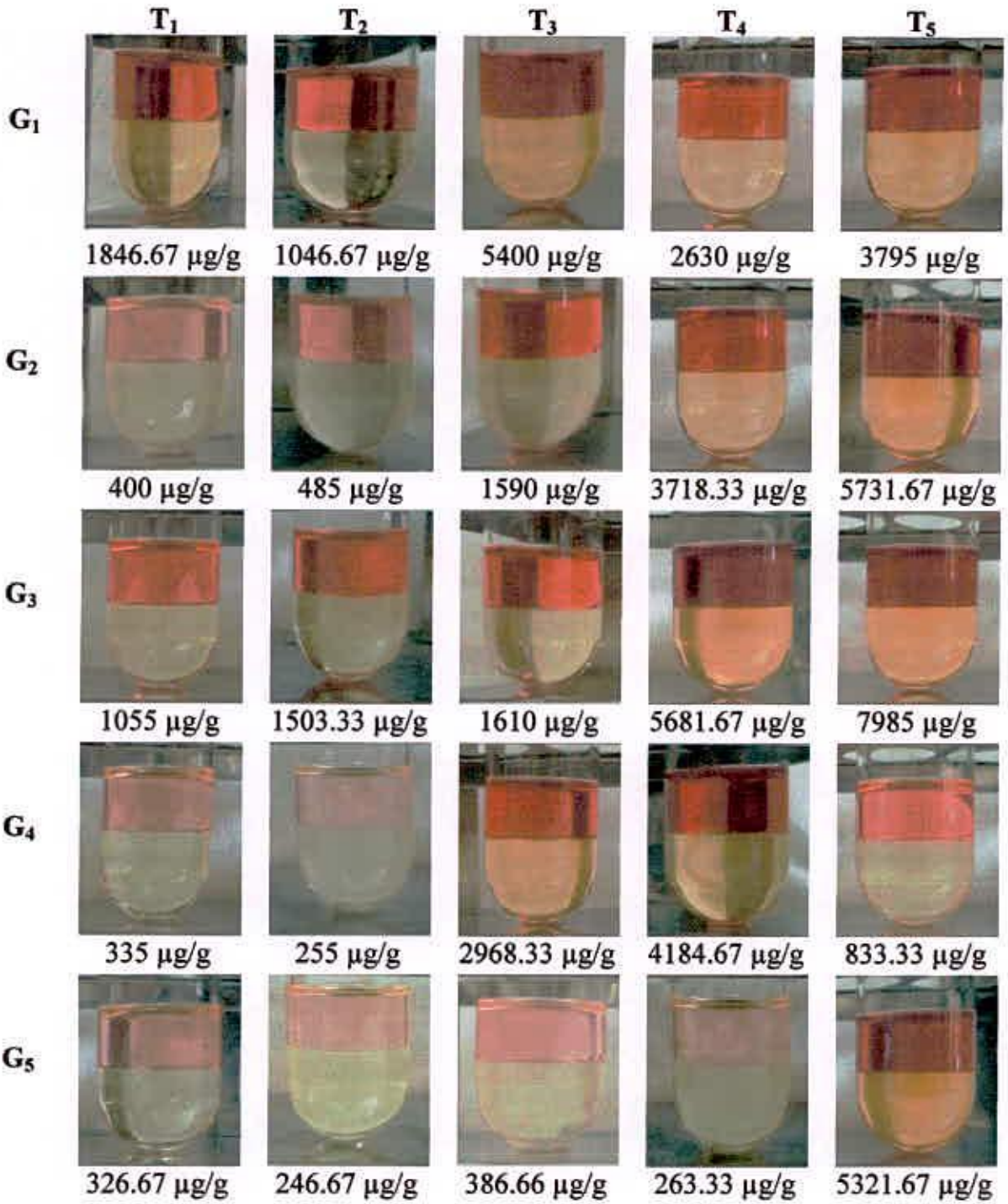


Plate 7. Difference of proline accumulation in calli under different NaCl treatments (T_1 = 0 mM, T_2= 50 mM, T_3=100 mM, T_4=150 mM, T_5=200 mM; G_1= CO 611, G_2 = CO 525, G_3 = SRC 02, G_4 = SR C05, G_5 = SRC 14)

treatment T_4, G_3 followed by G_4 showed better accumulation of proline (5681.67 µg/g and 4184.67 µg/g, respectively). Under treatment T_5 (200 mM), genotype G_3 (SRC 02) showed best accumulation of proline (7985 µg/g). These findings concluded that, G_3 (SRC 02) showed gradual increase of proline accumulation in calli under stress conditions which supported the totipotency trait of this genotype where the calli remained green, fresh and alive (Plate 6) compared to the other genotypes. Ashraf and Harris (2004) also used proline as a marker to select tolerant genotypes. Improved accumulation of proline conferred salt tolerance and this result supported that proline functions to regulate cellular osmotic balance. Additionally, proline is also utilized for protein synthesis. Proline serves to regulate osmotic adjustment. In this experiment proline levels increased with the increasing salt concentrations (Plate 7) which also corresponds with the result of Qasim *et al.* (2003), Ashraf *et al.* (1998, 1994), Dracup (1991). Lutts *et al.* (1999) and Chen *et al.* (2007), however contradicted with this result where they showed a negative correlation between the accumulation of proline and salt tolerance, indicating discrepancies in its function. By the increase or decrease of several enzymes viz., pyrroline-5-carboxylate synthetase, P5CS and pyrroline-5-carboxylate reductase, P5CR (increasing enzymes) or proline dehydrogenase or proline oxidase, PDH or POX, and P5C dehydrogenase, P5CDH (decreasing enzymes), accumulate proline (Huang *et al.*, 2013). Several genes like, *P5CS1* and *PDH* are positively and negatively regulated the accumulation of proline, respectively (Jaarsma *et al.*, 2013; Verslues and Sharma, 2010; Kishor *et al.*, 2005) by the increase in their expression and activity. Similar genes could be overexpressed to increase proline synthesis under salt stress to improve tolerance to salt.

Chapter V

Summary and Conclusion

CHAPTER V

SUMMARY AND CONCLUSIONS

This experiment was conducted under five different salinity conditions to study the tolerance of five *Capsicum* genotypes at *in vitro* celluar level as well as to investigate the underlying genetic mechanism for tolerance. Five *Capsicum* genotypes were selected on the basis of their germination ability. These genotypes were used as *in vitro* explant source in this study. The genotypes were collected from SRC, Bogra. From twenty days old seedling, cotyledonary leaves and nodal segment were excised as explants. Calli were developed from these explants using modified MS medium supplemented with 2 mg/L NAA, 0.5 mg/L KIN and 5 mg/L 2,4-D. The calli were then cut into pieces of same sizes and inoculated into callus induction medium supplemented with 0 mM, 50 mM, 100 mM, 150 mM and 200 mM NaCl. NaCl prevent plant cells from uptaking water necessary for physio-chemical activity. Salt tolerance assay of these calli in low to severe stresses has been carried out at different duration. The environment was controlled with 25°C and a 16 h photoperiod from white fluorescent lamps (40-50 $\mu mol\text{-}m^{-2}\text{-}s^{-1}$). Evaluation of response of calli was performed at 18 days and 36 days of inoculation. Initially the calli looked fresh and vigorous and it continued up to 18 days of stress period with some genotypic variation and almost all of them were whitish green. At prolonged stress period that is, from 18 days to 36 days they gradually became brown to dark brown and eventually died. Estimation of callus size and weight revealed that significant genotypic variation exist among the calli of five genotypes at 18 DAT and 36 DAT. The size and weight of calli were the indicators of salt tolerance. Regarding totipotency, G_3 showed better performance at moderate to severe stress and from short duration (18 DAT) to long duration (36 DAT) stress period. They remained whitish green, fresh and live for long prolonged stress period. For short duration salt stress (18 days) G_4 was the best performer at low to moderate salt stress whereas G_2 was the best performer under severe stress. At prolonged stress period i.e., at 36 DAT, also G_4 and G_2 showed better tolerance under low salt

stress, i.e., their callus increased in size under low salt stress (-5.57 mm and -1.25 mm, respectively). Upto 18 DAT (days after treatment) callus weight was not reduced at all at low salt stress (50 mM NaCl) but increased in case of G_2, G_4 and G_5 (-0.35 g, -0.11 g and -0.04 g respectively). At prolonged stress period (36 DAT), G_4 followed by G_2 were the best performer under low stress (-0.30 g and -0.09 g, respectively). Proline accumulation under salt stress is an indication of salt tolerance. In this experiment, G_3 showed gradual increase of proline accumulation in calli under stress condition. This genotype also performed better as compared to the other genotypes and this data supporter the totipotency trait of this genotype where they remained green, fresh and alive.

In this experiment a protocol for *in vitro* induction of salt tolerant calli using cotyledonary leaves and nodal segments was established. Significant genotypic variation was observed among the genotypes, treatments and genotype × treatment interaction. The genotypes G_2 (SRC 05) , G_3 (SRC 02) and G_4 (CO 525) showed tolerance to salt stress condition at different duration as the biomass changing of calli in *Capsicum* was comparatively better than those of other genotypes under stress condition. Proline accumulation continuously increased in G_3 up to 200 mM of NaCl which supports the totipotency for longer stress period in G_3. These results indicate the expression of functional gene occurs at high salt stress in case of G_2, G_3 and G_4 which accumulate proline gradually in the calli.

This investigation dealt with the *in vitro* selection of salt tolerant callus lines in *Capsicum* spp. and could be made the progress of regeneration followed by gene expression analysis. So, further experiment by RT-PCR or Northern hybridization is required. Field trial is quiet necessary of regenerated plants in coastal region of Bangladesh. The identification and isolation of the gene(s) involved in the process of salt tolerance is necessary for future genetic transformation or hybridization to other economically important crop plants.

References

REFERENCES

Aboshama, H.M.S. (2011). Direct somatic embryogenesis of pepper (*Capsicum annuum* L.). *World J. Agril. Sci.* **7**(6): 755-762.

Abu-Romman, S. (2010). Responses of cucumber callus to sorbitol-induced osmotic stress. *J. Genet. Eng. Biotechnol.* **8**(2): 45-50.

Adhikery, B. and Amin, M.H.A. (2011). Performance of cotyledonary explant of chilli and the effect of diffeient hom, ones on *in vitro* regenerations. *Bangladesh Res. Pub. J.* **6**(1): 57-64.

