IN VITRO REGENERATION OF ONION (*Allium cepa* L.) UNDER SALT STRESS CONDITION

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CERTIFICATE

*This is to certify that the thesis entitled "**IN VITRO REGENERATION OF ONION (Allium cepa L.) UNDER SALT STRESS CONDITION**" 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 BIOTECHNOLOGY,** embodies the result of a piece of bona-fide research work carried out by **MD. ASFIQUR RAHMAN PLABON** Registration No. **11-04569** under my supervision and guidance. No part of this thesis has been submitted for any other degree or diploma.*

I further certify that any help or sources of information as has been availed of during the course of this work has been duly acknowledged & style of the thesis have been approved and recommended for submission.

(Prof. Dr. Md. Ekramul Hoque)
Supervisor

Dated: *30 April, 2017*
Dhaka, Bangladesh

DEDICATED

TO

MY PARENTS

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IN VITRO REGENERATION OF ONION (*Allium cepa* L.) UNDER SALT STRESS CONDITION

ABSTRACT

The present research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of September 2016 to July 2017 for *in vitro* regeneration of onion (*Allium cepa* L.) under salt stress condition. Three genotypes namely Faridpuri, Taherpuri and Pusa red (Indian) were used as investigated genotypes in this study. Shoot tip segments of these genotypes were cultured in MS (Murashige and skoog, 1962) media having 0.0 (control), 25, 50, 75 and 100 mM NaCl. The shoot tips of experimental onion genotypes were not significantly affected upto 75 mM salinity level in comparison with control treatment. The experiment was conducted at two factorial (variety and Treatment) Completely Randomized Design (CRD) with 3 replications for each treatment. Salinity response on days required for shoot regeneration varied significantly from different salinity condition**.** The genotype Faridpuri was found maximum tolerant upto 100mM salinity level with 10.60 cm shoot length and 1.43 cm root length having the highest relative shoot and root growth. Besides, Pusa red was found as salinity sensitive genotype showing the lowest 7.03 cm shoot length and 0.60 cm root length at 100 mM NaCl treatment. However, Taherpuri was found tolerant up to 75 mM salinity level with 8.30 cm shoot length and 1.43 cm root length, respectively. The genotypes Faridpuri, Taherpuri and Pusa red (Indian) can be used for further investigation in field condition to evaluate their performance at various salinity levels. Therefore, a convenient *in vitro* regeneration protocol of onion genotypes under different salinity level has been developed which can be used for screening salinity tolerant variety of onion within a very short period of time.

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LIST OF ACRONYMS

AEZ = Agro-Ecological Zone
BARI = Bangladesh Agricultural Research Institute
BBS = Bangladesh Bureau of Statistics
ppm = parts per million
et al. = and others
n = Nitrogen
CRD = Completely Randomized Design
DAI = Days After Initiation
ha^{-1} = Per hectare
gm = Gram (s)
kg = Kilogram
µg = Micro gram
SAU = Sher-e-Bangla Agricultural University
no. = Number
wt. = Weight
^{0}c = Degree celsius
mm = Millimeter
max = Maximum
min = Minimum
% = Percent
NAA= 1-Naphthalene acetic acid
Nacl = Sodium chloride
LSD = Least significant difference
CV% = Percentage of coefficient of variance
hr = Hour
t = Ton
viz. = Videlicet (namely)
psi = Pounds per square inch

CHAPTER-I
INTRODUCTION

Onion (*Allium cepa* L.) is one of the most important bulb crops and popular vegetable grown for its pungent bulbs and flavorful leaves. It is a member of Alliaceae family (Hamlet, 1990) belonging to the genus *Allium*. There are more than 500 species within the genus *Allium*, of these most are bulbous plants. It is one of the most important spice as well as promising vegetable for Bangladesh. Central Asia is the primary center of its origin and the Mediterranean is the secondary center for large type of onion (Mc. Cullum, 1976).

In Bangladesh, it is mainly produced in winter season. Usually, it is sown in November and harvested mostly in the months from February to March. It produces 17.04 lakh metric tons of onion from 4.19 lakh acres area (BBS, 2015). The average yield of onion in Bangladesh is far below 11 t ha^{-1} (BBS, 2015) as compared to the world average of 19.32 t ha^{-1}. Onion is mainly produced in the winter season. But cultivation in summer season is constrained due to adverse weather and proper cultural practices (Islam *et al*. 2008). But demand for its use is ever increasing irrespective of season. In Bangladesh onion cultivation in commercial scale is found to be concentrated in the greater districts of Faridpur, Dhaka, Rajshahi, Comilla, Mymensingh, Rangpur and Pabna. About 11.5 lac metric tons onion is produced from 135721 hectare land in Bangladesh (BBS, 2012).

Among the spice crops grown in Bangladesh, the total area under onion cultivation is 117814 hectares with the total production of about 872000 metric tons (BBS, 2010b). To meet this shortage, Bangladesh has to import onion from India and China every year (Hossain and Islam, 2006). Onion is used in the preparation of soup, sauce, salad and for seasoning foods as well. The average consumption of onion in Bangladesh is 25 gm/head/day (BBS, 2015)

Salinity is one of the most brutal environmental factors limiting the productivity of crop plants because most of the crop plants are sensitive to salinity caused by high concentrations of salts in the soil. A considerable amount of land in the world is affected by salinity which is increasing day by day. On the other hand, increased salinity of agricultural land is expected to have destructive global effects, resulting in up to 50% loss of cultivable lands by the middle of the twenty first century (Mahajan and Tuteja 2005). In most of the cases, the negative effects of salinity have been attributed to increase in Na^+ and Cl^- ions in different plants hence these ions produce the critical conditions for plant survival by intercepting different plant mechanisms. Although both Na^+ and Cl^- are the major ions which produce many physiological disorders in plants, Cl^- is the most dangerous (Tavakkoli *et al.* 2010). Salinity at higher levels causes both hyperionic and hyperosmotic stress and can lead to plant demise. The outcome of these effects may cause membrane damage, nutrient imbalance, altered levels of growth regulators, enzymatic inhibition and metabolic dysfunction, including photosynthesis which ultimately leads to plant death (Mahajan and Tuteja 2005; Hasanuzzaman *et al*. 2012a). Biochemical and molecular studies of salt stress responses in plants have revealed significant increases of reactive oxygen species (ROS), including singlet oxygen, superoxide, hydroxyl radical and hydrogen peroxide (Tanou *et al*. 2009; Ahmad *et al*. 2012; Ahmad and Umar 2011). However, the effect of salt stress on plants depends on the concentration and time of exposure of salt, plant genotypes and environmental factors.

Crop production is hindered or restricted in several areas of the world by naturally saline soil. Salinity inhibits the growth of plants by affecting both water absorption and biochemical processes, such as nitrogen assimilation and protein biosynthesis (Dubey, 1994). Under saline conditions, the plants fail to maintain the required balance of organic constituents leading to suppressed growth and yield. In developing countries, the limited supply of good quality

water in many arid and semi-arid regions necessitates the use of saline water where available for crop production. This, in turn, requires the screening of crop plant varieties for their tolerance to salinity. Screening for improved salt tolerance is difficult in the field because of lateral, vertical and temporal variability in salt distribution within the soil profile. In addition, plant salt tolerance varies with ontogeny, the growth parameter measured and environmental factors.

Tissue culture techniques have been applied to the plant species in an attempt to screen salt tolerant genotypes within a very short period of time. In this respect, numbers of researchers have suggested that cultured tissues and cells may prove useful both in selections of the salt-tolerant plants and in studies of the physiological basis for salinity tolerance (Chen *et al*., 1980; Umiel *et al*., 1980). Easy manipulation of salt mixture concentrations in media, especially in suspension culture, also permits uniform and direct treatment on cell growth with a given salt stress level. Most efforts at selecting salt resistant cell lines have involved direct selection for capacity to grow on otherwise inhibitory levels of NaCl (Dix, 1985; Tal, 1983).

The onion crop is of great importance in Bangladesh and is one of the main spices crop. In cultivated onion, the development of new and improved genotypes are important for its growing in various ecological areas. Aseptic culture technique offers a potential for selection of salt-tolerant lines of onion. The present work was planned to study the influence of different salt levels on growth and chemical contents of onion tissue cultures and *in vitro* selection for salt stress tolerant genotypes.

Considering the above facts the present investigation was under taken with the following objectives:

1. Establishment of *in vitro* regeneration protocol for onion genotypes under salt stress condition.
2. Screening of the onion genotypes under different level of salinity.
3. Identification and selection of onion genotype under salt condition.
4. Identification of best salt tolerant genotype of onion *in vitro*.

CHAPTER II
REVIEW OF LITERATURE

Onion (*Allium cepa* L.) is one of the most important bulb crops and popular vegetable grown for its pungent bulbs and flavorful leaves. Bangladesh is an agriculture based country having 30% of her cultivated land located in the coastal areas where salinity is a critical environmental constraint to crop productivity (Mahmuduzzaman *et al*. 2014). Using the salt-tolerant crops is one of the most important strategies to solve the problem of salinity but salt tolerance does not appear to be conferred by unique gene(s) (Manchanda and Garg, 2008). Plant tissue culture techniques in assistance with conventional breeding and biotechnology have become latest approaches for rapid propagation, evaluation of potato cultivars against salt stress (Byun *et al*. 2007) as well as making them tolerant against environmental stresses especially for salt stress (Rahman *et al*. 2008). The study of plant salt tolerance to identify crop sensitivity seems to be a fruitful and short time approach (Zhu, 2007). Therefore the present study was undertaken to provide efficient information as much as possible on the maximum salinity tolerance of different salt tolerant onion cultivars against NaCl stress and their propagation efficiency using modified MS media composition under in vitro condition. But unfortunately, there is little research available on micropropagation of salt tolerant onion varieties in Bangladesh. However, the literatures which are most relevant to the present study are reviewed and cited here under the following headings-

2.1 Concept of plant tissue culture

Tissue culture technique is the back bone of plant biotechnology. Plant tissue culture is the collection of techniques used to regenerate or maintain plant cells, tissues or organs under aseptic and controlled environmental condition in an artificial and well defined liquid or semisolid nutrient medium. Plant tissue culture largely depends on the four fundamental abilities i.e. totipotency, dedifferentiation, competency and plasticity of plant. Among the different

cultural media MS media (Murashige and skoog, 1962) supplemented with or without different concentration of hormones or other elements have been used extensively since 1962 in plant tissue culture. Gottlieb Haberlandt (1902), an Austrian botanist, is recognized as father of plant tissue culture. On the other hand this technique was first introduced in Bangladesh with jute in late 1970s in the department of botany at Dhaka University and with potato at Tuber Crops Research Centre (TCRC) of Bangladesh Agricultural Research Institute (BARI) since 1983.

Various tissue culture techniques have been used in an attempt to develop *'in vitro*' propagation and breeding of the common onion, *Allium cepa*. Callus cultures had previously been found to be slow-growing; but a study of comparative growth rates on several standard media and the addition of nitrate, ammonium, and phosphate ions, resulted in the derivation of a medium producing a forty-fold increase in tissue fresh weight after eight week culture periods. Organogenesis from onion callus cultures has not been well documented, and in an attempt to study organogenic capacity from onion callus, several sources of callus were used. Callus originating from seedling radicles produced the largest numbers of shoots per callus explant (up to 6 shoots per 30 mg inoculum). Results using callus of bulb and set origin were more variable. A further source of shoot production has been found to occur from the onion capitulum in vitro. When plated onto agar media the capitulum responds by producing shoots of diverse origin. The hormone supplement of the culture medium, treatment method and age of inoculum have been studied, and results indicate the requirement for young flower-head material for optimal shoot production (about 10 shoots per flower-head) (Dunstan and Short, 1977).

