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Improvement of Androgenesis and Somatic Embryogenesis Using Physical and Chemical Pretreatment Factors in Barley (Hordeum Vulgare L.)

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IMPROVEMENT OF ANDROGENESIS AND SOMATIC EMBRYOGENESIS USING PHYSICAL AND CHEMICAL PRETREATMENT FACTORS IN BARLEY (*Hordeum vulgare* L.)

THESIS SUBMITTED FOR THE DEGREE
OF
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IN THE
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UNIVERSITY OF RAJSHAHI
BANGLADESH

BY

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B. Sc (Hons.), M. Sc (BOTANY)

DECEMBER 2015

PLANT GENETIC ENGENEERING LAB.
INSTITUTE OF BIOLOGICAL SCIENCES
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Dedicated

To

My Parents

DECLARATION

I hereby declare that the research work embodied in this thesis entitled **"Improvement of Androgenesis and Somatic Embryogenesis Using Physical and Chemical Pretreatment Factors in Barley (*Hordeum vulgare* L.)"** has been carried out by me for the degree of Doctor of Philosophy. The work was carried our under the guidance of Dr. S.M. Shahinul Islam, Associate Professor, Institute of Biological Sciences, University of Rajshahi, Bangladesh. I also declare that the result presented in this dissertation is my own investigation and any part of this thesis work has not submitted to elsewhere for any degree/diploma or for similar purpose.

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Certificate

This is to certify that **Md. Mozidul Haque** worked under my supervision as a Ph.D fellow, session 2011-2012, Institute of Biological Sciences (IBSc), University of Rajshahi, Bangladesh. It is my great pleasure to forward his thesis entitled **"Improvement of Androgenesis and Somatic Embryogenesis Using Physical and Chemical Pretreatment Factors in Barley (*Hordeum vulgare* L.)"** which is a bonafide record of research carried out at Plant Genetic Engeneering Laboratory, IBSc, RU. He has fulfilled all the requirements of the regulations relating to the nature and prescribed period of research submission of the thesis for the awared of the degree of **Doctor of Philosophy.**

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The Author

ABSTRACT

In vitro androgenesis and somatic embryogenesis are an essential tools for advance biotechnological research for barley and other crops improvement. To evaluate the growth and yield contributing characters six BARI barley and eight European genotypes were considered for this study. The major goals of this study was to screen a suitable genotype; optimization of media, plant growth regulators (PGRs), to evaluate the effect of salt and heat pretreatment factors on regeneration; various concentrations of copper sulphate and cobalt chloride added in medium to improve somatic embryogenesis. Various carbon sources were also evaluated with MS and other medium and standardized silver nitrate and amino acids concentration. Callus age and sizes were considered for their effects on plant regeneration. Different physical pretreatment factors such as cold and drought stresses also applied directly to the targeted explants (anthers, spikes) to improve anther culture responses of barley in Bangladesh. To assess the performance of the genotypes, variability and influence of sowing times on yield and yield contributing characters, six local and eight European barley genotypes were considered. Among the studied genotypes BB-5 showed earliest booting (59 days) and the maturity was found after 101 days from the date of sowing. Most yield contributing traits were found in Hor-9465, BB-5, Hor-291, Hor-9580 and BB-6 promising with good yield compared to other studied genotypes.

Six barley genotypes were tested and among them four (BB-6, BB-3, BB-1 and BB-2) showed significant results on callus induced and regeneration. Twelve different combinations of MS medium (CIM_1 – CIM_{12}) used for primary callus induction. It was observed that CIM_8 (4.0 mg/l 2,4-D, 200 mg/l L-proline and 300 mg/l casein) showed better performance on callus induction (38.17%) in BB-6. Nine different concentrations of plant growth regulators were used in MS medium and among them RM_7 (MS + 1.5 mg/l BAP + 30 g/l sucrose) showed better performance on plant regeneration in BB-6 (9.26%). Another attempt was taken about heat pretreatment factor along with various concentration of NaCl. For this experiment, mature embryos were used that derived from three barley genotypes (BARI barley-3, BARI barley-6 and BHL-18). In this case BB-6 showed highest viability on callus (14.72%) induction and regeneration (7.69%) with high concentrations

of NaCl (6.5 g/l). With high amount of NaCl (6.5 g/l) BB-6 exhibited maximum relative growth rate (0.91) and tolerance index (0.42) among the three studied genotypes. The result revealed that calli of BHL-18 performed highest desiccation (59.70%) when it was incubated at 40℃ and BB-6 gave the best regeneration (41.66%) when the calli incubated at 35℃ temperature.