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

Agarwal, D.C., Banerjee, R.R., Kolala, A.B., Dhage, A.V., Kulkarni, A.V., Nalawade, S.H. and Krishnamurthy, K.V. (1997). *In vitro* induction of multiple shoots and plant regeneration in cotton (*Gossypium hirsutum* L.). *Plant Cell Rep.* **16**: 647-652.

Agarwal, S. and Chandra, N. (1983). Differentiation of multiple shoot buds and plantlets in cultured embryos of *Capsicum annuum* L. *var.* Mathiana. *Cur. Sci.* **52:** 645-657.

Agarwal, S., Chandra, N. and Kothari, S.L. (1989). Plant regenerating in tissue cultures of peppers (*Capsicum annuum* L. *cv.* Mathania). *Plant Cell Tissue Organ Cult.* **16**: 47-55.

Agarwal, S., Chandra, N. and Lothari, S.L. (1988). Shoot tip culture of pepper for micropropagation. *Cur. Sci.* **57**: 1347-1349.

Alizah, Z. and Zamri, Z. (2013). Influence of genotype source on the *in vitro* regeneration ability of Malaysian chilli varieties. *American J. Res. Commun.* **1**(12): 1-6.

Amin, N.U., Sanga, G., Ara, N., Shah, S.H. and Farhatullah. (2013). Effect of various concentrations of calcium chloride oncallus growth and potassium nutrition of callicultures of potato (*Solanum tuberosum*). *Pakistan J. Bot.* **45**(1): 209-214.

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

Andres, M.S.A., Claver A.G., Chapapria, J.E., Peiro Abris, J.L., Palazon, C., Arteaga, L.M. and Ortega, G.R. (2004). Application of anther culture and molecular markers to a pepper breeding program for diseases resistance. *Capsicum Eggplant Newsl.* **23**: 105-108.

Anonymous. (2013). World Population Prospects, the 2012 Revision - "Low variant" and "High variant" values" UN. 2012. Retrieved June 15, 2013.http://esa.un.org/unpd/wpp/.

Anonymous. (2014). Distances From.com. http://www.distancesfrom.com/.

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

Arcos-Ortega, G.F., Chan-Kuuk, R.A., González-Kantún, W.A., Souza-Perera, R., Nakazawa-Ueji, Y.E., Avilés-Berzunza, E., Godoy-Hernández, G., Lawton, M.A. and Aguilar, J.J.Z. (2010). *Agrobacterium tumefaciens*-transient genetic transformation of Habaneropepper (*Capsicum chinense* Jacq.) leaf explants. *Elect. J. Biotechnol.* **13**(4): 1-9.

Arditti, J. and Ernst, R. (1984). Physiology of germinating orchid seeds. **In:** Orchid Biology -Reviews and Perspectives. J. Arditti. (ed.). Comstock Publishing, Cornell Univ. Press, Ithaca, London, pp. 177-222.

Arroya, R. and Revilla, M.A. (1991). *In vitro* plant regeneration from cotyledon and hypocotyls segments in two bell pepper cultivars. *Plant Cell Rep.* **10**: 414- 416.

Asakaviciute R. (2008). Androgenesis in anther culture of Lithuanian spring barley (*Hordeum vulgare* L.) and potato (*Solanum tuberosum* L.) cultivars. *Turkey J. Biol.* **32**: 155-160.

Ashraf, M. and Foolad, M.R. (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environ. Expt. Bot.* **59**: 206-216.

Ashraf, M. and Harris, P.J.C. (2004). Potential biochemical indicators of salinity tolerance in plants. *Plant Sci.* **166**: 3-16.

Ashraf, M.Y., Ali, Y. and Qureshi, T.M. (1998). Effect of salinity on photosynthetic efficiency and yield of rice genotypes. *Pakistan J. Biol. Sci.* **1**: 72-74.

Ashraf, M.Y., Azmi, A.R., Khan, A.H. and Ala, S.A. (1994). Effect of water stress on total phenol, peroxidase activity and chlorophyll contents in wheat (*Triticum aestivum* L.). *Acta Physiol. Plant.* **16**: 185-191.

Ashrafuzzaman, M., Hossain, M.M., Ismail, R.M., Haque, S.M., Shahidullah, S.M., and Shahin-uz-zaman. (2009). Regeneration potential of seedling explants of chilli (*Capsicum annuum*). *African J. Biotechnol.* **8**(4): 591-596.

Atanassov, A., Zagorska, N., Boyadjiev, P. and Djilianov, D. (1995). *In vitro* production of haploid plants. *World J. Microbiol. Biotechnol.* **11**: 400-408.

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

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

Bal, U. and Abak, K. (2007). Haploidy in tomato (*Lycopersicon esculentum* L. Mill.): a critical review. *Euphytica.* **158**: 1-9.

Ball, E. (1946). Development in sterile culture of stems tips and subjacent regions of *Tropaeolum malus* L. and of *Lupinus albus* L. *Amirican J. Bot.* **33:** 301-318.

Barakat, M.N. and Abdel-Latif, T.H. (1996). *In vitro* selection of wheat callus tolerant to high levels of salt and plant regeneration. *Euphytica.* **91**: 127-140.

Basay, S. and Ellialtıoglu, S.S. (2013). Effect of genotypical factors on the effectiveness of anther culture in eggplant (*Solanum melongena* L.). *Turkey J. Biol.* **37**: 499-505.

Basu, S., Gangopadhyay, G., Mukherjee, B.B. and Gupta, S. (1997). Plant regeneration of salt adapted callus of indica rice (var. Basmati 370) in saline conditions. *Plant Cell Tissue Organ Cult.* **50:** 153-159.

Basu, S.K. and De, A.K. (2003). *Capsicum*: historical and botanical perspectives. **In:** *Capsicum*: The genus *Capsicum.* A.K., De, (ed.). London: CRC Press. pp. 1-15.

Bates, L.S., Waldren, R.P. and Teare, I.D. (1973). Rapid determination of free proline for water stress studies. *Plant Soil.* **39**: 205-207.

BBS (2014). Report on the productivity survey of *Capsicum* crop. Area and household, pp. 20.

Ben-Hayyim, G. and Goffer, Y. (1989). Plantlet regeneration from a NaCl-selected salttolerant callus culture of shamouti orange (*Citrus sinensis* L. *Osbeck*). *Plant Cell Rep.* **7**: 680-683.

Benson, E. (2000). Special symposium: *In vitro* plant recalcitrance: An introduction. *In vitro Cell. Dev. Biol. Plant.* **36**: 141-148.

Bergmann, L. (1959). A new technique for isolating and cloning cells of higher piarits. *Nature.* **184**: 648-649.

Berljak, J. (1999). *In vitro* plant regeneration from pepper (*Capsicum annuum* L. cv. 'Soroksari') seedling explants. *Plant Physiol.* **39**(3): 289-292.

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

Binh, D.Q. and Heszky, L.E. (1990). Restoration of regeneration potential of long term cell culture in rice (*Oryza sativa* L.) by salt pre-treatment. *J. Plant Physiol.* **136**: 336-340.

Binzel, M.L., Sankhla, N., Joshi, S. and Sankhla, D. (1996). *In vitro* regeneration in chili pepper (*Capsicum annuum* L.) from half-seed explant. *Plant GrowthReg.* **20**: 287-293.

Bojórquez-Quintal, E., Velarde-Buendía, A., Ku-González, A., Carillo-Pech, M., Ortega-Camacho, D., Echevarría-Machado, I., Pottosin, I. and Martínez-Estévez, M. (2014). Mechanisms of salt tolerance in habanero pepper plants (*Capsicum chinense* Jacq.): Proline accumulation, ions dynamics and sodium root-shoot partition and compartmentation. *Frontiers Plant Sci.* **5**: 3-14.

Bonner J. (1937). Vitamin B_1, a growth factor for higher plants. *Science.* **85**: 183-184.

Bonner, J. (1938). Thiamin (vitamin B_1) and the growth of roots: the relation of chemical structure to physiological activity. *Amirican J. Bot.* **25:** 543-549.

Bonner, J. (1940). Specificity of nicotinic acid as a growth factor for isolated pea roots. *Plant Physiol.* **15**: 553-557.

Bonner, J. and Addicott, F. (1937). Cultivation *in vitro* of excised pea roots. *Bot. Gaz.* **99**: 144-170.

Borsani, O., Valpuesta, V. and Botella, M.A. (2003). Developing salt tolerant plants in a new century: a molecular biologyapproach. *Plant Cell Tissue Organ Cult.* **73**: 101-115.

Bray, E.A. (1993). Molecular response to water deficit. *Plant Physiol.* **103**: 1035-1040.

Bridson, E.Y. (1978). Diets, culture media and food supplements. **In**: CRC handbook series in nutrition and food. M.J., Rechcigl. (ed.). Section G. Vol. 3. pp. 91-281.

Burnet, G. and Ibrahim, R.K. (1973).Tissue culture of citrus peel and its potential for flavonoid synthesis. *Z. Pflanzenphysiol.* **69**: 152-162.

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

Caplin, S.M. and Steward, F.C. (1948). Effects of coconut milk on the growth of explants from carrot root. *Science.* **108**: 655-657.

Carimi, F., De-Pasquale, F. and Crescimanno, F.G. (1999). Somatic embryogenesis and plant regeneration from pistil thin cell layers of citrus. *Plant Cell Rep.* **18**: 935-940.

Carimi, F., De-Pasquale, F. and Puglia, A.M. (1998). *In vitro* rescue of zygotic embryos of sour orange, *Citrus aurantium* L., and their detection based on RFLP analysis. *Plant Breed.* **117**: 261-266.

Carlson, P.S., Smith, H.H. and Dearing, R.D. (1972). Parasexual interspecific plant hybridization. *Proc. Natl. Acad. Sci.* **69:** 2292-2294.

Chakraborty, K., Sairam, R.K. and Bhattacharya, R.C. (2012). Differential expression of salt overly sensitive pathway genes determines salinity stress tolerance in *Brassica* genotypes. *Plant Physiol. Biochem.* **51**: 90-101.

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

Chen, Y., Zahavi, E., Barak, P. and Ummiel, N. (1980). Effects of salinity stresses on tobacco. I. The growth of *Nicotiana tabacum* callus cultures under seawater, NaCl, and manitol stresses. *Z. Pflanzenphysiol.* **98**: 141-153.

Chen, Z., Cuin, T.A., Zhou, M., Twomey, A., Naidu, B.P. and Shabala, S. (2007). Compatible solute accumulation and stress-mitigating effects in barley genotypes contrasting in their salt tolerance. *J. Expt. Bot.* **58**: 4245-4255.