2.2 Effect of salinity on plant *in vitro*

Salinity limits the yield of crops by affecting the metabolism of plants and causes important modification in different biochemical and molecular processes (Allakhverdiev *et al*. 2000; Zhu 2007). It can activate certain

photosynthetic enzymes activity causing decomposition of membrane structures (Meloni *et al*. 2003). Rate of photosynthesis and respiration in crop plants is severely interfered causing reduced plant growth and low productivity at high salinity level (Silva *et al*. 2001; Zhang *et al*. 2005; Fidalgo 2004). Higher level of salinity disrupts plant roots making water deficiency, nutrients imbalance by altering uptake and transport, ionic stress by higher Na^+ and Cl^- accumulation, cell membrane ineffectiveness and interfering cellular processes like cell division and genotoxicity resulting in reduced plant growth, development and yield (Munns, 2008). Romero-Aranda and Syvertsen (1996) found that accumulation of Na^+ and Cl^- in the leaves caused stomatal closure and reduction of total chlorophyll content in leaves which ultimately limit the photosynthesis of plant.

Kiełkowska (2017) conducted to the effects of various concentrations of sorbitol (100, 200 and 360 mM) and NaCl (100, 200 and 300 mM) on root meristem cells of *in vitro*-cultured *Allium cepa* L. were analyzed after 10 and 20 days. Both root meristem cell cross-section area and nuclear volume decreased under osmotic and salt stress. The osmotic component of applied stresses had a greater impact on cell shrinkage, while ionic stress perturbed cell functioning, resulting in cell cycle arrest and various aberrations, affecting nucleus integrity. A concentration of 300 mM of NaCl in the culture medium caused complete inhibition of mitotic activity in onion root tip cells after 20 days of exposure. Analysis of the action of iso-osmotic concentrations of NaCl (200 mM) and sorbitol (360 mM) showed stronger mitodepressive effects of salt stress in comparison to osmotic stress.

The response of onion tissue cultures to salinity stress was investigated by Bekheet *et al*. (2006). Callus initiated from aseptic seedlings was exposed to different levles of salt mixture. Fresh weight and growth value of callus inoculums were decreased as salt mixture increased in culture medium. However, dry weight and dry matter increased as salt level increased up to

4000 ppm and then decreased. Total proteins of callus enhanced as salt mixture increased in culture medium. For selection under salt stress, regenerated shoot buds derived from tolerant callus cultures were exposed to the different levels of salts mixture. The number of proliferated shoot buds and their fresh weight and growth value were depressed upon increasing of salts in medium. The best results of salt tolerance ratio were reached at 2000 ppm salts. Although protein content took similar trend of callus, it was relatively higher than in callus cultures at the same salt levels. Esterase patterns showed new band with the tolerance lines. This band had different mobility and more intensity with the shoot bud lines exposed to 2000 ppm of salt mixture. The tolerant shoot buds were in vitro rooted and successfully adapted to free-living conditions..

2.3 Effect of Growth Regulators

Allium neapolitanum is a valuable species of snow-white flowers, which is suitable for cultivation in flowerbeds, rock gardens as well as in containers. Whole buds of *Allium neapolitanum* were excised from bulbs in the beginning of October and then they were cultured on Murashige and Skoog (MS) medium containing BA 2 $mg.dm^{-3}$ and NAA 0.1 $mg \cdot dm^{-3}$ for shoot initiation. After several passages on the same medium for shoot multiplication, bases of shoots were placed for 2 subcultures on MS medium supplemented with BA or 2-iP in concentration of 2 or 5 $mg.dm^{-3}$ separately or in combination with NAA in concentration of 0.1 or 1 $mg.dm^{-3}$ to obtain multiplication. MS medium without growth regulators was used as a control. The best results were obtained on the medium supplemented with BA 5 $mg \cdot dm^{-3}$ and NAA 0.1 $mg.dm^{-3}$. On average 5.7 shoots regenerated from 1 shoot base during 12 weeks. Three types of auxins, IAA, IBA and NAA in concentration of 0.5 $mg.dm^{-3}$, were used for rooting. It was observed that NAA enhanced root formation but reduced roots length. The best quality rooted shoots were obtained on medium supplemented with 0.5 $mg \cdot dm^{-3}$ IAA. The survival rate of the plantlets under *ex vitro* condition was 70% after 4 weeks (Stelmaszczuk and Kozak, 2013).

In an experimental micropropagation of onion cvs. 'Lietuvos didieji', 'Stutgarten Riesen' and 'Centurion' F1 after disinfection onions were split radially into four equal sectors and cross-section. Murashige and Skoog medium, supplemented with 1 mg L^{-1} naphthaleneacetic acid (NAA), 0.9, 4.4, 8.9, 13.1 μM concentrations of 6-benzylaminopurine (BAP), kinetin (1.1, 5.3, 10.6, 5.8 μM) and 30 g L^{-1}sucrose were used for plant micropropagation. The highest number of microshoots (1.8 to 2.4 microshoots per explant) was formed by 'Centurion' F1 and 'Lietuvos didieji' explants, containing stem dome plus basal plate. Experiments with growth regulators showed that the number of microshoots increased when the BAP concentration was raised from 0.9 to 4.4 μM, respectively from 1.0 to 2.1 microshoots per explant. The lower concentration of BAP had a higher efficiency while raised BAP concentration significantly decreased regeneration. The highest micropropagation frequency using kinetin (1.9 to 2.1 microshoots per explant) was obtained at a moderate (10.6 μM) concentration. The regeneration intensity (output of microshoots) was 68 % higher using kinetin in comparison with BAP (Kamstaityte and Stanys, 2004).

Khalid *et al*. (2001) conducted an experiment to study the effect of growth regulators on plantlet regeneration and bulbing of onion *in vitro*. Results from the experiment showed that both plantlet regeneration and bulbing were greatly affected by growth regulators or their combinations. It was found that shoot induced from twin scale in 0.1 mg/l NAA was with a percentage of 93% and per explant shoot rate of 0.84. A combination of 0.1 mg/I NAA+2 mg/I 4PU-30 induced 89% shoot regeneration from twin scale. The highest per explant shoot (1.25), however, was observed in 0.1 mg/I NAA+1 mg/I 4PU30. There was no significant difference between the effect of combining NAA+BA and NAA+4PU-30 on shoot production. The highest root percentage (89%) in twin scale was obtained with a combination of 0.1 NAA+3 mg/l BA, and highest per explant root (1.06) was observed in 0.1 mg/l NAA+1 mg/l BA treatment. The effect of 4PU-30 and sucrose on inducing bulb formation independently was

also studied. The highest bulb percent (84%) was induced in 0.8 mg/l 4PU-30. The mean bulb circumference at the same concentration was 2.6 cm after six weeks of culture. In sucrose treatment, the highest bulb percentage (81%) was observed in 100 g/l sucrose. Apparently, a slightly higher (2.9 cm) mean bulb circumference was observed compared to 4PU-30. There was, however, no significant difference in bulb circumference between the two treatments.

In a different work, Ayabe and Sumi (1998) used the stem disc (consisting in the apical meristem and the lateral buds of the clove) to regenerate plants of the cultivar *Fukuchihowaito*. When this was cut into various fragments and cultivated on a medium with BA (0.4 μM), 20-25 adventitious shoots were obtained. The same result was observed when protoplasts isolated from shoot primordia were cultured in the presence of NAA (0.5μM) and 2iP (2.4μM), adenine and coconut milk (Ayabe *et al*., 1995). Barandiaran *et al*. (1999) used immature bulbs of 23 accessions as a source of axillary buds, which were cultivated during six weeks on B5 medium with 2.5 μM 2iP and 0.55 μM NAA (establishment phase). Multiplication of regenerated shoots was done on the same culture medium and 20 weeks later shoot clusters were separated in order to cultivate them individually and to induce bulb formation at a low temperature (4°C). Although plants and bulbs were obtained for all accessions under tested conditions, response depended on genotype (accession). Three months later, 60% of bulbs that were transferred to soil survived and produced shoots. This protocol allowed the use of the same culture medium for all phases of micropropagation (establishment, multiplication and bulb formation) and for all accessions, which enabled the handling of all materials tested at the same time, as only three subcultures were required over a period of seven months. Primordial leaf obtained from cloves are also able to produce adventitious shoots when cultivated on a medium with 2,4-D (4.5 μM), and develop into plants when transferred onto a medium containing picloram (1.4μM) and BA (13.3 μM) (Myers & Simon, 1999). Haque *et al*. (2003) developed a protocol for plant regeneration and bulb formation from shoot and root meristems of the

cultivar *Bangladesh Local*. Meristems were cultivated on MS medium without growth regulators or containing various concentrations of BA (1-10μM) and NAA (1-5μM). None of the combinations of growth regulators tested produced a higher response than the one observed in their absence (95.5%). In fact, the presence of these compounds suppressed shoot formation in a directly proportional manner to concentration; 45% of root explants formed adventitious shoots, 60% of which produced bulbs. Although a higher number of buds resulted in shoot formation, the root meristems produced more shoots per explant (20). Bulbs derived from root meristems were smaller than the ones derived from bud meristems.

Besides, Luciani *et a*l. (2006) tested different explants for micropropagation of bulb crops variety, which were cultivated on BDS medium (Dustan and Short, 1977), supplemented with picloram, 2,4-D and BA. The basal plates and meristems resulted in the highest values of shoot regeneration, and 2,4-D proved to be better than picloram for inducing callus and shoot formation. By using a combination of 0.25 μM 2,4-D and 4.43 μM BA, 100% of explants were able to produce calli, which differentiated into both embryos and shoots. It is worth mentioning that *in vitro* propagation is frequently associated with a process known as hyperhydricity or vitrification, which is a physiological disorder caused by the *in vitro* culture conditions that affects the behavior of regenerated plants. This disorder promotes abnormalities at physiological, anatomical and morphological level, which limit the successful establishment of differentiated plants upon their transfer to greenhouse. Hyperhydric plants have a slow growth rate, thick and deformed stems. Their leaves are translucent, thick and wet (Olmos & Hellin, 1998; Kevers *et al*., 2004).

A study of biochemical and ultra-structural traits of hyperhydric garlic shoots regenerated *in vitro* was carried out by Wu *et al*. (2009), who observed that organelles such as mitochondria and chloroplasts were compressed against cell wall, in these shoots. In addition, protein content decreased significantly and O_2

and H_2O_2 generation rate increased 45.3% and 63.9%, respectively. Activity of oxidative stress-related enzymes (lipoxygenase, superoxide dismutase, peroxidase, catalase, ascorbate peroxidase) also increased. Moreover, a rise in the level of electrolytes lixiviation was observed, indicating a damage of membrane lipids. Authors concluded that hyperhydric condition of tissues is closely linked to oxidative stress.