To evaluate the effect of copper sulphate and cobalt chloride in medium, mature embryos of BARI barley-3 and BARI barley-6 were considered. The concentration of both chemicals at 2.5 to 7.5 mg/l was suitable for callus induction. In the case of plant regeneration T_{10} showed maximum plant regeneration (53.25%) in BARI barley-6 that was around 3 fold higher in comparison with control. For another experiment seeds of BARI barley-6 were pretreated with different concentrations of 2,4-D and various durations prior to culture. The highest frequency of callus induction (71.38%) was recorded with 3.5 mg/l 2,4-D that pretreated up to 4 days. To optimize a suitable carbon sources for callus induction various amount of sucrose, maltose and D-sorbitol were used either single or in combination with three media. The maximum percentage of primary callus (89.16%) was recorded in MS medium that supplemented with 60 g/l D-sorbitol (T_6). For another experiment various concentrations of PGRs were evaluated and the maximum embryogenic calli (70.0%) obtained when 2,4-D (2.0 mg/l) + BAP (0.5 mg/l) were used. Highest plant regeneration (47.40%) was recorded when the MS medium was supplemented with 0.5 mg/l NAA + 1.0 mg/l BAP. For rooting GM showed better performance in addition with 1.0 mg/l IAA.

In Exp. 6 (section 4.5) an efficient protocol has been established for callus induction and regeneration. In this case $AgNO_3$ and two different types of amino acids were used in addition to the medium. As explants immature embryos were used that derived from three genotypes *viz.* BB-1, BB-3 and BB-6. Here five doses of $AgNO_3$ either single or in combination were used along with two amino acids (L-proline, L-glutamine). The maximum callus were recorded for BB-6 (49.20%) and BB-3 (32.66%) when 2.0 mg/l $AgNO_3$ and 200 mg/l L-glutamine were added. Plant regeneration (37.20%) remarkably increased on MS medium that supplemented with 1.0 mg/l BAP + 1.5 mg/l $AgNO_3$ + 150 mg/l L-glutamine in BB-6. Other attempts (Exp. 7; section. 4.6) have been done using

callus size, age and their fresh weight to improve regeneration using mature embryos that derived from immature embryos (milky phases of seeds) in BB-6. It was observed that callusing and plant regeneration was better when 1.6-2.0 mm size of embryos, 4-6 weeks old calli and 151-200 mg weight were used.

To identify a suitable androgenetic genotype two BARI barley cultivars (BB-3 and BB-6) were used for anther culture. Under this study cold pretreatment for 8-12 days showed better performance on embryos formation in FHG medium. The highest level of embryoids (14.6%) was found at 10 days cold pretreatment and finally produced 13.8% plantlets and 10.72% green plants in BB-6. MSR medium showed better results on green plant regeneration. Another attempt was done for drought stress pretreatment factors that applied to the excised anthers of BB-3 and BB-6 (Expt. 9; section 5.2). It was observed that drought stress for 150 min (T_5) showed highest percentage of embryoids (26.50%) and green plants (13.58%) in BB-6. For all cases the number of androgenic embryos and regenerated green plants increased in liquid induction (FHG) medium. From these experimental findings it might be concluded that the cold and drought stress pretreatment factors enhanced anther culture responses. The protocol established under this study might be helpful for future advance level of biotechnological research for barley and other cereal crops in Bangladesh.