Chen, Z., Cuin, T.A., Zhou, M., Twomey, A., Naidu, B.P., and Shabala, S. (2007). Compatible solute accumulation and stress-mitigating effects in barley genotypes contrasting in their salt tolerance. *J. Expt. Bot.* **58:** 4245-4255.

Christopher, T. and Rajam, M.V. (1994). *In vitro* clonal propagation of *Capsicum* spp. *Plant Cell Tissue Organ Cult.* **38**: 25-29.

Christopher, T. and Rajam, M.V. (1996). Effect of genotype, explant and medium on *invitro* regeneration of red pepper. *Plant Cell Tissue and Organ Cult.* **46**: 245-250.

Christopher, T., Prolaram, B. and Subhash, K. (1991). Differential *in vitro* morphogenetic response in hypocotyl segments of *Capsicum annuum*. *Indian J. Expt. Biol.* **29**: 68-69.

Christopher, T., Prolaram, B., Rajam, M. V. and Subash, K. (1986). Plantlet formation from embryo cultures of *Capsicum annuum* L. *var.* G4. *Cur. Sci.* **55**: 1036-1037.

Chuah, A.M., Lee, Y.C., Yamaguchi, T., Takamura, H., Yin, L.J. and Matoba, T. (2008). Effect of cooking on the antioxidant properties of coloured peppers. *Food Chemist.* **111**(1): 20-28.

Chuang, C.C., Ouyang, T.W., Chia, H., Chou, S.M. and Ching, C.K. (1978). A set of potato media for wheat anther culture. Extended summary, Proc. Symp. Plant Tissue Cult. Sci. Press, Peking, China. pp. 51-56.

Cocking, E.C. (1960). A method for the isolation of plant protoplasts and vacuoles. *Nature.* **187**: 927-929.

Cohen, S.N., Chang, A.C.Y., Boyer, H.W. and Helling, R.B. (1973). Construction of biologically functional bacterial plasmids *in vitro*. *Proc. Natl. Acad. Sci.USA.* **70**: 3240-3244.

Comlekcioglu, N., Buyukalaka, S. and Abak, K. (2001). Effect of silver nitrate on haploid embryo induction by anther culture in pepper (*Capsicum annuum* L.). **In:** 11th EUCARPIA meeting on genetics and breeding of *Capsicum* and eggplant, Antalya, Turkey. pp. 133-136.

Csillery, G. (2006). Pepper taxonomy and the botanical description of the species. *Acta. Agron. Hung.* **54**: 151-66.

Das, T., Mitra, G.C. and Chatterjee, A. (1995). Micropropagation of *Citrus sinensis* var. mosambi: an important scion. *Phytomorphology.* **45**: 57-64.

Davenport, S.B., Gallego, S.M., Benavides, M.P. and Tomaro, M.L. (2003). Behaviour of antioxidant defense system in the adaptive response to salt stress in *Helianthus annuus* L. cells. *Plant Growth Regul.* **40**: 81-88.

De-Pasquale, F., Carimi, F. and Crescimanno, F.G. (1994). Somatic embryogenesis from styles of different cultivars of *Citrus limon* (L.) Burm. *Australian J. Bot.* **42**: 587-594.

Dita, M.A., Rispai, N., Prats, E., Rubiales, D. and Singh, K.B. (2006). Biotechnology approaches to overcome biotic and abiotic stress constraints in legumes. *Euphytica.* **147**: 1-24.

Dix, P.J. and Street, H.E. (1975). Sodium chloride-resistant cultured cell lines from *Nicotiana sylvestris* and *Capsicum annuum. Plant Sci. Lett.* **5**: 231-237.

Dracup, M. (1991). Increasing salt tolerance of plants through cell culture requires greater understanding of tolerance mechanisms. *Australian J. Plant Physiol.* **18**(1): 1-15.

Dumas-de-Vaulx, R. (1990). Haploid and pepper breeding: a review. *Capsicum Newsl.* **8-9**: 13-17.

Dziadczyk, P., Bolibok, H., Tyrka, M. and Hortynski, J.A. (2003). *In vitro* selection of strawberry (Fragaria×ananassa Duch.) clones tolerant to salt stress. *Euphytica.* **132**(1): 49-55.

Ebida, A.I.A. and Hu, C.Y. (1993). *In vitro* morphogenetic response and plant regeneration from pepper (*Capsicum annuum* L. *cv.* Earey California Wonder) seedling explants. *Plant Cell Rep.* **13**(2): 107-110.

Elavumoottil, O.C., Martin, J.P. and Moreno, M.L. (2003). Changes in sugars, sucrose synthase activity and proteins in salinity tolerant callus and cell suspension cultures of *Brassica oleracea* L. *Biol. Plant.* **46:** 7-12.

Elwan, M.W.M. (2009). *In vitro* shoot regeneration, elongation and rooting of pepper (*Capsicum Annuum* L.), **In**: Shoot regeneration as affected by PGR and explant type (59). 4th Conference on recent technologies in agriculture. pp. 564-576

Ercan, N. and Ayar-Şensoy, F. (2011). Androgenic responses of different (*Capsicum annuum* L.) cultivars. *Biyol. Bilimi. Arasti. Der.* **4**: 59-61.

Ercan, N., Sensoy, F.A. and Sensoy, A.S. (2006). Influence of growing season and donor plant age on anther culture response of some pepper cultivars (*Capsicum annuum* L.). *Sci. Hort.* **110**: 16-20.

Ernst R., Arditti, J. and Healey, P.L. (1971). Carbohydrate physiology of orchid seedlings. Hydrolysis and effects of oligosaccharides. *Amirican J. Bot.* **58:** 827-835.

Ezura, H., Nishimiya, S. and Kasumi, M. (1993). Efficient regeneration of plants independent of exogenous growth regulators in bell pepper (*Capsicum annuum* L.). *Plant Cell Rep.* **12**: 676-680.

FAO (2002). Crops and Drops. Making the best use of water for agriculture. Food and Agriculture Organization of the UN. Rome. p. 26.

Fari, M. and Czako. (1981). Relationship between positions and morphogenetic response of pepper hypocotyls explants cultured *in vitro*. *Sci. Hort.* **15**: 207-213.

Fari, M., Tury, Z., Csillag, F. and Peredia, B. (1990). Comparative studies on *in vitro* regeneration of seedling explants in chilli pepper (*Capsicum annuum* L.). *Acta. Hort.* **280**: 131-134.

Franck-Duchenne, M., Wang, Y., Tahar, B.S. and Beachy, R.N. (1998). *In vitro* stem elongation of sweet pepper in media containing 24-epi-brassinolide. *Plant Cell Tisssue Organ Cult.* **53**: 79-84.

Frearson, E.M., Power, J.B. and Cocking, E.C.(1973).The isolation, culture and regeneration of *Petunia* leaf protoplasts. *Dev. Biol.* **33**: 130-137.

Galiba, G. and Yamada, Y. (1988). A novel method for increasing the frequency of somatic embryogenesis in wheat tissues culture by NaCl and KCl supplementation. *Plant Cell Rep.* 7: 55-58.

Gamborg, O.L., Miller, R.A. and Ojima, O. (1968). Nutrient requirements of suspension cultures of soybean root cell. *Expt. Cell Res.* **50**: 151-158.

Gandonou, C.B., Errabii, T., Abrini, J., Idaomar, M. and Senhaji, N.S. (2006). Selection of callus cultures of sugarcane (*Saccharum* sp.) tolerant to NaCl and their response to salt stress. *Plant Cell Tissue Organ Cult.* **87**: 9-16.

Garcia, R.A. (1990). Tissue and cell culture of pepper (*Capsicum annuum* L. *cv.* Pico and cv. Piquillo). APHF/SECH. pp. 249-254.

Garciadeblas, B., Senn, M.E., Banuelos, M.A. and Rodriquez-Navarro, A. (2003). Sodium transport and HKT transporters: the rice model. *Plant J.* **34**: 788-801.

Gatz and Rogozinska, J. (1994). *In vitro* organogenesis potential of cotyledon and leaf explants of *Capsicum annuum* L. cv. Bryza. *Acta Soc. Bot.polon.* **63**: 255-258.

Gautheret, R. (1939). Sur la possibilité de réaliser la culture indéfinie des tissues de tubercules de carotte. *C. R. Soc. Biol. Paris.* **208**: 118-120.

Gautheret, R.J. (1934). Culture du tissus cambial. *C.R. Hebd. Seances Acad. Sci.* **198**: 2195-2196.

Gautheret, R.J. (1942). Hétéro-auxines et cultures de tissus végétaux. *Bull. Soc. Chem. Biol.* **24**: 13-41.

Ge, K.L., Saskuma, T. and Tanaka, M. (1991). Callus induction and plant regeneration from cultured hypocotyls and cotyledon explants of cultivated and wild *Capsicum* species. *Acta Agric. Shang.* 7(4): 10-16.

George, A. (2007). Principles of plant genetics and breeding. Blackwell Publishing Ltd. 350, Main Street, Malden, USA. pp.182-188.

Ghoulam, C., Ahmed, F. and Khalid, F. (2001). Effects of salt stress on growth, inorganic ions and proline accumulation in relation to osmotic adjustment in five sugar beet cultivars. *Environ. Expt. Bot.* **47**: 139-150.

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

Gorai, M. and Neffati, M. (2007). Germination responses of *Reaumuria vermiculata* to salinity and temperature. *Annu. App. Biol.* **151**: 53-59.

Govindarajan V.S. (1986). *Capsicum* - production, technology, chemistry and quality. Chemistry of the color, aroma, and pungency stimuli. *Crit. Rev. Food Sci. Nutr.* **4**: 245-355.

Gresshoff, P.M. and Doy, C.H. (1972). Development and differentiation of haploid *Lycopersicon esculentum* (tomato). *Planta*. **107**: 161-170.

Grigore, M.N., Boscaiu, M. and Vicente, O. (2011). Assessment of the relevance of osmolyte biosynthesis for salt tolerance of halophytes under natural conditions. *European J. Plant Sci. Biotech.* **5**: 12-19.

Grozeva, S., Rodeva, V. and Todorova, V. (2012).*In vitro* shoot organogenesis in Bulgarian sweet pepper (*Capsicum annuum* L.) varieties. *Elect. J. Biol.* **8**(3): 39-44.