2.4 Micropropagation

The genus *Allium*, consists of hundreds of medicinal plant species, is one of the most imperative sources of life supporting drugs. The *in vitro* biotechnological interventions are vital to choose, multiply, store up and improve the major *Allium* sp. *In vitro* culture of *Allium* has performed an incredibly crucial role in accelerated growth of several species with desirable traits and production of healthy and disinfectant propagules as well as paved the way towards cultivar improvement. During the last quite a few years, several moves have been made for *in vitro* propagation of *Allium*. *In vitro* regeneration via direct and indirect organogenesis using different explants and plant growth regulator formulations has been comprehensively covered in the literature. Recent challenges for establishment of protocols for genetic transformation have gained preference in the recent past reports. This review article comprehensively describes the exploitation of biotechnology for *in vitro* regeneration and genetic transformation for enhancement of the genus *Allium (*Gantait *et al*, 2010)**.**

Studies related to the application of tissue culture techniques such as micropropagation for garlic production started in 1970. This technique proved to be advantageous over clove reproduction, as it only requires cells or small tissue fragments to generate high number of plants. Micropropagation can be carried out via two morphogenetic ways: (1) organogenesis, which results in the formation of organs (shoots or roots), and (2) somatic embryogenesis, which leads to the formation of structures having a similar or equal morphology to that of a zygotic embryo. Both processes can involve (indirect)

or not (direct) a previous callus phase. Morphogenetic ability in garlic decreases as the callus grows older and the emergence of abnormal plants increases (Novak, 1990). For this reason, regeneration that does not involve a previous callus phase is preferred. Embryogenesis possesses a series of advantages over organogenesis, such as higher potential for high plant output, lower labor requirement and lower cost (Sata *et al*., 2001). Several micropropagation protocols have been established using both ways of morphogenesis and different explant types; however, most protocols have been developed following the organogenetic way.

CHAPTER III
MATERIALS AND METHOD

3.1 Time and location of the experiment:

The present research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of September 2016 to July 2017.

The materials and methods used in these investigations are described below under the following heads and sub-heads of this chapter.

3.2. Experimental materials

3.2.1 Source of plant materials

Three varieties namely Faridpuri, Taherpuri and Pusa red (Indian) were collected from the Bangladesh Agricultural research Institute (BARI), Gazipur-1701 and used as investigated materials in this present study.

3.2.2. Explant materials

The shoot tips of *Allium cepa* L. were used as experimental materials in the present investigation.

Plate 1: Preparation of explants for culture

3.2.3. Instruments and glassware

Metal instruments like forceps, scalpels, spatulas, aluminum foils etc. were sterilized in an autoclave at a temperature of 121^0C for 20 minutes at 1.06 kg/cm^2 (15 PSI) pressure. The Borosil glasswares were used in this present experiment. Pipette, test tube, beaker, flat bottom flask, petridishes, measuring cylinders (25 ml, 50 ml, 100 ml, 500 ml, 1000 ml) etc. were used for media preparation.

3.2.4. Culture media

For success in tissue culture media should contain all major and minor elements, vitamins, growth regulators which are essential for normal plant growth. Murashige and skoog (MS) (1962) medium have been used as culture medium supplemented with IBA (0, 1, 2, 3, 4) mg/L and NaCl (0, 25, 50, 75, 100) mM for micropropagation of onion plant. The compositions of MS medium has been presented in appendix I. In this study MS (Murashige and skoog, 1962) medium supplemented with NaCl for micro-propagation and subculture. The following treatments were applied in this study.

3.2.4.a. Treatment combination for the *in vitro* regeneration of onion in salinity are

Treatments Composition

1. T_0 =(Control) MS Media without NaCl
2. T_1 =25 mM NaCl in MS media (2.28 dsm^{-1})
3. T_2= 50 mM NaCl in MS media (4.57 dsm^{-1})
4. T_3= 75 mM NaCl in MS media (6.85 dsm^{-1})
5. T_4= 100 mM NaCl in MS media (9.13 dsm^{-1})

3.3 Preparation of stock solution

3.3. 1. Preparation of Murashige and Skoog (MS) stock solution

In appendix I chemical composition of MS (Murashige and Skoog, 1962) medium is tabulated. The preparation of stock solutions of macronutrients, Fe-

EDTA, vitamins, amino acids and growth regulators is the first step in the preparation of MS media. Stock solutions were prepared at first and stored appropriately for use. Stock solution of growth regulators were prepared separately by dissolving the desired quantity of ingredients in appropriate solvent and the required final volume was made with distilled water for ready use to expedite the preparation of medium wherever needed.

3.3.1. a. Macronutrients (stock solution, 10X)

The required quantities of major salts (Appendix I) were weighed and dissolved thoroughly in 800 ml of distilled water in a 1000 ml beaker and final volume was made to 1000 ml by further adding water. The strength of the solution was 10 timed of that used in culture medium. The solution was poured into a reagent bottle (Durand, Scotland) and stored at 4^{o} c.

3.3.1. b. Micronutrients (stock solution, 100X)

The required quantity of minor salts (Appendix I) were weighed and successively dissolved in 500 ml of distilled water in a 1000 ml beaker. Final volume was made to 1000 ml through adding distilled water. The strength of the solution was 100 times of that used in culture medium. The stock solution was also poured into a clean reagent bottle (Durand, Scotland) and stored at 4^{o}C.

3.3.1. c. Iron EDTA (stock solution, 100X)

The required quantities (Appendix I) of Na_2EDTA (Ethylene di-amine tetra acetic acid, di-sodium salt) and $FeSO_4.7H_2O$ were weighed and dissolved separately in a 500 ml beaker. Then mixed together in a100 ml beaker and was heated until yellowish color appears. Final volume was made to 1000 ml through adding distilled water. The strength of the solution was 100 times of that used in culture medium. The stock solution was also poured into an amber color bottle and storage at 4±1^{o}C.

3.3.1. d. Organics (stock solution, 100X)

The required quantities of organic constituents and vitamins except myoinositol (Appendix I) were weighed and dissolved in 750 ml of distilled water in a 1000 ml beaker and made up to 1000 ml by adding distilled water. The strength of the solution was 100 times of that used in culture medium. The stock solution was also poured into a clean reagent bottle (Durand Scotland) and stored in a refrigerator at 4±1ºC.

3.3.1. e. Myoinositol (stock solution, 100X)

The solution was made 100 times the final strength of the medium. Required amount of myoinsitol (Appendix I) was dissolved in 250 ml distilled water in a clean beaker until the salt dissolved completely. The final volume was made up to the mark by adding the distilled water. The solution was also filtered and stored in refrigerator at 4±1ºC.

3.3.2 Preparation of hormones (Hormonal stock solutions)

To prepare the stock solution of IBA, 1 mg powder was placed on a clean weighing boat and dissolved with 0.1 N NaOH solvent. The mixture was then washed off with distilled water and collected in a 100 ml measuring cylinder and was made up to the volume with distilled water. The solution was then poured into a reagent bottle (Durand, Scotland) and stored at 4±1ºC.

3.3.3 Preparation of chemical solutions

3.3.3. a. Preparation of 1N NaOH

To prepare of 1 N NaOH stock solution, 40 g of NaOH pellets were dissolved in 1 L of double distilled water. Prepared solution was stored in a glass bottle and kept in cool and dry place. This solution was used to adjust pH in culture media preparation by increasing the pH meter reading.

3.3.3. b. Preparation of 1N HCl

To prepare of 1 N HCl stock solution, 36.5 g of HCl substances were dissolved in 1L double distilled water. Prepared solution was stored in a glass bottle and kept in cool and dry place. This solution was used to adjust pH in culture media preparation by decreasing the pH meter reading.

3.3.3. c. Preparation of 70 % ethanol

70 ml 99.9 % ethanol was dissolved poured in a100 ml measuring cylinder. Then 30 ml double distilled water was added to make final volume (100ml) for 70% ethanol and stored in a glass bottle. This solution was made fresh each time before use. It was used for sterilization purpose.

3.4. Preparation of culture media from MS stock solution

To prepare 1 L of culture media the following steps were followed:

Step-1. 500 ml of sterile double distilled water was poured into in a 1000 mL beaker.

Step-2. 100 mL from prepared MS Stock I (10 x), 10 mL from prepared MS Stock II (100X),

10 mL from prepared MS Stock III (100X), 10 mL from prepared MS Stock IV (100x) were added in the beaker.

Step-3. 30g of sucrose was added and gently stirred to dissolve these ingredients completely with the help of a hot plate magnetic stirrer.

Step-4. Required amount of NaCl, prepared IBA stock or hormonal supplements of different concentration for different media were measured and added to the solution as required and mixed well.

Step-5. Finally the volume was made up to 1000 mL with addition of sterile double distilled water.

Step-6. The pH was adjusted at 5.8

Step-7. 8g agar was added to the mixture and heated for 10 minutes in an electric oven for melting of agar.

Step-8. The media were distributed in test tubes or other culture vessels.

Step-9. Then sterilization of media was done by autoclaving at 1.06kg/cm^2 (15 PSI) pressure with 121° C for 20 minutes.

Step-10. Finally the media were stored in culture room for future use.

3.5. Sterilization

Aseptic condition is the pre requisite for *in vitro* techniques and for that all instruments, glassware and culture media were sterilized.

3.5.1. Sterilization of culture media

The culture tubes containing prepared media were autoclaved at 1.06 kg/cm^2 (15 PSI) pressure with 121 ^{0}C for 20 minutes. In case of shoot regeneration, at first culture media were taken different conical flask and autoclaved at 1.06kg/cm^2 (15 PSI) pressure with 121^0 C for 20 minutes. Then autoclaved media were poured into sterile petri dishes under a Laminar Air Flow Cabinet and were allowed to cool before use. All the test tubes and petri dishes were parafilmed and marked with permanent marker or sticker to indicate specific treatment. After sterilization the media were stored at 21±2^{0}C for several hours to make them ready (semi-solid) for inoculation with explants.

3.5.2. Sterilization of glasswares and instruments

All types of glasswares and instruments viz. culture vessel, beaker, petri dishes, pipette, plastic caps, test tubes, conical flask, forceps, scalpels, needles, spatulas, etc. were first rinsed with liquid detergent (Trix) and washed thoroughly with tap water until the detergent was removed completely. Then they were rinsed with double distilled water.

Finally all the glasswares, instruments, H_2O, aluminum foil etc. were sterilized for two times in an autoclave at a temperature of 121° C for 20 minutes at 1.06kg/cm2 (15 PSI) pressure.

3.5.3. Sterilization of culture room and transfer area

The culture room was cleaned by washing the floor and walls with a detergent or Lysol (germicide) followed by wiping with 70 % ethyl alcohol. This process of sterilization of culture room is repeated at regular intervals. The Transfer area was also sterilized twice a month by 70 % ethyl alcohol. Laminar Air flow Cabinet was usually sterilized by switching on the cabinet and UV light was used for 20 minutes and after sterilization by UV light transfer work was delayed for at least 5 minutes to ensure safe environment. Then the working surface of Laminar Air flow Cabinet was sterilized by wiping with cotton soaked with 70% ethyl alcohol prior to start the transfer work.

3.6. Precaution to ensure aseptic condition

All inoculation and aseptic manipulation were carried out in laminar air flow cabinet. The cabinet was usually switched on half an hour before working with UV light for 20 minutes to kill the germs before use. The instruments like scalpels, forceps, needles etc. were pre sterilized by autoclaving and subsequent sterilization was done by dipping in 70% ethyl alcohol followed by flaming and cooling method inside the laminar air flow cabinet. Hands were also sterilized by wiping with cotton soaked with 70% ethyl alcohol. All glasswares and instruments except media were kept inside laminar air flow cabinet to reduce the chances of contamination. Glass plate, distilled water, petri dishes etc. were sterilized in autoclave by following the same method of media sterilization. The neck of the culture vessels were flamed before closing it with the cap. Aseptic conditions were followed in each and every operation to avoid the contamination of cultures.