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LIST OF ABBREVIATIONS

The following abbreviations have been used through the text:

ABA	:	Abscisic acid
AN	:	Anther culture
ANOVA	:	Analysis of variance
BA	:	Benzyl adenine
BAP	:	6-benzylaminopurine
BARI	:	Bangladesh Agricultural Research Institute
BB	:	BARI Barley
B5	:	Gamborg et al. (1968) medium
°C	:	Celsius
CH	:	Casein hydrolysate
cm	:	Centimetre
DH	:	Doubled haploids
DW	:	Distilled water
EDTA	:	Ethylenediaminetetraacetic acid
et al.	:	Et alia = and others
Fig.	:	Figure (s)
g	:	Gram
g/l	:	Gram per litre
h	:	Hour (s)
HCl	:	Hydrochloric acid
IAA	:	Indole- 3-acetic acid

IBA	:	Indole-3- butyric acid
i.e	:	That is (to say)
Kin.	:	Kinetin
mg	:	Milligram
LSD	:	Least significant difference
Mg/l	:	Milligram per litre
ME	:	Mature embryo (s)
MS	:	Murashige and Skoog (1962) medium
NAA	:	1-Napthaleneacetic acid
N6	:	Chu (1975) medium
PGRs	:	Plant growth regulators
P^H	:	Negative logarithm of hydrogen ion (H-) concentration
SE	:	Somatic embryogenesis
sp.	:	Species
v/v	:	Volume by volume
viz.	:	Videlicet (L.); namely
w/v	:	Weight by volume
0.1N	:	0.1 Normal
2,4-D	:	2,4-dichloro phenoxy acetic acid
%	:	Percent

Chapter I

General Introduction

1. General Introduction

1.1 The genus *Hordeum*

Barley (*Hordeum vulgare* L.) is one of the earliest global domesticated crop plants and belongs to the family Poaceae (Gramineae). There are 32 species within the *Hordeum* genus, all with a basic chromosome number of x = 7. *Hordeum vulgare* is divided into two sub-species: *vulgare* and *spontaneum* C. Koch (Bothmer and Jacobsen, 1985; Nevo et al. 2012). Both taxa are diploid (2n = 14) with a large haploid genome of 5.1 gigabases (Gb) which was completely sequenced in 2012 and barley contains approximately 26,000 genes (Mayer et al. 2012). *Hordeum vulgare* is predominantly self-pollinated and fully inter-fertile (Jakob et al. 2014; Zohary and Hopf, 2000). Wild and domesticated barley differ in several phenotypic characteristics, collectively referred to as the domestication syndrome (Jakob et al. 2014). Wild barley naturally found in Southwest Asia, from the eastern Mediterranean coasts to the semi-deserts of Afghanistan (Harlan and Zohary, 1966).

Today, barley represents the fourth most abundant cereal both in area of cultivation and in grain output (http://faostat.fao.org). Among barley global yield, approximately 75% is used as feed, 20% as raw material for beverages and 5% as food (Sreenivasulu et al. 2008). Barley is regarded as an inferior staple compared to wheat, and is considered as the poor people's bread. It has a comparably stable yield in spite of climatic variation within the growing season. In this respect wheat and other small grain cereals cannot compete with barley (Goyal and Ahmed, 2012; Usubaliev, 2013). It is widely adapted to adverse environmental conditions and displays much higher tolerance to different environmental stresses than its close relative wheat (Nevo et al. 2012). Barley serves as an important model crops for studies in malting and brewing industry, in the field of plant breeding, genetics and biotechnology (Nilan and Ullrich, 1993).

In recent years, barley attracts as a model crop because of its broad natural diversity including geographically diverse elite varieties, landrace and wild accessions and

tremendous achievements in genomics such as rapid accumulation of EST sequences (http://barleygenomics.wsu.edu) and great efforts on barley genome sequencing and physical mapping (Freialdenhoven et al. 1994; Close et al. 2004; Varshney et al. 2007; Harwood et al. 2009; Mayer et al. 2012).