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

Guerrier, G. (1998). Proline accumulation in salt-treated tomato: Different proline precursors in *Lycopersicon esculentum* and *Lycopersicon pennellii*. *J. Plant Nutr.* **21**: 505-513.

Guha, S. and Maheshwari, S.C. (1966). Cell division and differentiation of embryos in the pollen grains of *Datura in vitro*. *Nature.* **212**: 97-98.

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

Gunay, A.L. and Rao, P.S. (1978). *In vitro* plant regeneration from hypocotyl and cotyledon explants of red pepper (*Capsicum*). *Plant Sci. Letts.* **11**: 365-372.

Gupta, B.and Huang, B. (2014). Mechanism of salinity tolerance in plants: physiological, biochemical, and molecular characterization. *Int. J. Genome.* **2014**: 18.

Gupta, P., Sharma, S. and Saxena, S. (2014). Effect of salts (NaCl and Na_2CO_3) on callus and suspension culture of *Stevia rebaudiana* for Steviol glycoside production. *Appl. Biochem. Biotechnol.* **172**(6): 2894-906.

Haberlandt, G. (1902). Kulturversuche mit isolierten Pflanzenzellen. Sitzungsber. Akad. Wiss. Wien. Math.-Naturwiss. Kl. *Abt. J.* **111**: 69-92.

Hannig, E. (1904). Zur Physiologie pflanzlicher Embryonen. I. Über die Kultur von Cruciferen-embryonen ausserhalb des Embryosacks. *Bot. Ztg.* **62**: 45-80.

Haque, S.A. (2006). Salinity problem and crop production in coastal regions of Bangladesh. *Pakistan J. Bot.* **38**(5): 1359-1365.

Harini, I. and Sita, G.L. (1993). Direct somatic embryogenesis and plant regeneration from immature embryos of chilli (*Capsicum annuum* L.). *Plant Sci. Lim.* **89**(1): 107-112.

Harvais, G. (1982). An improved medium for growing the orchid *Cypripedium reginae* axenically. *Canadian J. Bot.* **60**: 2547-2555.

Hasanuzzaman, M., Nahar, k. and Fujita, M. (2013). Plant response to salt stress and roleof exogenous protectants to mitigatesalt-induced damages. **In**: P. Ahmad, M.M., Azooz, and M.N.V., Prasad, (eds.). **15**: 510.

Hasegawa, P.M., Bressan, R.A., Zhu, J.K. and Bohnert, H.J. (2000). Plant cellular and molecular responses to high salinity. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **51**: 463-499.

Hassan, N.M., Serag, M.S., El-Feky, F.M. and Nemat-Alla, M.M. (2008). *In vitro* selection of mung bean and tomato for improving tolerance to NaCl. *Annu. Appl. Biol.* **152**: 319-330.

Hassan, N.S. and Wilkins, D.A. (1988). *In vitro* selection for salt tolerant lines in *Lycopersicon peruvianum*. *Plant Cell Rep.* **7:** 463-466.

He, S., Han, Y., Wang, Y., Zhai, H. and Liu, Q. (2009). *In vitro* selection and identification of sweetpotato (*Ipomoea batatas* L.) Plants tolerant to NaCl. *Plant Cell Tissue Organ Cult.* **96**: 69-74.

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

Hmida-Sayari, A., Gargouri-Bouzid, R., Bidani, A., Jaoua, L., Savouré, A., and Jaoua, S. (2005). Overexpression of 1-pyrroline-5-carboxylate synthetase increases proline production and confers salt tolerance in transgenic potato plants. *Plant Sci.* **169**: 746-752.

Hoq, M. S., Hossain, T.M.B., Matin, M.A. and Hossain, S. (2013). Existing value chain assessment of *Capsicum* marketing in selected areas of Bangladesh. Agricultural Economics Division, BARI, Gazipur. Annual Res. Rep. p. 33.

Hossain, Z., Mandal, A.K.A., Datta, S.K. and Biswas, A.K. (2007). Development of NaCl tolerant line in *Chrysanthemum morifolium* R. through shoot organogenesis of selected callus line. *J. Biotechnol.* **129**: 658-667.

Huang, Z., Zhao, L., Chen, D., Liang, M., Liu, Z. and Shao, H. (2013). Salt stress encourages proline accumulation by regulating proline biosynthesis and degradation in Jerusalem artichoke plantlets. *PLoS ONE.* **8**: 62085.

Husain, S., Jain, A. and Kothari, S.L. (1999). Phenylacetic acid improves bud elongation and *in vitro* plant regeneration efficiency in *Capsicum annuum* L. *Plant Cell Rep.* **19**: 64-68.

Hyde, C. and Phillips, G.C. (1996). Silver nitrate promotes shoot development and plant regeneration on chile pepper (*Capsicum annuum* L.) via organogenesis. *In vitro Cell Dev. Biol. Plant.* **32**: 72-80.

Irikova, T. (2008). Obtaining and genotype investigations of androgenic haploids from Bulgarian pepper (*Capsicum annuum* L.) varieties. PhD thesis, University of Plovdiv, Plovdiv, Bulgaria. p. 199.

Jaarsma, R., de Vries, R. S. M., and de Boer, A. H. (2013). Effect of salt stress on growth, Na^+ accumulation and proline metabolism in potato (*Solanum tuberosum*) cultivars. *PLoS ONE.* **8**: e60183.

Jain, R.K., Jain, S., Nainawatee, H.S. and Chowdhury, J.B. (1990). Salt tolerance in *Brassica juncea* L.: *In vitro* selection, agronomic evaluation and genetic stability. *Euphytica.* **48**: 141-152.

Joshi, A. and Kothari, S.L. (2006). High copper levels in the medium improve shoot bud differentiation and elongation from the cultured cotyledons of *Capsicum annuum* L. *Plant Cell Tissue Organ cult.* **12**: 71-76.

Jumin, H.B. (1995). Plant regeneration via somatic embryogenesis in citrus and its relatives. *Phytomorphology.* **45**: 1-8.

Kanta, K., Rangaswamy, N.S., and Maheshwari, P. (1962). Test-tube fertilization in flowering plants. *Nature.* **194**: 1214-1217.

Kao, K.N. and Michayluk, M.R. (1975). Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. *Planta.* **126**: 105-110.

Kaparakis, G. and Alderson, P.G. (2008). Role of cytokininsin somatic embryogenesis of pepper (*Capsicum annuum* L.). *J. Plant Growth Regul.* **27**: 110-114.

Karadimova, M. and Djambova, G. (1993). Increased NaCl-tolerance in wheat (*Triticum aestivum* L. and *T. durum* desf.) through *in vitro* selection. *In vitro Cell Dev. Biol. Plant.* **29**: 180-182.

Kehie, M., Kumaria, S. and Tandon, P. (2011). *In vitro* plantlet regeneration from nodal segments and shoot tips of *Capsicum chinense* Jacq. cv. Naga King Chili. *Springer Biotechnol.* **2**: 31-35.

Khan, H., Siddique, I. and Anis, M. (2006). Thidiazuron induced somatic embryogenesis and plant regeneration in *Capsicum annuum*. *Biol. Plant.* **50**: 789-92.

Kim, M., Jang, I.C., Kim, J.A., Park, E.J., Yoon, M. and Lee, Y. (2008). Embryogenesis and plant regeneration of hot pepper (*Capsicum annuum* L.) through isolated microspore culture. *Plant Cell Rep.* **27**: 425-434.

Kim, S., Kim, S.R., An, C.S., Hang, Y.N. and Lee, K.W. (2001). Constitutive expression of rice MAPS box gene using leaf explants in hot pepper (*Capsicum annuum* L.). *Mol. Cells.* **12**(2): 221-226.

Kim, S.H., Ahn, Y.O., Ahn, M.J., Lee, H.S. and Kwak, S.S. (2012). Down-regulation of β-carotene hydroxylase increases β-carotene and total carotenoids enhancing salt stress tolerance in transgenic cultured cells of sweetpotato. *Phytochemistry.* **74**: 69-78.

Kintzios, S., Drossopoulos J.B., Shortsianitis, E. and Peppes, D. (2000). Induction of somatic embryogenesis from young, fully expanded leaves of chilli pepper (*Capsicum annuum* L.): Effect of leaf position, illumination and explant pre-treatment with high cytokinin concentration. *Sci. Hort.* **85**: 137-144.

Kintzios, S., Drossopoulos, J.B. and Lymperopoulos, C. (2001). Effect of vitamins and inorganic micronutrient on callus growth and somatic embryogenesis from leaves of chilli pepper. *Plant Cell Tissue Organ Cult.* **67**: 55-62.

Kirti, P.B., Hadi, S., Kumar, P.A. and Chopra, V.L. (1991). Production of sodium-chloride-tolerant *Brassica juncea* plants by *in vitro* selection at the somatic embryos level. *Theor. Appl. Genet.* **83**: 233-237.

Kishor, P.B.K., Sangam, S., Amrutha, R.N., Laxmi, P.S., Naidu, K.R., Rao, K.R.S.S. (2005). Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. *Cur. Sci.* **88**: 424-438.

Kishore, B., Hong, Z., Miao, G., Hu, C. and Verma, D. (1995). Overexpression of delta-pyrroline-5-carboxylatesynthetase increase proline production and confers osmotolerance in transgenic plants. *Plant Physiol.* **108**: 1387-1394.

Kniitel, N., Escancon, A.S. and Hane, G. (1991). Plant regeneration at high frequency from mature sunflower cotyledons. *Plant Sci.* **73**: 219-226.

Knop, W. (1884). Bereiting einer concentrierten Nàhrstoffl6sung für Pflanzen. *Landw. Vers. Stn.* **30**: 292-294.

Koc, N.K., Bas, B., Koc, M. and Kusek, M. (2009). Investigations of *in vitro* selection for salt tolerant lines in sour orange (*Citrus aurantium* L.). *Biotechnol.* **8**: 155-159.

Kothari, S.L., Joshi, A., Kachhwaha, S. and Ochoa-Alejo, N. (2010). Chilli peppers-a review on tissue culture and transgenesis. *Biotechnol. Adv.* **28**: 35-48.

Kotte, W. (1922). Kulturversuch isolierten wurzelespitzen. *Beitr. Allg. Bot.* **2**: 413-434.

Kripkyy, O., Kerkeb, L., Molina, A., Belver, A., Rodrigues Rosales, P. and Donaire, P.J. (2001). Effects of salt-adaptation and salt-stress on extracellular acidification and microsome phosphohydrolase activities in tomato cell suspensions. *Plant Cell Tissue Orgnan Cult.* **66**: 41-47.