3.7. Explant preparation and culture

3.7.1. a. Preparation of explants

The shoot tip segment was the starting material. It was obtained from developing shoots of onion grown under field conditions and was brought to the preparation room .The shoot tips were washed with water in a beaker. The segment then cut in a optimum size required for inoculation in culture vial.

3.7.1. b. Sterilization of explants

The shoot tip segment of 2 to 3 cm size was taken in a beaker. Surface sterilization of explants was done as follows:

i. The shoots were cut as small size (2 to 3 cm) and rinsed with water.

ii. The shoot tips were soaked with Tween-20 solution having 10% concentration for 5 min.

iii. Washing with distilled water was done for several times.

iv. The explants were sterilized with 70% ethanol for 1min.

v. Then the explants were sterilized with 0.2% $HgCl_2$ for 2 min.

vi. The explants were rinsed with sterilized distilled water for at least 4 times.

vii. The final size of explants were made 0.5-1.0 cm.

viii. Finally the explants were transferred to the MS media carefully.

3.7.1. c. Inoculation of explants

The shoot tips were separately arranged horizontally on each culture vial having Murashige and Skoog (MS, 1962) medium supplemented with NaCl and 0.5 mg/L IBA along with the 8 g/L agar and 30 g/L sucrose .Inoculation of explants was done in Laminar Air Flow Cabinet. Explants were placed vertically on medium and mouth of the bottle was quickly flamed and capped tightly. After proper labeling mentioning treatment code, inoculation date etc. culture jars were transferred incubation room.

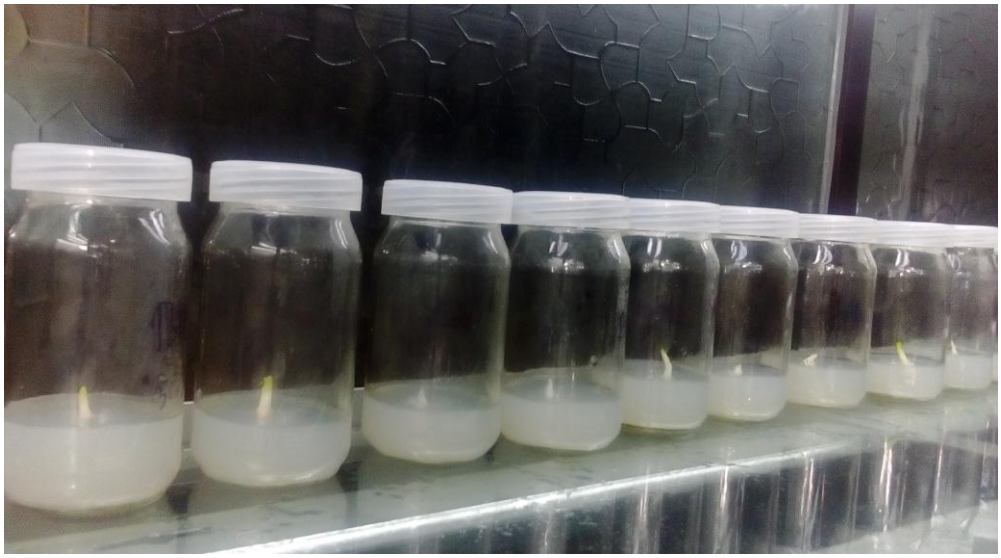


Plate 2: Inoculation of explants

3.7.1. d. Incubation of culture

The culture jars containing explants were incubated in a growth room with 21 ±1^{o}C under 2500 lux of fluorescent light with 16/8 hours photoperiod for regeneration. The culture jars were checked daily to note the growth response and contamination.

3.7.1. e. Maintenance of proliferating shoots

Initial sub-culturing was done after 15-25 days when the explants had produced some shoots. For sub-culturing, the entire samples of *in vitro* shoot were cut into small pieces so that each piece would contain about one shoot. Leaf was removed. Each piece was inoculated into a similar fresh medium. It was practiced at the interval of 10-20 days.

3.7.2. a. Root induction of regenerated shoots

When the shoots grew about 4-5 cm in length with 3-4 well developed leaves, they were removed aseptically from the culture vials and were separated from each other and again cultured on freshly prepared medium containing different combinations of salt for root induction.

3.7.2. b Treatments

Two sub-experiments were conducted to assess the effect of salt on shoot proliferation and subsequent rooting of the multiplied shoot. Treatment combination for the *in vitro* regeneration of onion in salinity were given below:

Treatments Composition

NaCl treatment (25, 50, 75 and 100) mM and 0.0 (control) for screening of salinity tolerance.

3.8. Calculation of Data

Development of plantlets was observed after two, three, four weeks of initiation and the following data were recorded for over all experiment:

3.8.1. **Days required for shoot and root initiation:** The cultures were observed at alternate days starting from 3rd day of inoculation and continued up to 28th day for shooting. Any change or development in culture when observed was recorded as days to shoot initiation or appearance and any development or outcome of root was recorded as days to root initiation.

3.8.2. **Number of shoots per explant:** Multiple shoots found in cultures after shoot initiation were counted from 5 days after culture and recorded as Number of shoots per explant.

3.8.3. Length of the shoot (cm.): The length or height of the plantlet was measured against a ruler in cm. upto 28^{th} days after culture. The length from the base of plantlet to the tip of the plantlet was considered as height of the plant. In case of multiple shoot, the length of the tallest plant was considered as plant height and measured in cm.

3.8.4. Number of leaves per plant: The number of leaves of plant was counted upto 28^{th} day after culture. In case of single stem plantlet, al the leaves were counted from base to tip of the plantlet as 1 to 15 and in case of multiple stem plantlet, all the leaves counted in plantlet were divided by the total number of stems of the planlet.

3.8.5. Number of roots per explant: It was counted at 28th day after culture. All roots were counted in number as 1 to 8 and sum total of each was divided by the sum total of plantlet for average Plate.

3.8.6. Length of the root (cm.): Root length of the plantlet was measured against a ruler in mm. upto 28^{th} day after culture. The length from the base of plantlet to the tip of the root was considered as length of the plant. In case of multiple root, the length of the longest root was considered as root length and measured in mm.

3.8.7. Fresh weight of the root (mg): The fresh weight of root were measured in mm. by a digital balance at 28th day after culture using following formula:

$$\textbf{Fresh weight of root / shoot (mg)} = \frac{\text{Total weight of root}}{\text{Total number of root measured}}$$

3.8. 8 Dry weight of the root (mg): Root were collected at 28th day after culture and dried in an oven at 300 C for 48 hours. Then dry weight of the shoot and shoot was measured in mg by a digital balance using following formula:

$$\textbf{Dry weight of root / shoot (mg)} = \frac{\text{Total weight of root}}{\text{Total number ofroot measured}}$$

3.9. Experimental design and statistical analysis

All the experiments were conducted under laboratory conditions where all the factors were homozygous. For this reason, experiments were arranged in two factorial (variety and Treatment) Completely Randomized Design (CRD) with 3 replications for each treatment. Data for the characters under the study were statically analyzed following MSTATE-C (1990) package computer program for *in vitro* shoot and root bioassay for salinity. The analysis of variance (ANOVA) for different characters was performed and means were compared by the Duncan's Multiple Range Test (DMRT) for *in vitro* shoot and root bioassay for salinity at 5% probability level.

CHAPTER IV

RESULT AND DISCUSSION

Two sub-experiments were conducted sequentially under the laboratory condition to study the in *vitro* regeneration of onion (*Allium cepa* L.) under salt stress condition. The results have been presented and discussed and possible interpretations are given experiment wise under the following headings:

4.1. Sub-experiment 1: *In vitro* plantlet regeneration of different genotypes of onion

4.1.1 Days required for shoot initiation

Varietal response on days required for shoot initiation of three onion genotypes varied significantly from one another under *in vitro* condition (Fig. 1). The lowest time (6.53 days) was needed in case of Faridpuri which was statistically significant from rest of the varieties followed by Taherpuri (7.7 days). On the other hand Pusa red required highest time (8.87 days) for initiation.

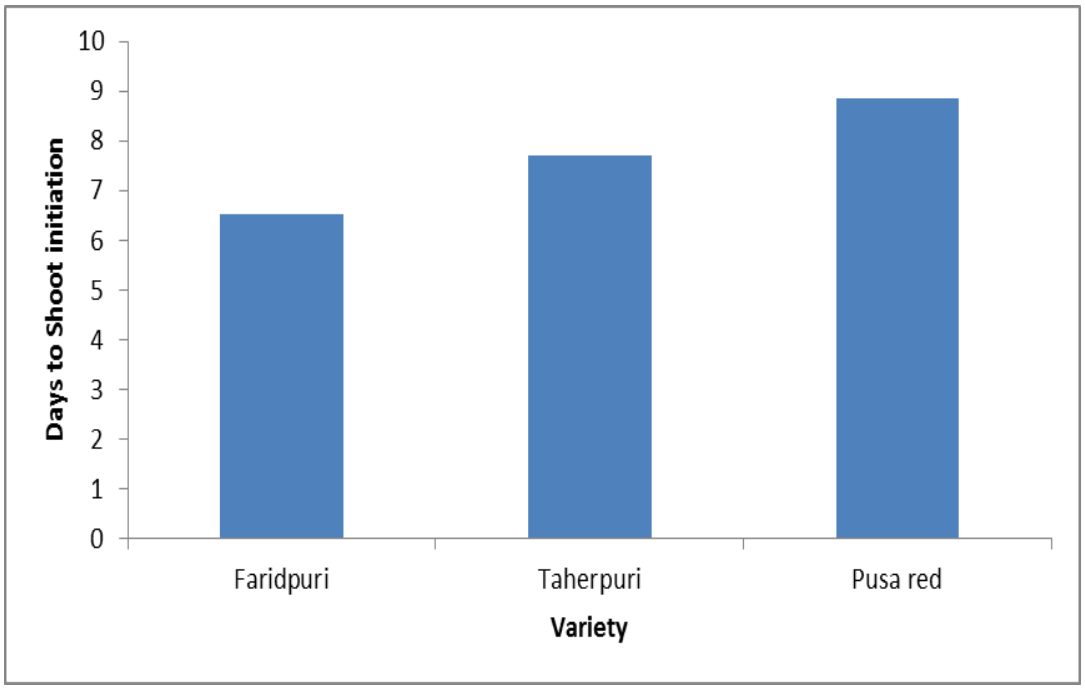


Fig. 1: Effect of variety on days required for shoot initiation of onion

4.1.2 Number of shoot per explant

Significant difference was found among the genotypes in respect of number of shoot per explant at 7, 14, 21 and 28 days after initiation (DAI) . The genotype Faridpuri produced maximum number of shoot (2.6, 4.07, 4.27, 5.8) followed by Taherpuri (1.3, 2.9, 3.2, 4.0) at 7, 14, 21 and 28 DAI, respectively. On the other hand, minimum number of shoots (0.93, 2.12, 2.52, 3.13) at 7, 14, 21 and 28 DAI, respectively were produced by Pusa red (Fig. 2).