1.2 Origin and distribution

Till the origin of barley is not exactly well known, presumed to be originated either in Egypt, Ethiopia, the Near East/Fertile Crescent region or Tibet (Badr et al. 2000). There is, however, compelling evidence of the possibilities of multi-centers of origin of barley, initiating in the Iberian Peninsula, extending across of North Africa. But it can be surely said that it was one of the earliest cultivated grains. Barley was grown in the Middle East prior to 10,000 BC, but its cultivation in China, India and Bangladesh probably occurred later. Archaeologists think barley was more important than wheat in the early days of agriculture-based civilization. In ancient Egypt (3200 BC to 30 BC) barley bread and beer (made from malt) constituted a complete diet. Indeed, the archeological data gathered from the large project where 367 barley accessions were obtained from the International Center for Agricultural Research in Dry Areas (ICARDA), Aleppo, Syria. Now the main barley growing countries in the world are Russia, Canada, Germany, France, Ukraine, Spain, Turkey, UK, Australia, USA and Denmark. In Tibet, Nepal, Ethiopia, and the Andes, farmers cultivate barley on the mountain slopes at elevations higher than other cereals. In areas with little irrigation in the dry regions of North Africa, the Middle East, Afghanistan, Pakistan, Eritrea, and the Yemen, barley is often the only suitable cereal. Developing countries account for about 18% of global production and 25% of the harvested area of barley.

1.3 Morphology

Barley is an annual plant that stands 60-120 cm tall and has a cylindrical stem or tiller which is hollow, except at the nodes which bear the leaves. The ability of the barley plant to drive up new tillers in response to favorable environmental conditions is a useful mechanism for adapting to changes during the growing season.

1.4. Agronomical traits

Barley is commonly classified according to different agronomic or quality traits, such as growth habit, spike morphology, grain morphology, seasonal growth habit, etc.

1.4.1 Nature of inflorescence and spikes

The inflorescence of barley is referred to as the ear, head or spike. The spike or head is composed of spikelets attached to the nodes of a zigzag structure known as a rachis. Each spikelet consists of two husks or hulls, enclosing male and female floral parts. The rachis nodes may have either one or 3 spikelets, giving the spike the appearance of having 2 or 6 rows of kernels; hence, the names 2-rowed and 6-rowed barley.

Barley has a single floret in each spikelet. There are three spikelets at each node, alternating on opposite sides of the barley head or spike. In two-rowed barley, the central floret is fertile and the two lateral florets are sterile, resulting in a single seed at each node, giving the head a flat appearance. In six-rowed barley, all of the florets are fertile. The central seeds are round and fat, but the laterals tend to be slightly asymmetric. On the basis of spikelets barley cultivars are divided into two sections that are mentioned below:

1.4.2 Six-row barley

In this case spikelets of barley are arranged in triplets that alternate along the rachis. In six row barley, all of the spikelets in triplets are fertile and able to develop into grains. Each spike may carry 25-60 grains in six rows varieties.

1.4.3 Two-row barley

In two row barley, only the central spikelet is fertile, the other two lateral spikelets are sterile. This condition is retained in certain cultivars known as two-row barley. In this case each spike may carry 15-30 grains. Two-row barley has lower protein content and having more fermentable sugar (Vasan et al. 2014).

1.4.4 Type of grains

On the basis of hulls, barley cultivars are divided into two groups that are described below:

1.4.4.1 With hulled

Hulled barley is covered and that has been minimally processed to remove only the tough inedible outer hull. In this case it is challenging to remove the hull carefully. In market, hulled barley may be purchased in several forms including kernels (berries), cut (grits), flaked or ground (meal or flour).

1.4.4.2 Without hull

This type of barley has an outer hull that is so loosely attached to the kernel and generally falls off during harvesting. Processors often refer to this type of barley as ‘naked’ barley. Hulless is a form of domesticated barley and an ancient food crop, but a new industry has developed around uses of selected hulless barley to increase the digestible energy of the grain, especially for swine and poultry. Hullless barley contains more protein, starch and β-glucan. It is potential also for new applications as whole grain, and for its value-added products.

1.5 Application of barley as food crop

1.5.1 Feed barley

Barley mainly cultivated and used for human food, but it is now used for various reasons like mainly for livestock and poultry feed. Among barley global yield, approximately 75% is used as feed. Whole grain barley is commonly used as a forage source for dairy cattle in the world.

1.5.2 Pearl barley

Pearl barley, the most popular form in many parts of the world consists of whole kernels from which the outer husk and part of the bran layer have been removed by a polishing process. If it's lightly pearled, pearl barley will be tan colored; if it's heavily pearled, barley will be quite white. Most of the pearl barley found in the typical supermarket. Although it is technically a refined grain, it's much healthier than other refined grains because, a) some of the bran may still be present and, b) the fiber in

barley is distributed throughout the kernel, and not just in the outer bran layer. Pearl barley cooks more quickly than whole grain and it is used for soups and dressing flour also.