Kumar, O.A. and Tata, S.S. (2010). *In vitro* shoot bud differentiation from hypocotyls explants of chili peppers (*Capsicum annuum* L.). *Not. Sci. Biol.* **2**(1): 66-69.

Kumar, O.A., Rupavathi, T. and Tata, S.S. (2012). Adventitious shoot bud induction in chili pepper (*Capsicum annuum* L. Cv. X-235). *Int. J. Sci. Nat.* **3**(1): 192-196.

Kumar, V., Shriram, V., Nikam, T.D., Jawali, N. and Shitole, M.G. (2008). Sodium chloride induced changes in mineral nutrients and proline accumulation in indica rice cultivars differing in salt tolerance. *J. Plant Nutr.* **31**: 1999-2017.

Lantos, C., Gemes, A.J., Somogyi, G., Otvos, K., Vagi, P., Mihaly, R., Kristof, Z., Somogyi, N. and Pauk, J. (2009). Improvement of isolated microspore culture of pepper (*Capsicum annuum* L.) via co-culture with ovary tissues of pepper or wheat. *Plant Cell Tissue Organ Cult.* **97**: 285-293.

Lebrun, L., Rajasekaran, K. and Mullins, M.G. (1985). Selection *in vitro* for NaCl tolerance in vitis rupestris Scheele. *Annu. Bot.* **56**: 733-740.

Lee, S.Y., Lee, J.H. and Kwon, T.O. (2003). Selection of salt-tolerant doubled haploids in rice anther culture. *Plant Cell Tissue Orgnan Cult.* **74**: 143-149.

Lim, G.H., Zhang, X., Chung, M.-S. Lee D.J., Woo, Y.M. and Cheong, H.S. (2010). A putative novel transcription factor, AtSKIP, is involved in abscisic acid signalling and confers salt and osmotic tolerance in *Arabidopsis*. *New Phytol.* **185**(1): 103-113.

Liu, J., Ishitani, M., Halfter, U., Kim, C.S. and Zhu, J.K. (2000). The *Arabidopsis thaliana SOS2* gene encodes a protein kinase that is required for salt tolerance. *Proc. Natl. Acade. Sci. USA.* **97**(7): 3730-3734.

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

Liu, T. and Staden, J.V. (2000). Selection and characterization of sodium chloride-tolerant callus of *Glycine max* (L.) Merr cv. Acme. *Plant Growth Regul.* **31**: 195-207.

Liu, W., Parroti, W.A., Hildebrand T.D.F., Collins, G.B. and Williams, E.G. (1990). *Agrobacterium* induced gall formation in bell pepper *(Capsicum annuum* L.) and formation of shoot like structures expressing introduced genes. *Plant Cell Rep.* **9**: 360-364.

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

Lu, S., Peng, X., Guo, Z., Zhang, G., Wang, Z., Wang, C., Pang, C., Fan, Z. and Wang, J. (2007). *In vitro* selection of salinity tolerant variants from triploid bermudagrass (*Cynodon transvaalensis* and *C. dactylon*) and their physiological responses to salt and drought stress. *Plant Cell Rep.* **26**: 1413-1420.

Lutts, S., Kinet, J.M. and Bouharmont, J. (1996). Effects of salt stress on growth, mineral nutrition and proline accumulation in relation to osmotic adjustment in rice (*Oryza sativa* L.) cultivars differing in salinity resistance. *Plant Growth Regul.* **19**: 207-218.

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

Madhuri, V. and Rajam, M.V. (1993). Apical shoot meristem culture in red pepper. *J. Plant Biochem. Biotechnol.* **2**: 67-68.

Malik, A.A., Li, W.-G., Lou, L.-N., Weng, J.-H. and Chen, J.-F. (2010).Biochemical/ physiological characterization andevaluation of *in vitro* salt tolerance in cucumber. *African J. Biotechnol.* **9**(22): 3284-3292.

Mangang, R.K.J. (2014). *In vitro* callus induction of placental tissues of *Capsicum chinense Jacq.cv.*'Umorok' usingdifferent concentrations and combinations of growth hormones. *Int. J. Interdiscip. Multidiscip. Stu.* **1**(8): 63-66.

Manoharan, M., Sreevidya, C.S. and Sita, L. (1998). *Agrobacterium*-mediated genetic transformation in hot chilli *(Capsicum annuum* L. *var. Pusa Jwala). Plant Sci.* **131**: 77-83.

Marcum, K.B. (2002). Growth and physiological adaptations of grasses to salinity stress. **In:** M. Pessarakli, (ed.) Handbook of plant and crop physiol. New York Marcel Dekker. pp. 623-636.

Mathew, D. (2002). *In vitro* shoot and root morphogenesis from cotyledon and hypocotyls explants of hot pepper cultivars Byadgi Dabbi and Arka Lohit. *Capsicum Eggplant Newsl.* **21**: 69-72.

McCoy, T.J. (1987). Characterization of alfalfa (*Medicago sativa* L.) plants regenerated from selected NaCl tolerant cell lines. *Plant Cell Rep.* **6**: 417-422.

McGregor, L.J. and McHughen, A. (1990). The influence of various cultural factors on anther culture of four cultivars of spring wheat (*Triticum aestivum* L.). *Canadian J. Plant Sci.* **70**: 183-192.

McHughen, A. (1987). Salt tolerance through increased vigor in a flax line (STS-II) selected for salt tolerance *in vitro*. *Theor. Appl. Genet.* **74**: 727-732.

Meena, R.K., Gour, K. and Patni, V. (2014). Production of leaf curls virus-free chilli by meristem tip culture. *Int. J. Pharm. Sci. Rev. Res.* **25**(2): 67-71.

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

Mohammed, W. and Mohammed, E. (2007). Explant type, regeneration stage and pre conditioning affect in vitro salinity tolerance in sweet pepper (*Capsicum annuum* cv. California wonder). *African Crop Sci. Conf. Proc.* **8**: 1951-1956.

Montoliu, A., López-Climent, M., Arbona, V., Pérez-Clemente, R. and Gomez-Cadenas, A. (2009). A novel *in vitro* tissue culture approach to study salt stress responses in citrus. *Plant Growth Regul.* **59**: 179-187.

Morel, G. and Muller, J.F. (1964). *In vitro* culture of the apical meristem of the potato. *Compt. Rend.Acad. Sci.* **258**: 5250-5252.

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

Muir, W.H., Hildebrandt, A.C. and Riker, A.J. (1954). Plant tissue cultures produced from single isolated plant cells. *Science.* **119**: 877-878.

Mukherjee, A. (2002). Effect of NaCl on *in vitro* propagation of sweet potato (*Ipomoea batatas* L.). *Appl. Biochem. Biotechnol.* **102-103**: 431-441.

Munns, R. (2002a). Comparative physiology of salt and water stress. *Plant Cell Environ.* **25**: 239-250.

Munns, R. (2002b). Salinity, growth and phytohormones. **In:** Salinity: environment-plants-molecules. A., Läuchli and U., Lüttge, (eds.). Kluwer, Netherlands. pp. 271-290.

Munns, R. (2005). Genes and salt tolerance: bringing them together. *New Phytol.* **167**: 645-663.

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

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

Murphy, J.F. and Kyle, M. (1994). Isolation and viral infection of *Capsicum* leaf protoplasts. *Plant Cell Rep.* **13**: 397-400.

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

Nagata, T. and Tabake, I. (1971). Planting of isolated tobacco mesophyll protoplast on agar medium. *Planta.* **99**: 12-20.

Naidoo, G. and Kift, J. (2006). Responses of the saltmarsh rush *Juncus kraussii* to salinity and waterlogging. *Aquat. Bot.* **84**: 217-225.

Naik, P.S. and Widholm, J.M. (1993). Comparison of tissue culture and whole plant responses to salinity in potato. *Plant Cell Tissue Organ Cult.* **33**: 273-280.

Nanakorn, M. and Namwongprom N. (1994). Application of tissue culture technique for production and micropropagation in pepper (*Capsicum frutescence* L.). Proc. second symposium on vegetable and legume res. Kasetsart Univ., Bangkok (Thailand). Res. and Dev. Inst. Tropic. Vegetable Res.Center, Bangkok (Thailand). pp. 164-177.

Nanakorn, M., Jiamjetjaroon, W., Suwanawong, S., Wongwattana, C. and Shim, I.S. (2003). *In vitro* selection of salt-tolerant cell lines in kallar grass *(Diplachne fusca* L.). *Weed Biol. Manage.* **3**: 49-52.

Nervo, G., Azzimonti, M.T., Bonelly, A. and Tamietti, G. (2007). Application of *In vitro* anther culture methods to a pepper breeding program for disease resistance. *Phytomorphology.* **19**: 389-404.

Neumann, P.M. (1995). Inhibition of root growth by salinity stress: Toxicity or an adaptive biophysical response. **In:** Structure and Function of Roots. F.

M. Baluska, Ciamporova, O. Gasparikova, P.W. Barlow, (eds.). Kluwer Academic Publishers. pp. 299-304.

Nickell, L.G. and Maretzki, A. (1969). Growth of suspension cultures of sugarcane cells in chemically defined media. *Physiol. Plant.* **22**: 117-125.

Nitsch, J.P. and Nitsch, C. (1969). Haploid plants from pollen grains. *Science.* **163**: 85-87.

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

Nobecourt, P. (1937). Cultures en série de tissus végétaux sur milieu artificiel. *C.R. Seanc. Soc. Biol.* **205**: 521-523.

Nobecourt, P. (1939). Sur la pérennité et l'augmentation de volume des cultures de tissues végétaux. *C.R. Seances Soc. Biol. Ses. Fil.* **130**: 1270-1271.

Nowaczyk, P. and Kisiala, A. (2006). Effect of selected factors on the effectiveness of *Capsicum annuum* L. anther culture. *J. Appl. Genet.* **47**: 113-117.

Nowaczyk, P., Olszewska, D. and Kisiala, A. (2009). Individual reaction of *Capsicum* F_2 hybrid genotypes in anther cultures. *Euphytica.* **168**: 225-233.

Oboth, G. and Rocha, J.B.T. (2007). Distribution and antioxidant activity of polyphenols in ripe and unripe tree pepper (*Capsicumm pubescens*). *J. Food Biochem.* **31**: 456- 473.

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

Ochoa-Alejo, N. and Ireta, M.L. (1990). Cultivar differences in shoot forming capacity of hypocotyl tissue of red pepper pepper (*Capsicum annuum*) cultured *in vitro*. *Sci. Hort.* **42**: 21-28.