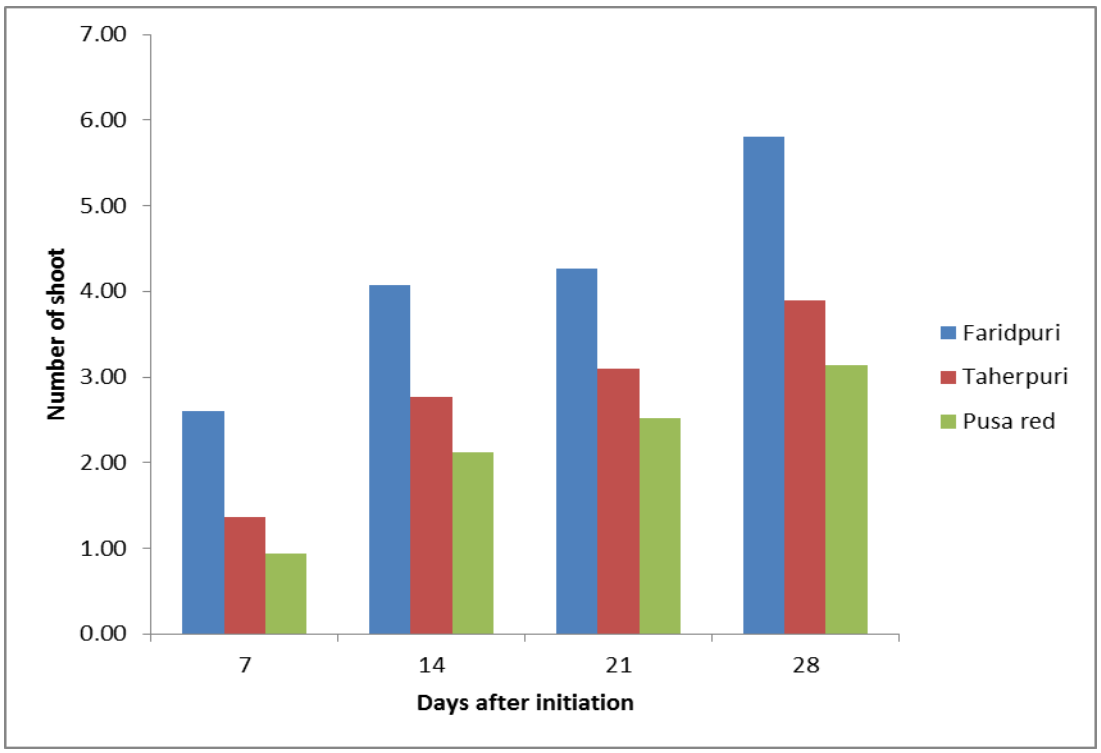


Fig. 2: Effect of variety on number of shoot per explant of onion

4.1.3 Length of shoot per plant (cm)

Different genotypes showed significant variations to the length of shoot (cm) per plant at 7, 14, 21 and 28 days after initiation (DAI) (Table 1). Faridpuri showed the longest plant height (7.95, 9.49 and 12.48) at 14, 21 and 28 DAI, respectively which was statistically significant from rest of the varieties followed by Taherpuri. Besides, Pusa red produced lowest shoot length (7.00, 7.8 and 8.91) at 14, 21 and 28 DAI, respectively.

Table 1. Effect of variety on shoot length of onion

Treatment	Shoot length (cm)		
	14 DAI	21 DAI	28 DAI
Faridpuri	7.95 a	9.49 a	12.48 a
Taherpuri	7.21 b	8.05 b	9.98 b
Pusa red	7.00 c	7.38 c	8.91 c
$LSD_{(0.05)}$	0.19	0.59	0.31
CV(%)	11.53	12.21	10.09

Figures in the columns followed by different letters are significantly different by DMRT at p=0.05, LSD = Least significant difference, CV% = Percentage of coefficient of variance

4. 1.4 Number of leaves per plant

Significant difference was found among the varieties in respect of number of leaves per plant at 14, 21 and 28 DAI. The maximum number of leaves per plant (3.2, 3.67 and 4.27 at 14, 21 and 28 DAI, respectively) was produced from Faridpuri genotype. The minimum number of leaves (1.74, 2.52, and 3.22 at 14, 21 and 28 DAI, respectively) was in Pusa red (Table 2).

Table 2. Effect of variety on number of leaves per plant of onion

Genotype	Number of leaves per plant		
	14 DAI	21 DAI	28 DAI
Faridpuri	3.20 a	3.67 a	4.27 a
Taherpuri	1.87 b	2.57 b	3.33 b
Pusa red	1.74 b	2.52 b	3.22 b
$LSD_{(0.05)}$	0.35	1.08	1.16
CV(%)	9.21	5.71	11.53

Figures in the columns followed by different letters are significantly different by DMRT at p=0.05, LSD = Least significant difference, CV% = Percentage of coefficient of variance

4.1.5 Days required for root initiation

There was no significant variation among the three onion genotypes in respect of days required for root initiation (Table 3)**.** Maximum time (14.73 days) was recorded in case of Pusa red. The minimum 14.27 day time was needed for Faridpuri genotype.

Table 3. Effect of variety on days to root initiation and number of root of Onion

Genotype	Days to root initiation	Number of root		
		14 DAI	21 DAI	28 DAI
Faridpuri	14.27 a	4.60 a	5.33 a	8.07 a
Taherpuri	14.62 a	2.70 b	3.83 b	6.33 b
Pusa red	14.73 a	1.93 c	2.97 c	5.62 c
$LSD_{(0.05)}$	2.23	0.51	0.59	0.27
CV(%)	5.34	4.40	12.16	10.55

Figures in the columns followed by different letters are significantly different by DMRT at p=0.05, LSD = Least Significant Difference, CV% = Percentage of coefficient of variance

4.1.6 Number of root

Prominant variation was found among the genotypes in term of root number per explant at 14, 21 and 28 days after inoculation (Table-3). Maximum number of root (4.6, 5.33, 8.07) at 14, 21 and 28 DAI, respectively was produced by Fridpuri which was statistically significant from rest of the varieties followed by Taherppuri. The minimum number of roots was produced by Pusa red (1.93, 2.97 and 5.62 at 14, 21 and 28 DAI, respectively).

4.1.7 Length of root (cm)

After four weeks of inoculation remarkable variation was observed among the genotypes in terms of root length (Table 4). The longest root length (1.78, 2.08, 2.39) at 14, 21 and 28 DAI, respectively was observed in Faridpuri. The shortest root length (0.93, 1.20 and 1.51) at 14, 21 and 28 DAI, respectively was found in pusa red**.**

4.1.8 Fresh weight of root (mg)

Highly significant differences were found among the investigated genotypes for fresh weight of root per explant (Table 4). The highest fresh weight of root (49.11) was found in Faridpuri, which was statistically different from Taherpuri. Besides, minimum fresh weight of root (42.66) was found from Pusa red.

Table 4. Effect of variety on length of root, Fresh weight of root and dry weight of root of onion

Genotype	Length of root (cm)			Fresh weight of root (mg)	Dry weight of root (mg)
	14 DAI	21 DAI	28 DAI		
Faridpuri	1.78 a	2.08 a	2.39 a	49.11 a	37.35 a
Taherpuri	1.08 b	1.38 b	1.70 b	44.71 b	33.35 b
Pusa red	0.92 b	1.20 b	1.51 b	42.66 c	31.40 c
$LSD_{(0.05)}$	0.22	0.19	0.42	0.38	1.28
CV(%)	9.42	5.51	3.19	2.23	2.68

Figures in the columns followed by different letters are significantly different by DMRT at p=0.05, LSD = Least significant difference, CV% = Percentage of coefficient of variance

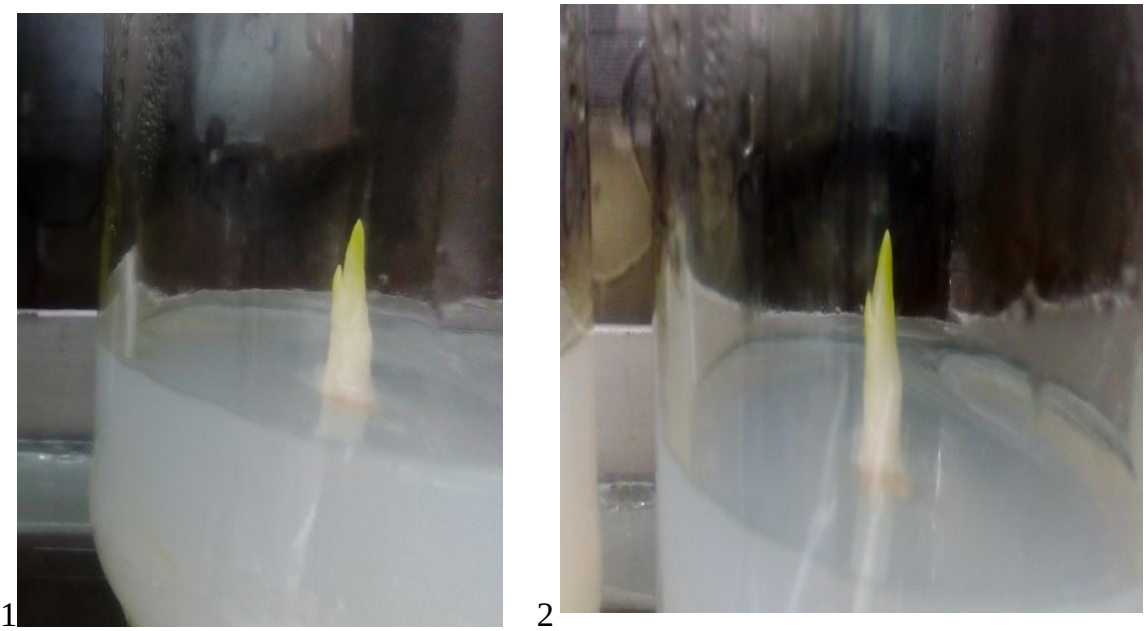
4.1.9 Dry weight of root (mg)

Dry weight of root significantly varied in different genotype of onion. Concerning the dry weight of root Faridpuri had the highest value (37.35) as compared to the other genotypes while Pusa red had the lowest value (31.4) (Table 4).

4.2. Sub-experiment 2: *In vitro* plantlet regeneration of onion of different level of salinity

4.2.1 Days required for shoot initiation

Combined effect of varieties and salinity level varied significantly for days required to shoot regeneration (Table 5). The lowest time (6.33 days) was needed in case of Faridpuri with 25 mM NaCl treatment. Pusa red required maximum 9.67 days with 100 mM and 50 mM NaCl treatment.(Plate-3)



1 2

Plate 3: Days of shoot initiation(1-Pusa red, 2-Faridpuri)

4.2.2 Number of shoot per plant

Significant difference was found among the combined effect of genotypes of onion and different salinity levels on number of shoot per plantlet at 14, 21 and 28 (DAI) (Table 5). The treatment of 25 mM NaCl showed maximum number of shoots (4.67, 4.67, 6.0) at 14, 21 and 28 DAI followed by Taherpuri. The minimum numbers of shoots (1.22, 1.22, 1.33) at 14, 21 and 28 DAI, respectively were produced from Pusa red in 100 mM NaCl (Plate-4). Hence, The number of proliferated shoots and their growth were depressed upon increasing of salts in medium as investigated by Bekheet (2006).