1.5.3 Malt Barley

It is made by drying barley kernels. It is important for beer production and also used in extracts and syrups for adding flavor, colour or sweetness to commercially prepared foods such as cereals, baked goods, confections and beverages. Potential uses of malt barley are given below:

- ⟐ Malt grains for dairy feeds
- ⟐ Distiller's alcohols, spirits, whiskies
- ⟐ Infant baby food, chocolate from malt
- ⟐ Malted syrups, textile use, baking uses, candles
- ⟐ Dextrin for breakfast cream, coffee substitute
- ⟐ Milk based beverage and malt milk
- ⟐ Malted sprouts, vinegars, non-beverage products
- ⟐ Health tonics and chocolate

1.6 Growth season

One of the main classifications attends to the seasonal growth habit of the cultivars, for which two main such as winter and spring.

1.6.1 Winter barley

Winter barley is sown in autumn. It is tolerant to low temperature, it requires vernalization to promote flowering, and commonly displays a strong promotion to flowering in response to long days.

1.6.2 Spring barley

Spring barley is essentially the opposite of the winter barley. It usually has minimal low temperature tolerance, does not require vernalization, and is insensitive to long photoperiods.

1.7 Growth temperature of barley

Generally barley grows under cool conditions, but it is not required hardy winter for its usual grown. So it is currently popular in temperate and tropical areas (Bothmer et al. 1995). Spring-sown barley is especially successful in the cooler, moist areas of Western Europe and North America. The climate of Bangladesh (sub-tropical country) is favorable for barley production, where commonly spring barley is grown in Rabi season (October to March), which becoming mature within 80-90 days. This is because the optimum temperature for the growth of this crop is 10-20°C (Fisher, 1981) which eventually prevails in Bangladesh. It can be grown in regions where annual precipitation ranges from 250-1750 mm; about three-fourth of the land area based for barley production revise and average of 375-875 mm annually (Briggle and Curtis, 1987). Recently barley cultivation in Bangladesh is increasing for late harvesting of preceding crops, excessive soil moisture after rainy season and increasing cropping intensity have pushed a sizable barley area under moderately late to late sown condition (Alam et al. 2007).

1.8 Worldwide barley production

Barley has a considerable economic importance in agriculture and industry in many countries. The total barley grain production around the world accounted for 134 million tonnes in 2013 (FAOSTAT, 2014). The name of top ten barley producing countries and production (2013-2014) in the world are shown in **Table 1**.

Table 1: Top producing countries of barley in the world (Million metric tons)

Country	2013	2014
Australia	7.9	7.3
Canada	9.5	7.6
France	12.9	10.1
Germany	12.3	10.4
Russia	17.9	8.4
Spain	7.4	8.2
Turkey	7.3	7.2
Ukraine	11.8	8.5
United Kingdom	6.8	5.3
United States	5.0	3.9
World total	151.8	123.7

Source: Food and Agriculture Organization (FAO) of the United Nations.

1.8.1 Barley production in Bangladesh

Barley ranks fourth among the cereals in worldwide production but still it is a minor cereal in Bangladesh. It can play an important role in enhancing the food security of the country and in drainage of foreign currency (Yesmin et al. 2014). In Bangladesh, the climate and epidemic condition are suitable for barley cultivation except some costal districts. Barley cultivation in Bangladesh is increasing using hulless varieties for high yielding. It is an important crop to use such fallow, char and marginal lands in Bangladesh, but there are some constraints, such as socio-economic condition, lack of irrigation facilities, high cost of fuel, lack of stress tolerant varieties etc. Till Bangladesh Agricultural Research Institute (BARI), Gazipur has released a good number of barley varieties namely BARI barley-1, BARI barley-2, BARI barley-3, BARI barley-4, BARI barley-5 and BARI barley-6. The production of barley in Bangladesh is shown in **Table 2**.