Ochoa-Alejo, N. and Ramirez-Malagon, R. (2001). *In vitro* chili pepper biotechnology. *In vitro Cell. Dev. Biol. Plant.* **37**(6): 701-729.

Otroshy, M., Moradi, K. and Nekouei, M. K. (2011a). The effect of different cytokenins in propagation of *Capsicum annuum* L. by *in vitro* nodal cutting. *Turkey J. Sci.* **9**(3): 21-30.

Otroshy, M., Moradi, M., Nekouei, M.K. and Struik, P.C. (2011b). Micropropagation of pepper (*Capsicum annuum* L.) through *in vitro* direct organogenesis. *Asian J. Biotechnol.* **3**(1): 38-45.

Parvaiz, A. and Satyawati, S. (2008). Salt stress and phyto-biochemical responses of plants - a review. *Plant Soil Environ.* **54**: 89-99.

Patil, R.H. (2001). Dry chilli production in Karnataka. *Kisan World.* **29**: 49-50.

Patnaik, J. and Debata, B.K. (1997a). *In vitro* selection of NaCl tolerant callus lines of *Cymbopogon martinii* (Roxb.) *Wats. Plant Sci.* **124**: 203-210.

Patnaik, J. and Debata, B.K. (1997b). Regeneration of plantlets from NaCl tolerant callus lines of *Cymbopogon martinii* (Roxb.) *Wats. Plant Sci.* **128**: 67-74.

Pauk, J., Lantos, C., Somogyi, G., Vagi, P., Abraham, T.Z., Gemes, J.A., Mihaly, R., Kristof, Z., Somogyi, N. and Timar, Z. (2010). Tradition, quality and biotechnology in Hungarian spice pepper (*Capsicum annuum* L.) breeding. *Acta. Agron. Hung.* **58**(3): 259-266.

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

Petersen, L. and Shireen, S. (2001). Soil and water salinity in the coastal area of Bangladesh, SRDI.

Piqueras, A., Hernandez, J.A., Olmos, E., Hellin, E. and Sevilla, F. (1996). Changes in antioxidant enzymes and organic solutes associated with adaptation of citrus cells to salt stress. *Plant Cell Tissue Orgnan Cult.* **45**: 53-60.

Power, J.B. and Chapman, J.V. (1985). Isolation, culture and genetic manipulation of plant protoplasts. **In**: Plant cell Culture.R.A., Dixon, (ed.). Oxford, Washington DC, IRL Press. pp. 37- 66.

Prakash, A.H., Sankararao, K. and Udayakumar, M. (1997). Plant regeneration from protoplasts of *Capsicum annuum* L. *cv.* California Wonder. *J. Biosci.* **22**(3): 334-339.

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

Pundeva, R. and Simeonova, N. (1992a). Induction of callus and organogenesis in pepper, *Capsicum fruitescens. Cur. Sci.* **57**: 99-120.

Pundeva, R. and Simeonova, N. (1992b). Preliminary study on the morphogeneric response of several pepper varieties. *Capsicum Newsl.* **Special issue**: 238-242.

Purohit, M., Srivastava, S. and Srivastava, P.S. (1998). Stress tolerant plants through tissue culture. **In:** Plant tissue culture and molecular biology: application and prospects. P.S., Srivastava, (ed.). Narosa Publishing House, New Delhi. pp. 554-578.

Qasim, M., Ashraf, M., Jamil, M.A., Ashraf, M.Y. and Shafiq-ur-Rehman, E.S.R. (2003). Water relations and leaf gas exchange properties in some elite canola (*Brassica napus*) lines under salt stress. *Ann. App. Biol.* **142**: 307-316.

Queiros, F., Fidalgo, F., Santos, I. and Salema, R. (2007). *In vitro* selection of salt tolerant cell lines in *Solanum tuberosum* L. *Biol. Plant.* **51**: 728-734.

Rabbani, G., Rahman, A. and Mainuddin, K. (2013). Salinity-induced loss and damage to farming households in coastal Bangladesh. *Int. J. Global Warming.* **5**(4): 400-415.

Rahman, M.H., Krishnaraj, S. and Thorpe, T.A. (1995). Selection for salt tolerance *in vitro* using microspore-derived embryos of *Brassica napus* cv. Topas, and the characterization of putative tolerant plants. *In vitro Cell. Dev. Biol. Plant.* **31**: 116-121.

Raia, M.K., Kaliaa, R.K., Singha, R., Gangolaa, M.P. and Dhawana, A.K. (2011). Developing stress tolerant plants through *in vitro* selection - An overview of the recent progress. *Environ. Expt. Bot.* **71**: 89-98.

Rakshit, A., Rakshit, S., Deokar, A. and Dasgupta, T. (2007). Effect of different explant and hormones on *in vitro* callus inductionand regeneration of pepper (*Capsicum annuum* L.). *Asian J. Biosci.* **3**(1): 180-183.

Rama, N. (1997). Stand ardization of protocols for *Agrobacterium*-mediated gene transfer in chilli (*Capsicum annuum* L.). M.Sc. (Agri.) Thesis, University of Agricultural Sciences, Bangalore.

Ramirez-Malagon, R. and Ochoa-Alejo, N. (1996). An improved and reliable chilli pepper (*Capsicum annuum* L.) plant regeneration method. *Plant Cell Rep.* **16**: 226-231.

Rangan, T.S. (1984). Clonal propagation. **In:** Cell culture and somatic cell genetics of plants. I.K., Vasil, (ed.). vol. 1. Acad. Press, New York, pp. 68-73.

Rani, C.R., Reema, C., Alka, S. and Singh, P.K. (2012). Salt tolerance of *Sorghum bicolor* cultivars during germination and seedling growth. *Res. J. Recent Sci.* **1**(3): 1-10.

Ravishankar, G.A., Suresh, B., Giridhar, P., Rao, S.R. and Johnson, T.S. (2003). Biotechnological studies on *Capsicum* for metabolite production and plant improvement. **In:** *Capsicum*: The genus *Capsicum*. D.E.A., Krishna, (ed.). Harwood Academic Publishers, UK, pp. 96-128.

Reddy, E.N.P., Anuradha, M., Vauterin, M. and Jacobs, M. (2002). Analysis of dihydropicolinate synthase promoter activity in *Arabidopsis thaliana* by *In-planta* transformation. *J. Plant Biochem. Biotechnol.* **11**: 73-77.

Reddy, M.K., Srivastava, A., Kumar, S., Kumar, R., Chawda, N., Ebert, A.W. and Vishwakarma, M. (2014). Chilli (*Capsicum annuum* L.) breeding in India: An overview. *SABRAO J. Breed. Genet.* **46**(2): 160-173.

Rhitu, R. and Shastri, L.B. (2007). Genetics and plant breeding: Introduction to plant biotechnology. New Delhi-110012, pp. 7-10.

Rhoads, D. (2007). History of cell biology. http://bitesizebio.com/166/history-of-cell-biology.

Robbins, W.J. (1922). Cultivation of excised root tips and stems tips under sterile conditions. *Bot. Gaz.* **73**: 376-390.

Robbins, W.J. and Bartley, M.A. (1937). Vitamin-B and the growth of excised tomato roots. *Science.* **85**: 246-247.

Rodeva, V., Koleva-Gudeva, L., Grozeva, S. and Traikova, F. (2007). Obtaining of haploids in anther culture of pepper *Capsicum annuum* L. and their including in the breeding process. *Goce. Delchev. Univ. Stip. Fac. Agric. Yearbook.* 7: 7-17.

Roshandel, P. and Flowers, T. (2009). The ionic effects of NaCl on physiology and gene expression in rice genotypes differing in salt tolerance. *Plant Soil.* **315**(1-2): 135-147.

Rout, G.R., Senapati, S.K. and Panda, J.J. (2008). Selection of salt tolerant plants of *Nicotiana tabacum* L. through *in vitro* and its biochemical characterization. *Acta Biol. Hung.* **59**: 77-92.

Roychoudury, A., Basu, S., Sarkar, S.N. and Sengupta, D.N. (2008). Comparative physiological and molecular responses of a common aromatic indica rice cultivar to high salinity with non aromatic indica rice cultivars. *Plant Cell Rep.* **27**(1): 395-1410.

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

Sabbah, S. and Tal, M. (1990). Development of callus and suspension cultures of potato resistant to NaCl and mannitol and their response to stress. *Plant Cell Tissue Orgnan Cult.* **21**: 119-124.

SADC (2004). *Capsicum* trade information brief. sadctrade.org/TIB/*Capsicum*.

Safarnejad, A., Collin, H.A., Bruce, K.D. and McNeilly, T. (1996). Charactrization of alfalfa (*Medicago sativa* L.) following *in vitro* selection for salt tolerance. *Euphytica.* **92**: 55-61.

Sagawa, Y. and Kunisaki, J.T. (1982). Clonal propagation of orchids by tissue culture. **In**: Plant tissue culture. A. F., Maruzen, (ed.). Tokyo. pp. 683-684.

Sajid, A.Z. and Aftab, F. (2014). Plant regeneration from *in vitro*-selected salt tolerantcallus cultures of *Solanum tuberosum* L. *Pakistan J. Bot.* **46**(4): 1507-1514.

Sakhanokho, H.F. and Kelley, R.Y. (2009). Influence of salicylic acid on *in vitro* propagation and salt tolerance in *Hibiscus acetosella* and *Hibiscus moscheutos* (cv. 'Luna Red'). *African J. Biotechnol.* **8**: 1474-1481.

Sanatombi, K. and Sharma, G.J. (2006). *In vitro* regeneration and mass multiplication of *Capsicum annuum* L. *J. Food Agric. Environ.* **4**(1): 205-208.

Sanatombi, K. and Sharma, G.J. (2007). Micropropagation of *Cpsicum annuum* L. *Not. Bot. Hort. Agrobot. Cluj.* **35**(1): 57-64.

Santana-Buzzy, N., Pez-Puc, G.L., Canto-Flick, A., Barredo-Pool, F., Balam-Uc, E., Aviles-Vinas, S., Solis-Marroquin, D., Lecona-Guzman, C., Bello-Bello, J.J., Gomez-Uc, E. and Mijangos-Cortes, J.O. (2009). Ontogenesis of the somatic embryogenesis of habanero pepper (*Capsicum chinense* Jacq.). *Hort. Sci.* **44**(1): 113-118.