1 2 3

Plate 4: Number of shoot per plant (1-Tahirpuri, 2-Faridpuri, 3-Pusa red)

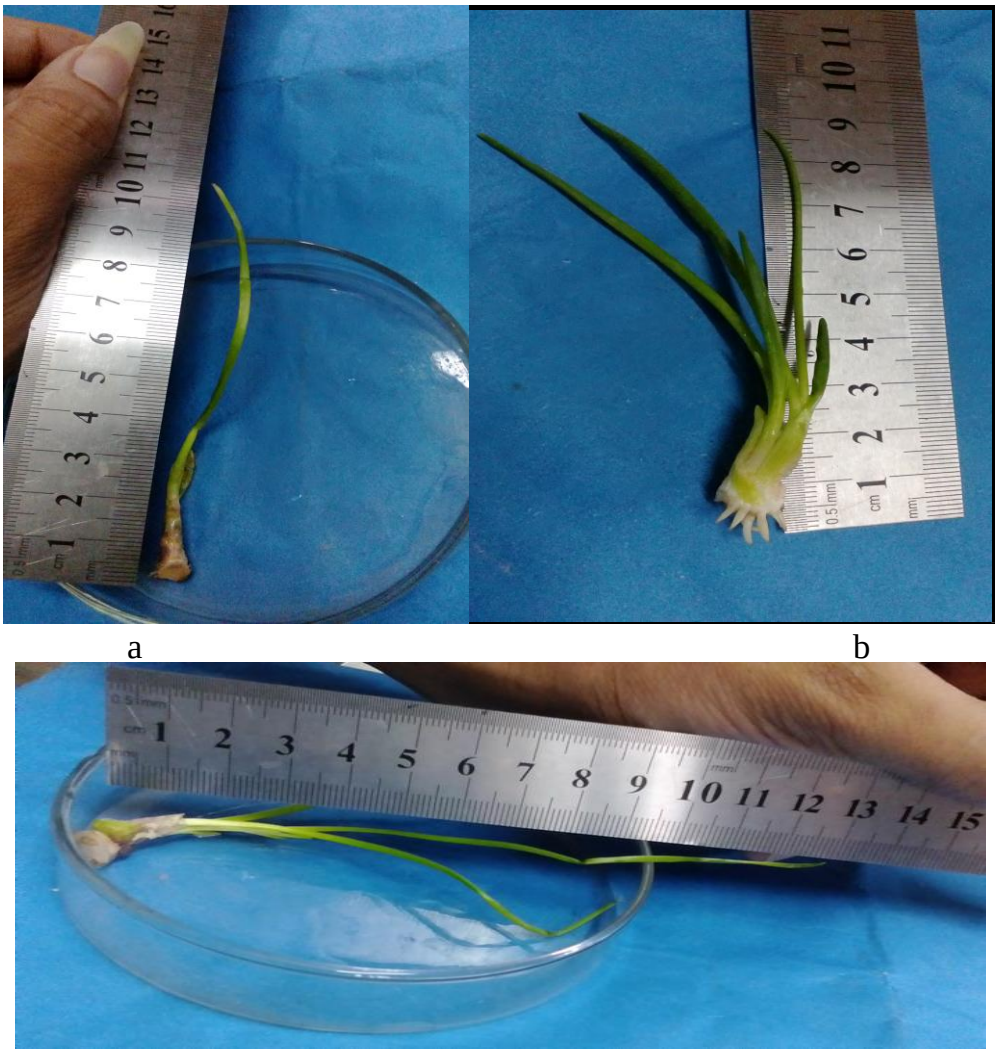
Table 5. Combined effect of variety and salinity level on days to shoot initiation and number of shoot per plant of Onion

Genotypes	Treatments (mM)	Days to shoot initiation	Number of shoot per plant		
			14 DAI	21 DAI	28 DAI
Faridpuri	25	6.33 de	4.67 ab	4.67 bc	6.00 bc
	50	7.33 bcde	3.67 bc	3.67 bcdef	6.00 bc
	75	6.33 de	3.67 bc	3.67 bcdef	5.00 bcde
	100	7.33 bcde	2.67 cde	2.67 defgh	4.00 cdef
Taherpuri	25	7.50 bcd	3.43 bcd	4.00 bcde	4.10 cdef
	50	8.50 abc	2.43 cde	3.00 cdefgh	4.10 bcdef
	75	7.50 bcd	1.93 de	2.17 efgh	3.10 efg
	100	8.50 abc	1.60 e	1.33 gh	2.10 fg
Pusa red	25	8.67 ab	2.72 cde	3.22 bcdefg	3.33 ef
	50	9.67 a	1.72 e	2.22 efgh	3.33 def
	75	8.67 ab	1.22 e	1.72 fgh	2.33 fg
	100	9.67 a	1.22 e	1.22 h	1.33 g
$LSD_{(0.05)}$		1.83	1.39	1.71	1.77
CV(%)		4.26	7.98	11.14	4.75

Figures in the columns followed by different letters are significantly different by DMRT at p=0.05, LSD = Least significant difference, CV% = Percentage of coefficient of variance

4.2.3 Length of shoot per plant (cm)

Combined effect of genotype and different salt stress showed significant variations to the length of shoot (cm) per plantlet at 14, 21 and 28 (DAI) (Table 6). Under salinity level, 50 mM NaCl treatment generateded the maximum shoot length (8.87, 10.37, 13.50 cm) at 14, 21 and 28 DAI in Faridpuri followed by Taherpuri and Pusa red with 100 mM NaCl treatment produced lowest shoot length (5.52, 5.66 and 7.03) at 14, 21 and 28 DAI, respectively (Plate-5).



a b

c

Plate 5: Combined effect of genotypes (a-Pusa red, b-Taherpuri, c-Faridpuri) and salinity level on length of shoot per plant

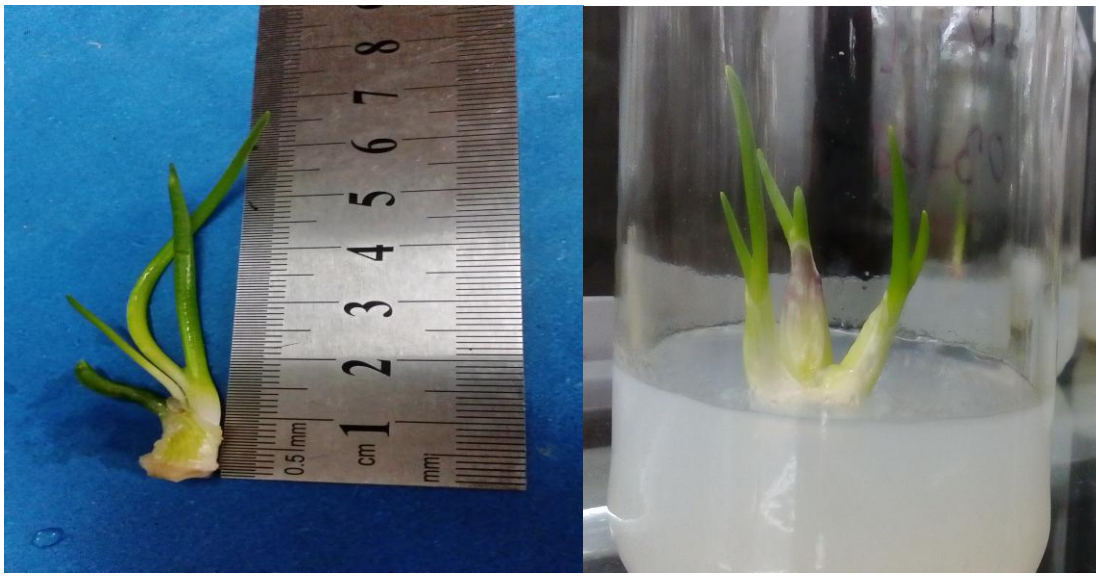
Table 6. Combined effect of genotype and salinity level on Shoot length of Onion

Genotypes	Treatments (mM)	Shoot length (cm)		
		14 DAI	21 DAI	28 DAI
Faridpuri	25	8.57 a	9.77 abc	12.00 bc
	50	8.87 a	10.37 abc	13.50 b
	75	6.77 bcd	8.57 bcde	10.80 cd
	100	6.47 cd	7.77 defg	10.60 cd
Taherpuri	25	8.13 ab	8.93 bcde	11.00 cd
	50	7.83 abc	8.33 cdef	9.50 de
	75	6.03 d	7.13 efgh	8.30 ef
	100	5.73 d	6.33 gh	8.10 ef
Pusa red	25	7.92 abc	8.26 cdef	9.93 de
	50	7.62 abc	7.66 defg	8.43 ef
	75	5.82 d	6.46 fgh	7.23 f
	100	5.52 d	5.66 h	7.03 f
$LSD_{(0.05)}$		1.42	1.69	1.76
CV(%)		11.53	12.21	10.09

Figures in the columns followed by different letters are significantly different by DMRT at p=0.05, LSD = Least significant difference, CV% = Percentage of coefficient of variance

4.2.4 Number of leaves per plant

Significant difference was found in combined effect of genotypes and salt stress in number of leaves per plant at 14, 21 and 28 DAI. The maximum number of leaves per plant (3.33, 3.67, 4.67 at 14, 21 and 28 DAI, respectively) was produced from Faridpuri with 25mM NaCl treatment followed by Taherpuri genotype (Plate-6). The minimum number of leaves (0.67, 1.22, 1.22 at 14, 21 and 28 DAI, respectively) was produced from Pusa red with 75 mM NaCl treatment (Table 7).



a **b**

Plate 6: combined effect of genotypes (a-Pusa red, b-Faridpuri) and salinity level on number of leaf per plantlet

Table 7. Combined effect of genotype and salinity level on number of leaves per plant of onion

Genotypes	Treatments (mM)	Number of leaves per plant		
		14 DAI	21 DAI	28 DAI
Faridpuri	25	3.33 b	3.67 bc	4.67 bc
	50	2.33 c	2.67 cde	3.67 cd
	75	2.33 c	3.67 bc	3.67 cd
	100	2.33 c	2.67 cde	2.67 de
Taherpuri	25	2.17 c	3.27 bcd	4.33 bc
	50	1.67 cd	2.27 cdef	3.33 cd
	75	1.17 de	1.77 ef	2.33 def
	100	1.17 de	1.27 ef	1.33 ef
Pusa red	25	2.13 c	3.22 bcd	4.22 bc
	50	1.63 cd	2.22 def	3.22 cd
	75	0.67 e	1.22 f	1.22 f
	100	1.13 de	1.72 fe	2.22 def
$LSD_{(0.05)}$		0.70	1.25	1.30
CV(%)		9.21	5.71	11.53

Figures in the columns followed by different letters are significantly different by DMRT at p=0.05, LSD = Least significant difference, CV% = Percentage of coefficient of variance

4.2.5 Days required for root initiation

There was significant variation among combined effect of genotypes and salt stress in respect of days required for root initiation. Maximum period (16.33 days) was recorded in case of Taherpuri with 100 mM NaCl treatment which was statistically similar with Pusa red with 100 mM NaCl. The minimum days (14.67 days) was needed for Faridpuri with 25 mM NaCl treatment (Table-8).