Table 2: Barley production in recent years in Bangladesh (2012-2014)

Production	2012	2013	2014
Area (hector)	6130	6040	6000
Per metric ton (mt)	7008	6950	7000
Per hector yield (mt)	1.14	1.15	1.17

Source: BBS (2014).

1.9 Uses of Barley

Barley is an important food grain among working-class people in Europe until the end of the 19th century, when other grains such as wheat, rye and oats became more abundant, replacing barley in the diet. Throughout historical and archaeological reports, barley is referred to as a source of health, strength and stamina for athletes and manual laborers. The health benefits and medical aspects of barley foods are also referred to ancient Arabic, Chinese, Egyptian, Ethiopian and Greek literature, and have been reported by more recent civilizations from Asia to Europe (Newman and Newman, 2008). There are some countries where barley remains an important food crops namely Tibet, Korea, Mongolia, and many African and Asian countries (McIntosh et al. 1995). For example, Morocco has the highest per-capita consumption of food barley, where it is incorporated into soups, bread and porridge (Ashman and Beckley, 2006). In Japan, barley is used to produce miso, tea and shochu, and is used as a rice extender (Ashman and Beckley, 2006). In Western countries, barley is increasing in popularity as a food grain and is used in flours for bread making or other specialties such as baby foods, health foods and thickeners. Bangladesh and India are preferred barley as flour with molasses and its flavor and nutrients are very important specially for poor people. They are mainly using it with milk or ripe mango.

1.10 Nutrient composition and health benefits

Barley is almost similar to other cereal grains in terms of caloric value and protein content, but contains higher levels of β-glucan soluble fibre than other cereal grains. **Table 3** shows the macronutrient composition of the common grains. There is strong evidence that barley β-glucans can lower blood cholesterol levels, thereby reducing the risk of coronary heart disease (Jebor et al. 2013). Research has also shown that barley β-glucans lower blood glucose levels, which is important in the prevention and management of type 2 diabetes (Tosh, 2013) and increases satiety, which aids in weight management (El-Khoury, 2012). Considereing high level of β-glucan soluble fibre, barley is an excellent source of insoluble fibre and important in maintaining digestive health and protecting against colon cancer (Aune et al. 2011).

Table 3: Macronutrient compositions of the common whole grain crops

Grain	Energy	Total carbohydrate	Protein	Total fat	Total dietary fiber
	Kilojoules (KJ)/100 g		Calorie/100 g		
Barley	1480	73.5	12.48	2.3	17.3
Corn	1526	74.3	9.4	4.7	7.3
Millets	1580	72.8	11.0	4.2	8.5
Oat	1626	66.3	16.89	6.90	10.6
Rice	1547	77.2	7.9	2.9	3.5
Rye	1413	75.9	10.3	1.6	15.1
Triticale	1404	72.1	13.0	2.1	Not available
Sorghum	1413	74.6	11.3	3.3	6.3
Wheat	1421	75.4	10.7	1.99	12.7

Source: USDA (2014).

1.10.1. Nutritional value

Barley is a good source of many essential vitamins and minerals including thiamin, niacin, folate, riboflavin, iron, phosphorus, magnesium, zinc and selenium-all of which are important in maintaining good health. Barley contains similar levels of fat to other cereal grains, with the exception of oats, which has higher levels than all other cereal grains (USDA, 2014) (**Table 4**).

Table 4: Nutritional value per 100 g of raw barley

Energy	1474 kilojoules	Vitamin B_6	0.3 mg (23%)
Carbohydrates	77.7 g	Folate (Vit. B_9)	23 µg (6%)
Sugars	0.8 g	Vitamin C	0.0 mg (0%)
Dietary fiber	15.6 g	Calcium	29.0 mg (3%)
Fat	1.2 g	Iron	2.5 mg (19%)
Protein	9.9 g	Magnesium	79.0 mg (22%)
Thiamine (Vit. B_1)	0.2 mg (17%)	Phosphorus	221 mg (32%)
Riboflavin (Vit. B_2)	0.1 mg (8%)	Potassium	280 mg (6%)
Niacin (Vit. B_3)	4.6 mg (31%)	Zinc	2.1 mg (22%)
Pantothenic acid (B_5)	0.3 mg (6%)	-	-

Source: USDA (2014).