Saxena, P.K., Rashid, G.R. and Maheshwari, S.C. (1981). Isolation and culture of protoplast of *Capsicum annuum* L. and their regeneration into plants flowering *in vitro*. *Protoplasm.* **108**: 357-360.

Schleiden, M.J. and Schwann, T. (1839). Contributions to phytogenesis. https://en.wikipedia.org/wiki/Cell_theory.

Segui-Simarro, J.M., Corral-Martinez, P., Parra-Vega, V. and Gonzalez-Garcia, B. (2011). Androgenesis in recalcitrant solanaceous crops. *Plant Cell Rep.* **30**: 765-778.

Sengupta, S. and Majumder A.L. (2009). Insight into the salt tolerance factors of a wild halophytic rice, *Porteresia coarctata*: a physiological and proteomic approach. *Planta.* **229**: 911-929.

Shanker, A.K. and Venkateswarlu, B. (2011). Abiotic stress in plants-mechanisms and adaptations. Intech Publisher, Janeza Tridne Rijeka, Croatia. p. 428.

Shankhdhar, D., Shankhdhar, S.C., Mani, S.C., Pant, R.C., (2000). *In vitro* selection for salt tolerance in rice. *Biol. Plant.* **43**: 477-480.

Shen, H., Wang, Z.Y. and Jiang. (1994). Regeneration and variation of pepper. **In**: Advances in horticulture, Dong, G. and Meng, L.Y. (eds.). Beijing, China. pp. 295-299.

Shi, H., Ishitani, M., Kim, C. and Zhu, J.K. (2000). The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na^+/H^+ antiporter. *Proc. Natl. Acad. Sci. USA*. **97**(12): 6896-6901.

Shivegowda, S.T., Mythili, J.B., Lalitha, A., Saiprasad, G.V.S., Gowda, R. and Gowda, T.K.S. (2002). *In vitro* regeneration and transformation in chilli pepper *(Capsicum annuum* L.). *J. Horti. Sci. Biotechnol*. **77**(5): 629-634.

Shreya, S., Krupakar, A., Surendra, Chandran, D. and Koshy, P. (2014). Direct regeneration protocols of five *Capsicum annuum* L. varieties. *African J. Biotechnol*. **13**(2): 307-312.

Siddiquei, A.N. and Anis, M. (2006). Improved plant regeneration in *Capsicum annuum* L. from nodal segments. *Biol. Plants*. **50**(4): 701-704.

Simon, E. (1908). Étude sur les espèces de la famille des eresidae qui habitent l'Égypte. *Bull. Soc. Ent. Egypte*. **1**: 77-84.

Simonne, A.H., Simonne, E.H., Eitenmiller, R.R., Mills, H.A. and Green, N.R. (1997). Ascorbic acid and provitamin-A contents in unusually colored bell peppers (*Capsicum annuum* L.). *J. Food Comp. Anal*. **10**: 299-311.

Singh, M., Jaiswal, U. and Jaiswal, V.S. (2003). *In vitro* selection of NaCl-tolerant callus line and regeneration of plantlets in a bamboo (*Dendrocalamus strictus* Nees.). *In vitro Cell Dev. Biol. Plant*. **39**: 229-233.

Skinner, J.C. and Street, H.E. (1954). Studies on the growth of excised roots: observations on the growth of excised groundsel roots. *New Phytol*. **53**: 44-67.

Skoog, F. and Miller, C.O. (1957). Chemical regulation of growth and organ formulation in plant tissue cultures *in vitro. Symp. Soxen Exptal. Biol*. **11**: 118-131.

Skoog, F. and Tsui, C. (1948) Chemical control of growth and bud formation in tobacco stem segments and callus cultured *in vitro*. *Amirican J. Bot*. **35:** 782-787.

Smith, H.O. and Nathans, D. (1973). A suggested nomenclature for bacterial host modification and restriction systems and their enzymes. *J. Mol. Biol*. **81**(3): 419-423.

Solis-Marroquin, D., Lecona-Guzmán, C.A., Canto-Flick, A., Bello-Bello, J.J. and Santana-Buzzy, N. (2011). *In vitro* selection of a salt-tolerant embryogenic line in habanero pepper (*Capsicum chinense* Jacq.). *Hort. Sci*. **46**(12): 1666-1671.

Song, S.Y., Chen, Y., Chen, J., Dai, X.Y. and Zhang, W.H. (2011). Physiological mechanisms underlying OsNAC5-dependent tolerance of rice plants to abiotic stress. *Planta.* **234**(2): 331-345.

Sopory, S.K., Jacobsen, E. and Wenzel, G. (1978). Production of monohaploid embryoids and plantlets in cultured anthers of *Solanum tuberosum*. *Plant Sci. Lett.* **12**: 47-54.

Stasolla, C. and Yeung, E.C. (2003). Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. *Plant Cell Tissue Organ Cult.* **74**: 15-35.

Steinitz, B., Wolf, D., Matzevitch-Josef, T. and Zelcer, A. (1999). Regeneration *in vitro* and genetic transformation of pepper (*Capsicum* spp.): the current state of the art. *Capsicum Eggplant Newsl.* **18**: 9-15.

Steward, F.C. (1968). Totipotency of angiosperm cells and its significance for morphology and embryology. *Phytomorphology.***17**: 499-507.

Steward, F.C. and Caplin, S.M. (1952). Investigations on growth and metabolism of plant cells: Evidence on the role of the coconut milk factor. *Annu. Bot.* **16**: 491-504.

Stewart, G.R. and Lee, J.A. (1974). The role of proline accumulation in halophytes. *Planta.* **120**: 279-289.

Straus, J. and La-Rue, C.D. (1954). Maize endosperm tissue grown *in vitro* culture requirements. *Amirican J. Bot.* **41**: 687-694.

Subhash, K. and Christopher, T. (1988). Direct plantlet formation in cotyledon cultures of *Capsicum frutescens. Cur. Sci.* **57**: 99-100.

Sunkar, R., Chinnusamy, V., Zhu, J. and Zhu, J.K. (2007). Small RNAs as big players in plant abiotic stress responses and nutrient deprivation. *Tren. Plant Sci.* **12**(7): 301-309.

Supena, E.D., Suharsono, S., Jacobsen, E. and Custers, J.B. (2006). Successful development of a shed-microspore culture protocol for doubled haploid production in Indonesian hot pepper (*Capsicum annuum* L.). *Plant Cell Rep.* **25**: 1-10.

Swamy, S., Krupakar, A., Ran, S.C.D. and Koshy, E.P. (2014). Direct regeneration protocols of five *Capsicum annuum* L. varieties. *African J. Biotechnol.* **13**(2): 307-312.

Szabados, L. and Savouré, A. (2010). Proline: A multifunctional amino acid. *Tren. Plant Sci.* **15**: 89-97.

Szasz, A., Nervo, G. and Fasi, M. (1995). Screening for *in vitro* shoot forming capacity of seedling explants in bell pepper *(Capsicum annuum* L.)

genotypes and efficient plant regeneration using thidiazuron. *Plant Cell Rep.* **14**: 666-669.

Tal, M. (1994). *In vitro* selection for salt tolerance in crop plants: Theoretical and practical considerations. *In vitro Cell Dev. Biol. Plant.* **30:** 175-180.

Tavakkoli, E., Rengasamy, P. and McDonald, G.K. (2010). High concentrations of Na^+ and Cl^- ions in soil solution have simultaneous detrimental effects on growth of faba bean under salinity stress. *J. Expt. Bot.* **61**: 4449-4459.

Tester, M. and Davenport, R. (2003). Na^+ tolerance and Na^+ transport in higher plants. *Annu. Bot.* **91**: 503-527.

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

Thom, M., Maretzki, A., Komor, E. and Sakai, W.S. (1981). Nutrient uptake and accumulation by sugarcane cell cultures in relation to the growth cycle. *Plant Cell Tissue Organ Cult.* **1**: 3-14.

Touraev, A. and Heberle-Bors, E. (2003). Anther and microspore culture in tobacco. **In:** Double haploid production in crop plants. M. Maluszynski, K.J. Kasha, B.P. Forster, I. Szarejko, (eds.). Kluwer, Dordrecht. pp. 223-228.

Turkan, I. and Demiral, T. (2009). Recent developments in understanding salinity tolerance. *Environ. Expt. Bot.* **67**: 2-9.

Unnikrishnan, S.K., Prakash, L., Josekutty, P.C., Bhatt, P.N. and Mehta, A.R. (1991). Effect of NaCl salinity on somatic embryo development in *Sapindus trifoliatus* L. *J. Expt. Bot.* **42**: 401-406.

Urechean, V. (2003). The influence of stress induced by NaCl on morphogenetic aspects of the callus initiated from immature maize embryos. *Bulg. J. Plant Physiol.* **Special Issue**: 336-352.

Vacin, E.F. and Went, F.W. (1949). Some pH changes in nutrient solutions. *Bot. Gaz.* **110**: 605-613.

Vajrabhaya, M., Thanapaisal, T. and Vajrabhaya, T. (1989). Development of salt tolerant lines of KDML and LPT rice cultivars through tissue culture. *Plant Cell Rep.* **8**: 411-414.

Valera-Montero, L.L. and Ochoa-Alejo, N. (1992). A novel approach for chilli pepper *(Capsicum annuum* L.) plant regeneration: Shoot induction in rooted hypocotyls. *Plant Sci.* **84**: 215-219.

Van-Overbeek, J., Conklin, M.E. and Blakeslee, A.F. (1942). Cultivation *in vitro* of small *Datura* embryos. *American J. Bot.* **29**: 472-477.

Vasil, I.K. and Hildebrandt, A.C. (1966). Variations of morphogenetic behavior in plant tissue cultures. *American J. Bot.* **53**: 869-874.

Vaziri, A., Ahmadi, A.R., Fahimi, H., Bernard, F. and Shaker, H. (2004). The effect of different concentrations of NaCl on the callus induction and lipids of soybean. *Plant Tissue Cult.* **14**(21): 75-77.

Venkataiah, P., Christopher, T. and Subhash, K. (2003). Thiadiazuron induced high frequency adventitious shoot formation and plant regeneration in *Capsicum annuum* L. *J. Plant Biotechnol.* **5**: 245-50.

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

Verbruggen, N. and Hermans, C. (2008). Proline accumulation in plants: a review. *Amino Acids.* **35**: 753-59.

Verma, S., Dhiman, K. and Srivastava, D.K. (2013). Efficient *in vitro* regeneration from cotyledon explants in bell pepper (*Capsicum annuum* L. cv. California Wonder). *Int. J. Adv. Biotechnol. Res.* **4**(3): 391-396.