Table 8. Combined effect of variety and salinity level on days to root initiation of onion

Genotypes	Treatments (mM)	Days to root initiation	
Faridpuri	25	14.67	bc
	50	14.67	bc
	75	14.67	bc
	100	14.67	bc
Taherpuri	25	14.33	bc
	50	14.33	bc
	75	15.33	ab
	100	16.33	a
Pusa red	25	14.22	bc
	50	14.22	bc
	75	15.22	ab
	100	16.22	a
$LSD_{(0.05)}$		1.29	
CV(%)		5.34	

Figures in the columns followed by different letters are significantly different by DMRT at p=0.05, LSD = Least significant difference, CV% = Percentage of coefficient of variance

4.2.6 Number of root

Combined effect of genotypes and salt stress varied significantly for total number of root per explant at 14, 21 and 28 days after inoculation (Table 9). The highest number of root (5.0, 5.33, 9.67) was obtained from Faridpuri genotype with 25mM NaCl treatment at 14, 21, 28, DAI respectively (Plate-7).

The minimum number of roots was produced by Pusa red with 50 mM NaCl treatment (1.33, 1.33 and 1.17) at 14, 21 and 28 DAI, respectively.

Table 9. Combined effect of genotype and salinity level on number of root per plantlet of Onion

Genotypes	Treatments (mM)	Number of root per plant		
		14 DAI	21 DAI	28 DAI
Faridpuri	25	5.00 ab	5.33 b	9.67 b
	50	4.00 bc	5.33 b	7.67 c
	75	4.00 bc	5.33 b	6.67 cd
	100	4.00 bc	4.33 cd	4.67 ef
Taherpuri	25	3.10 bcd	3.83 de	7.93 c
	50	2.10 cd	3.83 de	5.93 de
	75	2.10 cd	3.83 de	4.93 ef
	100	2.10 cd	2.83 f	2.93 g
Pusa red	25	2.33 cd	3.13 ef	7.22 c
	50	1.33 d	1.33 g	2.22 g
	75	1.33 d	3.13 ef	4.22 f
	100	1.33 d	3.13 ef	5.22 ef
$LSD_{(0.05)}$		1.77	0.82	1.17
CV(%)		4.40	12.16	10.55

Figures in the columns followed by different letters are significantly different by DMRT at p=0.05, LSD = Least Significant Difference, CV% = Percentage of coefficient of variance

a **b**

Plate 7. Combined effect of variety and salinity level on number of root (a-Faridpuri, 25mM & b-Pusa red, 50 mM) NaCl per plant of Onion

4.2.7 Length of root (cm)

Combined effect of genotypes and salt stress was observed significant due to root length (Table 10). The height root length (1.90, 2.30, and 2.23) cm at 14, 21 and 28 DAI, respectively was observed in Faridpuri with 25 mM NaCl treatment. The lowest root length (0.63, 0.93 and 0.60) cm at 14, 21 and 28 DAI, respectively) was found Pusa red with 100 mM NaCl treatment**.**

Table 10. Combined effect of genotype and salinity level on length of root of onion

Genotypes	Treatments (mM)	Length of root (cm)		
		14 DAI	21 DAI	28 DAI
Faridpuri	25	1.90 ab	2.30 ab	2.23 abc
	50	1.70 abc	2.00 bc	1.93 abcd
	75	1.60 abcd	1.80 cd	1.73 bcde
	100	1.50 abcde	1.80 cd	1.43 de
Taherpuri	25	1.20 bcdef	1.60 cdef	1.93 abcd
	50	1.00 cdef	1.30 efgh	1.63 bcde
	75	0.90 cdef	1.10 gh	1.43 de
	100	0.80 def	1.10 gh	1.13 ef
Pusa red	25	1.03 cdef	1.43 defg	1.83 bcde
	50	0.83 def	1.13 fgh	1.53 cde
	75	0.73 ef	0.93 h	1.33 de
	100	0.63 f	0.93 h	0.60 f
$LSD_{(0.05)}$		0.70	0.44	0.67
CV(%)		9.42	5.51	3.19

Figures in the columns followed by different letters are significantly different by DMRT at p=0.05, LSD = Least significant difference, CV% = Percentage of coefficient of variance

4.2.8 Fresh weight of root (mg)

Combined effect of varieties and salt stress varied significantly for fresh weight in root per plant. The highest fresh weight of root (54.77) was found in Faridpuri with 25 mM NaCl treatment. The minimum fresh weight of root (30.32 mg) was found Pusa red with 75 mM NaCl treatment (Table no. 11).

4.2.9 Dry weight of root (mg)

Dry weight of root was significantly varied from combined effect of varieties and salt stress. The maximum dry weight of root (41.37 mg) was obtained from Faridpuri with 25 mM NaCl treatment l. Pusa red with 50 mM NaCl treatment was produced in the lowest value of dry weight of root (23.12 mg) (Table no. 11).

Table 11. Combined effect of genotype and salinity level on fresh weight of root and dry weight of root of Onion

Genotypes	Treatments (mM)	Fresh weight of root (mg)	Dry weight of root (mg)
Faridpuri	25	54.77 c	41.37 b
	50	52.07 d	38.57 c
	75	41.07 h	33.57 ef
	100	36.77 i	29.07 gh
Taherpuri	25	50.37 e	37.37 c
	50	47.67 f	34.57 de
	75	36.67 i	29.57 g
	100	32.37 k	25.07 i
Pusa red	25	48.32 f	35.42 d
	50	45.62 g	23.12 j
	75	30.32 i	27.62 h
	100	34.62 j	32.62 f
$LSD_{(0.05)}$		1.69	1.52
CV(%)		2.23	2.68

Figures in the columns followed by different letters are significantly different by DMRT at p=0.05, LSD = Least significant difference, CV% = Percentage of coefficient of variance

CHAPTER V

SUMMARY AND CONCLUTION

The present research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of September 2016 to July 2017.

Three genotypes namely Faridpuri, Taherpuri and Pusa red (Indian) were used as investigated genotypes in this present study. Shoot tip segments of these genotypes were cultured in MS (Murashige and skoog, 1962) media having 0.0 (control), 25, 50, 75 and 100 mM NaCl. The shoot tips of experimental onion genotypes were not significantly affected up to 75 mM salinity level in comparison with control treatment. The experiment was conducted at two factorial (variety and Treatment) Completely Randomized Design (CRD) with 3 replications for each treatment..

Varietal response on days required for shoot initiation of three onion genotypes varied significantly from one another under *in vitro* condition**.** The lowest time (6.53 days) was needed in case of Faridpuri which was statistically significant from rest of the varieties followed by Taherpuri (7.7 days). On the other hand Pusa red required highest time (8.87 days) for initiations under salinity condition.

Significant difference was found among the genotypes in respect of number of shoot per explant at 7, 14, 21 and 28 days after initiation (DAI). The genotype Faridpuri produced maximum number of shoot (2.6, 4.07, 4.27, 5.8) followed by Taherpuri (1.3, 2.9, 3.2, 4.0) at 7, 14, 21 and 28 DAI, respectively. On the other hand minimum numbers of shoots (0.93, 2.12, 2.52, 3.13) at 7, 14, 21 and 28 DAI, respectively were produced by Pusa red.

Different genotypes showed significant variations as to the length of shoot (cm) per plant at 7, 14, 21 and 28 days after initiation (DAI). Faridpuri showed the longest plant height (7.95, 9.49 and 12.48) at 14, 21 and 28 DAI, respectively which was statistically significant from rest of the varieties followed by Taherpuri. Besides, Pusa red emerged as most sensitive variety to salt stress with lowest shoot length (7.00, 7.8 and 8.91) at 14, 21 and 28 DAI, respectively.

Significant difference was found among the varieties in respect of number of leaves per shoot at 14, 21 and 28 DAI. The maximum number of leaves per plant (3.2, 3.67 and 4.27) at 14, 21 and 28 DAI, respectively) was produced from Faridpuri genotype. The minimum number of leaves (1.74, 2.52, and 3.22) at 14, 21 and 28 DAI, respectively was produced by Pusa red.

There was not significant variation among the three onion genotypes in respect of days required for root initiation**.** Optimum time (14.73 days) was recorded in case of Pusa red. The minimum 14.27 day time was needed for Faridpuri genotype.

Pronounced variation was found among the genotypes in terms of root number resulting total number of root per explant at 14, 21 and 28 days after inoculation. Maximum number of root (4.6, 5.33, 8.07) at 14, 21 and 28 DAI, respectively was produced by Fridpuri in which was statistically significant from rest of the varieties followed by Taherppuri. The minimum number of roots was produced by Pusa red (1.93, 2.97 and 5.62) at 14, 21 and 28 DAI, respectively.

After four weeks of inoculation remarkable variation was observed among the genotypes in terms of root length. The longest root length (1.78, 2.08, 2.39) at 14, 21 and 28 DAI, respectively was observed in Faridpuri. The shortest root

length (0.93, 1.20 and 1.51) at 14, 21 and 28 DAI, respectively was found pusa red**.**

Highly significant differences were found among the investigated genotypes for fresh weight of root per explant. The highest fresh weight of root (49.11) was found in Faridpuri, which was statistically different from Taherpuri. Besides, minimum fresh weight of root (42.66) was found Pusa red.

Dry weight of root was significantly varied from different genotype of onion. Concerning the dry weight of root Faridpuri had the highest value (37.35) as compared to the other genotypes while Pusa red had the lowest value (31.4).

Combined effect of varieties and salinity level varied significantly for days required to shoot regeneration. The lowest time (6.33 days) was needed in case of Faridpuri with 25 mM NaCl treatment. Pusa red required maximum 9.67 days with 100 mM and 50 mM NaCl treatment.

Significant difference was found among the combined effect of genotypes of onion and different salinity level of number of shoot per plantlet at 14, 21 and 28 (DAI). The treatment of 25 mM NaCl showed maximum number of shoots (4.67, 4.67, 6.0) at 14, 21 and 28 DAI followed by Taherpuri. The minimum numbers of shoots (1.22, 1.22, 1.33) at 14, 21 and 28 DAI, respectively were produced from Pusa red in 100 mM NaCl.Hence, The number of proliferated shoots and their growth were depressed upon increasing of salts in medium which was investigated by Bekheet.

Combined effect of genotype and different salt stress showed significant variations as to the length of shoot (cm) per plantlet at 14, 21 and 28 (DAI). Under salinity level, 50mM NaCl treatment produced the maximum shoot length (8.87, 10.37, 13.50) at 14, 21 and 28 DAI in Faridpuri followed by

Taherpuri. Pusa red with 100 mM NaCl treatment produced lowest shoot length (5.52, 5.66 and 7.03) at 14, 21 and 28 DAI, respectively.

Significant difference was found combined effect of genotypes and salt stress of number of leaves per plant at 14, 21 and 28 DAI. The maximum number of leaves per plant (3.33, 3.67, 4.67) at 14, 21 and 28 DAI, respectively was produced from Faridpuri with 25mM NaCl treatment followed by Taherpuri genotype. The minimum number of leaves (0.67, 1.22, 1.22) at 14, 21 and 28 DAI, respectively was produced from Pusa red with 75 mM NaCl treatment.

There was significant variation among combined effect of genotypes and salt stress in respect of days required for root initiation. Maximum period (16.33 days) was recorded in case of Taherpuri with 100 mM NaCl treatment which was statistically similar with Pusa red with 100 mM NaCl. The minimum days (12.67 days) was needed for Faridpuri with 25 mM NaCl treatment.

Combined effect of genotypes and salt stress was varied significantly of total number of root per explant at 14, 21 and 28 days after inoculation . The highest number of root was obtained from Faridpuri genotype with 25mM NaCl treatment (5.0, 5.33, 9.67) at 14, 21, 28 DAI respectively.The minimum number of roots was produced by Pusa red with 50 mM NaCl treatment (1.33, 1.33 and 1.17) at 14, 21 and 28 DAI, respectively.