Verslues, P.E., and Sharma, S. (2010). Proline metabolism and its implications for plant-environmental interaction. *Arabidopsis Book.* **8**: e0140.

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

Wang, Y.Y., Sun, C.S., Wand, C.C. and Chien, N.F. (1973). The induction of the pollen plantlets of triticale and *Capsicum annuum* from anther culture. *Sci. Sinic.* **16**: 147- 157.

Went, F.W. (1926). On growth-accelerating substances in the coleoptile of *Avena sativa*. *Proc. Kon. Ned. Akad. Wet.* **30**: 10-19.

Went, F.W. (1929). On a substance causing root formation. *Proc. Kon. Akad. Wet.* **32**: 35-39.

White, P.J. and Broadley, M.R. (2001). Chloride in soils and its uptake and movement within the plant: a review. *Annu. Bot.* **88**: 967-988.

White, P.R, (1963). The cultivation of animal and plant cells. Ronald Press Co, New York. p. 228.

White, P.R. (1934). Potentially unlimited growth of excised tomato root tips in a liquid medium. *Plant Physiol.* **9**: 585-600.

White, P.R. (1937). Survival of isolated tomato roots at sub-optimal and supra-optimal temperatures. *Plant Physiol.* **12**: 771-776.

White, P.R. (1943). Nutrient deficiency studies and an improved inorganic nutrient for cultivation of excised tomato roots. *Growth.* **7**: 53-65.

White, P.R. (1939). Controlled differentiation in plant tissue and organs. *Annu. Rev. Plant Physiol.* **2**: 231-244.

Willenborg, C.J., Gulden, R.H., Johnson, E.N. and Shirtliffe, S.J. (2004). Germination characteristics of polymer-coated canola (*Brassica napus* L.) seeds subjected to moisture stress at different temperatures. *Agron. J.* **96**(3): 786-791.

Winicov, I. (1996). Characterization of rice (*Oryza sativa* L.) plants regenerated from salt-tolerant cell lines. *Plant Sci.* **13**: 105-111.

Woodward, A.J. and Bennett, I.J. (2005). The effect of salt stress and abscisic acid on proline production, chlorophyll content and growth of *in vitro* propagated shoots of *Eucalyptus camaldulensis*. *Plant Cell Tissue Orgnan Cult.* **82**: 189-200.

Xu, G., Magen, H., Tarchitzky, J. and Kafkafi, U. (2000). Advances in chloride nutrition of plants. *Adv. Agron.* **68**: 97-150.

Yamaguchi, T. and Blumwald, E. (2005). Developing salt-tolerant crop plants: challenges and opportunities. *Tren. Plant Sci.* **10**: 615-620.

Ye, J.M., Kao, K.N., Harvey, B.L. and Rossnagel, B.G. (1987). Screening salt-tolerant barley genotypes via F_1 anther culture in salt stress media. *Theor. Appl. Genet.* **74**: 426-429.

Yen, H. E., Wu, S.M., Hung, Y.H. and Yen, S.K. (2000). Isolation of 3 salt-induced low-abundance cDNAs from light-grown callus of Mesembryanthemum crystallinum by suppression subtractive hybridization. *Physiol. Plant.* **110**(3): 402-409.

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

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

Zadeh, H.M. and Naeni, M.B. (2007). Effects of salinity stress on the morphology and yield of two cultivars of canola (*Brassica napus* L.). *J. Agron.* **6**: 409-414.

Zagorska, N.A., Dimitrov, B.D., Ilieva, I., Gadeva, P. (2002). *In vitro* induction of androgenesis and gynogenesis in *Rubus caesius* L. and *Rubus idaeus* L. *Genet. Breed.* **31**: 3-13.

Zair, I., Chlyah, A., Sabounji, K., Tittahsen, M. and Chlyah, H. (2003). Salt tolerance improvement in some wheat cultivars after application of *in vitro* selection pressure. *Plant Cell Tissue Organ Cult.* **73**: 237-244.

Zeba, N. (2009). Functional analysis of abiotic stress-inducible genes encoding RING zinc finger protein and an adenylate kinase from *Capsicum annuum*. PhD. Thesis.

Zenk, M.H. (1974). Haploids in physiological and biochemical research. **In:** Haploids in higher plants. K.J. Kasha, (ed.). Canada University Guelph Press. pp. 339-353.

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

Zhang, S. and Duijn, B.V. (2014). Review: Cellular auxin transport in algae. *Plants*. **3**: 58-69.

Zhao, G.Q., Ma, B.L. and Ren, C.Z. (2007). Growth, gas exchange, chlorophyll fluorescence and ion content of naked oat in response to salinity. *Crop Sci.* **47**(1): 123-131.

Zhu, Y.X., Ougang, W.J., Zang, Y.F. and Chen, Z.L. (1996). Transgenic sweet pepper plants from *Agrobacterium* mediated transformation. *Plant Cell Rep.* **16**: 71-75.

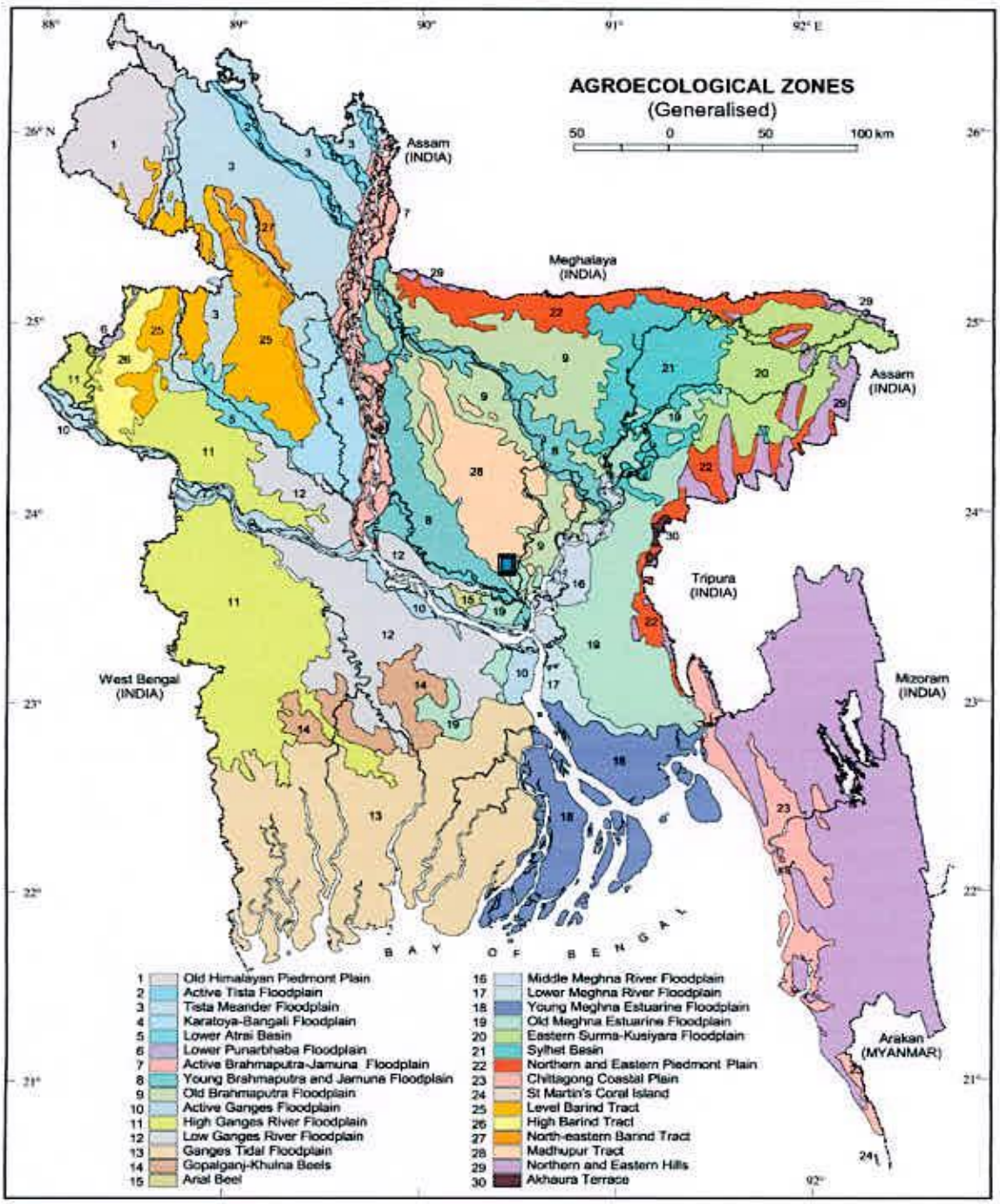
Zinnah, K.M.A., Zobayer, N., Sikdar, S.U., Liza, L.N., Chowdhury, M.A.N. and Ashrafuzzaman, M. (2013). *In vitro* regeneration and screening for salt tolerance in rice (*Oryza sativa* L.). *Int. Res. J. Biol. Sci.* **2**(11): 29-36.

Zou, J., Liu, A., Chen, X. Chen, X., Zhou, X., Gao, G., Wang, W. and Zhang, X. (2009). Expression analysis of nine rice heat shock protein genes under abiotic stresses and ABA treatment. *J. Plant Physiol.* **166**(8): 851-861.

Zou, J., Liu, C., Liu, A., Zou, D. and Chen, X. (2012). Overexpression of OsHsp 17.0 and OsHsp 23.7 enhance drought and salt tolerance in rice. *J. Plant Physiol.* **169**(6): 628-635.

Appendices

Appendix I. Map showing the experimental site under the study



■ The experimental site under study

Appendix II. 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 sulfate heptahydrate ($MgSO_4.7H_2O$)	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 III: 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	18 DAT	36 DAT	0 DAT	18 DAT	36 DAT
Factor A (genotype)	4	0.000^{NS}	43.134^{*}	95.056^{*}	0.000^{NS}	0.115^{*}	0.257^{*}
Factor B (salinity treatments)	4	0.000^{NS}	37.316^{*}	108.268^{*}	0.000^{NS}	0.123^{*}	0.722^{*}
A×B	16	0.000^{NS}	8.508^{*}	13.425^{*}	0.000^{NS}	0.045^{*}	0.062^{*}
Error	50	1.000	0.371	0.499	1.000	0.001	0.001

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

Appendix IV. Proline standard curve