Combined effect of genotypes and salt stress was observed significantly due to terms of root length. The height root length (1.90, 2.30, and 2.23) at 14, 21 and 28 DAI, respectively was observed in Faridpuri with 25 mM NaCl treatment. The lowest root length (0.63, 0.93 and 0.60) at 14, 21 and 28 DAI, respectively) was found Pusa red with 100 mM NaCl treatment**.**

Combined effect of varieties and salt stress was varied significantly for fresh weight of root per explant. The highest fresh weight of root (54.77) was found in Faridpuri with 25 mM NaCl treatment. The minimum fresh weight of root (30.32 mg) was found Pusa red with 75 mM NaCl treatment.

Dry weight of root was significantly varied from combined effect of varieties and salt stress. The maximum dry weight of root (41.37 mg) was obtained from Faridpuri with 25 mM NaCl treatment l. Pusa red with 50 mM NaCl treatment was produced in the lowest value of dry weight of root (23.12 mg).

RECOMMENDATIONS

Based on above discussion the following recommendations can be written as follows:

1. Further study may be conducted with more genotypes.
2. More explants (seed, root tip etc.) may be used for *in vitro* salinity tolerance study.
3. Combination of different growth regulators can be used for *in vitro* root and shoot development.

REFERENCES

Ahmad, P., Hakeem, K.R., Kumar, A., Ashraf, M., and Akram, N.A., (2012). Salt-induced changes in photosynthetic activity and oxidative defense system of three cultivars of mustard (*Brassica juncea* L.). *Afr. J. Biotechnol.* **(11):**2694–2703.

Ahmad, P., Jaleel, C.A., Salem, M.A., Nabi, G., Sharma, S. (2010a). Roles of Enzymatic and non-enzymatic antioxidants in plants during abiotic stress. *Crit. Rev. Biotechnol.* **30(3):**161–175.

Ahmad P., and Umar, S. (2011) Oxidative stress: role of antioxidants in plants. Studium Press, New Delhi.

Ayabe, M., and Sumi, S. (1998). Establishment of a novel tissue culture method, stem-disc culture, and its practical application to micropropagation of Garlic (*Allium sativum* L.). *Plant Cell. Rep*., **(17):** pp. 773-779.

Ayabe, M., Taniguchi, K. and Sumi, S. (1995). Regeneration of whole plants from protoplasts isolated from tissue-cultured shoot primordial of Garlic (*Allium sativum* L.). *Plant Cell. Rep*. **(15):** pp. 17-21.

Barandiaran, X., Martín, N., Alba, C., Rodríguez-Conde, M. F., Di Pietro, A. & Martin, J. (1999). An efficient method for the *in vitro* management of multiple Garlic accessions. *In vitro Cell. Dev. Biol. Plant*. **(35):** pp. 466-469.

BBS (Bangladesh Bureau of Statistics), (2015). Statistics Division, Ministry of planning, Govt. of the People's Republic of Bangladesh. Dhaka. pp: 140.

BBS. (2012). Hand Book of Agricultural Statistics, Bangladesh Bureau of Statistics Statistics and Informatics Division (SID) Ministry of Planning Government of the People's Republic of Bangladesh. P.97.

BBS. (2010a). Bangladesh Bureau of Statistics. Statistical Pocket Book of Bangladesh. Statistics Divin, Ministry of Planning, Govt. of the People's Republic of Bangladesh. Dhaka, p-199.

BBS.(2010b). Statistical Year Book of Bangladesh. Statistics Divin, Ministry of Planning, Govt. of the People's Republic of Bangladesh. Dhaka, p.135.

Bekheet, S.A, Taha, H.S. and Solliman, M.E. (2006). Salt tolerance in tissue culture of onion (*Allium cepa* L.). *Arab. J. Biotech.* **9 (3):** 467-476.

Bockish, T., Saranga, Y. and Altman, A. (1997). Garlic micropropagation by somatic embryogenesis. *Acta Hort.* **44(7):** 241-242.

Chen, Y., Zahavi, E., Barek, P. and Umiel, N. (1980). Effect of salinity stresses on tobacco 1.The growth of *Nicotiana tabacum* callus cultures under water,NaCl and Mannitol stress. *Z. Pflanzenphysiol*. **98:** 141-143.

Dix, P.J. (1985)**.** Cell line selection. In: Plant Cell Culture Technology, (M.M.Yeoman, Ed.). Pp.141-199. Oxford, Blackwell, Scientific.

Dubey, R. (1994). Protein synthesis by plants under stressful conditions. In: **"**Handbook of Plant and Crop Stress", pp.277- 299. P. Mohammed(ed.) marcel ,Dekker, New York.

Dunstan, D.I. and Short, K.C. (1977). *In vitro* studies on organogenesis and growth in *allium cepa* tissue cultures. *Acta. Hortic.* **(78):** 139-148

Gantait, S., Mandal , N. and Das, P.K. (2010). Ano on *in vitro* culture of genus *Allium*. *American J. Plant Physiol.* **(5):** 325-337.

Hamlet, P. (1990). Taxonomy, evolution and history. In: Rabinowitch, H.D. and Brewster (eds), J.L, onions and Allied crops. CRC press, Boca Raton, Florida. **(1):**1-26.

Haque, M. S., Wada, T. and Hattori, K. (2003). Shoot regeneration and Bulblet formation from shoot and root meristem of Garlic cv Bangladesh Local. *Asian J. Plant Sci.* pp 23-27.

Hasanuzzaman, M. and Fujita, M. (2012) Selenium and plant health: the physiological role of selenium. In: Aomori, C. Hokkaido, M. (eds) Selenium: sources, functions and health effects. Nova Publishers, New York.

Hossain, A.K.M.A. and J. Islam. (2006). Status of *Allium* Production in Bangladesh. *Acta Hort*. **35(8):** 33-36.

Kamstaityte, D. and Stanys, V. (2004). Micropropagation of onion (*Allium cepa* L.). *Acta. Universitatis Latviensis. Biology.* **67(6):** *pp. 173–176.*

Kevers, C., Franck, T., Strasser, R. J., Dommes, J. and Gaspar, T. (2004). Hyperhydricity of micropropagated shoots: a typically stress-induced change of physiological state. *Plant Cell Tiss. Org. Cult.*, **(77):** pp. 181-191.

Khalid, A., De-ping, G. and Zhu-jun, Z. (2001). Effect of growth regulators on plantlet regeneration and bulbing in Onion (*Allium cepa* L.) *in vitro. Pakistan J. Biol. Sci.* **4 (3):** 374-377.

Kiełkowska, A. (2017). *Allium cepa* root meristem cells under osmotic (sorbitol) and salt (NaCl) stress *in vitro. Acta Bot. Croat.* **76 (2):** 146–153.

Lee, S.Y., Kim, H.H., Park, N.I. and Park, S.U. (2009). Plant regeneration of garlic (*Allium sativum* L.) via somatic embryogenesis. *Scientific Res. Essay*. **(4):** 1569-1574.

Luciani, G. F., Mary, A. K., Pellegrini, C. and Curvetto, N. R. (2006). Effects of explants and growth regulators in garlic callus formation and plant regeneration. *Plant Cell Tiss. Org. Cult.*, (**87):** pp. 139-143.

Ma, Y., Wang, H., Zahang, C. and Kang, Y. (1994). High rate of virus-free plantlet regeneration via garlic scape-tip culture. *Plant Cell Rep.* **(14):** 65-68.

Mahajan S, Tuteja N. (2005). Cold, salinity and drought stresses: an overview. *Arch Biochem. Biophys.* **44(4):**139–158.

Martin-Urdiroz, N.J. Gala, G. Martin, J. and Barandiaran, X. (2004). Effect of light on the orpanogenic ability of garlic roots using a one-step *in vitro* system. *Plant Cell Rep.* **(22):** 721-724.

Mc. Collum, G.D. (1976). Evalution of crop plants. Ed. N.W. Simmonds, Lonfman, London and New York. pp. 186-190.

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

Myers, J. M. and Simon, P. W. (1999). Regeneration of Garlic callus as affected by clonal variation, plant growth regulators and culture conditions over time. *Plant Cell Rep.* **(19):** pp. 32-36.

Nagakubo, T., Nagasawa, A. and H. Ohkawa. (1993). Micropropagation of garlic through *in vitro* bulblet formation. *Plant Cell Tis. Organ Cul.* (**32):** 175-183.

Olmos, E., and Hellin, E. (1998). Ultrastructural differences of hyperhydric and normal leaves from regenerated carnation plants. *Sci. Hort.* **(75):** pp. 91-101.

Sata, S. J., Bagatharia, S. B., and Thaker, V. S. (2001). Induction of direct somatic embryogenesis in Garlic (*Allium sativum*). *Meth. Cell. Sci*. **(22):** pp. 299-304.

Stelmaszczuk, M. and Kozak, D. (2013). Micropropagation of *Allium neapolitanum* Cirillo. *Acta Sci. Pol. Hortorum Cultus,* **12(5):** 193-206.

Tal, M. (1983). Selection for stress tolerance. In: Handbook of Plant Cell Culture, Volume 1. D.A. Evans; W. R. Sharp P.V. Ammirato and Y. Yamada, (eds) New York, Macmillan pp. 481-488.

Tanou, G, Molassiotis, A. and Diamantidis, G. (2009). Induction of reactive oxygen species and necrotic death-like destruction in strawberry leaves by salinity. *Environ. Exp. Bot.* **(65):**270–281.

Tavakkoli, E. Rengasamy, P. 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. Exp. Bot.* **(61):**4449–4459.

Umiel, N., Zahavi, E. and Chen,Y.(1980). Effect of salinity sresses on tobacco, 2.Short term kinetics of Na+ and K+ uptke by callus cultures grown on media containing NaCl. *Z.* Pflanzenphysiol. (**100):** 363-367.

Wu, Z., Chen, L. J. and Long, Y. J. (2009). Analysis of ultrastructure and reactive oxygen species of hyperhydric Garlic (*Allium sativum* L.) shoots. *In vitro Cell Dev. Biol. Plant*. **(45):** pp. 483-490.

APPENDICES

Appendix I. Composition of Duchefa Biochemic MS (Murashige and Skoog, 1962) medium including vitamins

Components	Concentrations (mg/L)	Concentrations
Micro Elements	**mg/L**	**µM**
$CoCl_2.6H_2O$	0.025	0.11
$CuSO_4.5H_2O$	0.025	0.10
Fe Na EDTA	36.70	100.00
H_3BO_3	6.20	100.27
KI	0.83	5.00
$MnSO_4.H_2O$	16.90	100.00
$Na_2MoO_4.2H_2O$	0.25	1.03
$ZnSO_4.7H_2O$	8.60	29.91
Macro Elements	**mg/L**	**mM**
$CaCl_2$	332.02	2.99
KH_2PO_4	170.00	1.25
KNO_3	1900.00	18.79
$MgSO_4$	180.54	1.50
NH_4NO_3	1650.00	20.61
Vitamins	**mg/L**	**µM**
Glycine	2.00	26.64
Myo-Inositol	100.00	554.94
Nicotinic acid	0.50	4.06
Pyridoxine HCl	0.50	2.43
Thiamine HCl	0.10	0.30

Total concentration of Micro and Macro elements including vitamins: 4405.19 mg/L
Manufacturing Company: Duchefa Biochem