1.10.2 Medicinal value in barley

Recent research suggests that using whole grains can be reduced cholesterol levels and the risk of heart disease (Jebor et al. 2013). Studies have also shown that whole grain barley products with slow glycemic response and rich in dietary fibre and resistant starch in test meals significantly improved insulin sensitivity in type 2 diabetic and play an important role in reducing the risk of type 2 diabetes and certain cancers (Jonnalagadda et al. 2011). Barley grain also successfully used in molecular farming as a promising bioreactor adapted for production of human therapeutic proteins or animal vaccines (Mrízová et al. 2014). The medicinal uses of barley have been summarized in **Table 5**.

Table 5: The medicinal uses of barley

Disease		Application
Anti-cough	:	Decoction of *H. vulgare* seeds with apples, dried figs and pears.
Bladder inflammation	:	A decoction of dried seeds is used orally for bladder inflammation in Iran.
Blood glucose level	:	Seeds of *H. vulgare* 125 gm are roasted and mixed with each of 50 gm of *Cicer arietinum* and *Elettaria cardamomum* and used half teaspoon with water thrice a day to control blood glucose level.
Cholera	:	Powdered flower of *Calotropis procera*, fruits of *Piper nigrum*, seed ash of *H. vulgare* and rose water are taken orally for cholera in India.
Dermatitis	:	Hot water extract of dried seeds is also used externally for dermatitis in Guatemala.
Diabetes	:	This remedy is used as dietary supplement to control diabetes.
Inflammations	:	Hot water extract of dried seeds is used externally for inflammations.

Source: Jebor et al. (2013).

1.11 Biotechnological approaches for barley improvement

Biotechnology is a field of applied biology of scientific techniques to modify and improve plants, animals and microorganisms to enhance their value. Recently barley has been used as a genetic model species because of its true diploidy along with the similarity of its genome to that of other small-grain cereals (Nagy et al. 2011). A further advantage is that the barley genome is very similar to the D genome of wheat, so the results with barley should be relevant to transgenic wheat as well (Éva et al. 2008). For barley improvement tissue culture systems have a great importance than

other procedure (Holme et al. 2008). The biotechnology tools that are important for agricultural biotechnology include:

- Conventional plant breeding
- Tissue culture and micropropagation
- Molecular breeding or marker assisted selection
- Genetic engineering and GM crops
- Molecular diagnostic tools.

Barley is a model crop because of its short life cycle and its morphological characteristics are simple. For barley, efficient and reproducible plant regeneration protocols have been developed mostly based on somatic embryogenesis. Transgenic plants could be generated by gene transfer using somatic and gametic embryos that derived from mature and immature seeds and also by anther/microspores (Islam and Tuteja, 2012). To the best of my knowledge, there is no report on *in vitro* plant regeneration using barley genotypes in Bangladesh, and none of the cultivars used in this study have been considered previously for analysis of *in vitro* plant regeneration capacity.

1.11.1 *In vitro* somatic embryogenesis

The process of embryo development is called embryogenesis. Somatic embryogenesis is a unique pathway for asexual propagation or somatic cloning in plants. The developmental process of somatic embryogenesis shares considerable similarity with that of zygotic embryogenesis and this is likely due to the conservation in the underpinning cellular and molecular mechanisms between the two processes (Zimmerman, 1993). Therefore, somatic embryogenesis provides an attractive tool for studying zygotic embryogenesis, particularly because zygotic embryos are encased by maternal tissues and difficult to access by biochemical and molecular tools. Somatic embryogenesis has been induced in variety of explants including embryo (Amali et al. 2014). There are two major systems of embryo culture, e.g. i) mature embryo (it is derived from ripe seeds) and, ii) immature embryo (to rescue the embryos of wild crosses is used to avoid embryo abortion and produce viable plants). Different explants such as immature embryos, immature inflorescence, coleoptiles, mature

embryo and seedling explants have been used for callus induction and plant regeneration in barley (Sahrawat and Chand, 2004; Abumhadi et al. 2005; He and Jia, 2008). The success of plant tissue culture as a means of plant regeneration is greatly influenced by the nature of the culture medium.

1.11.2 Using explants for somatic embryogenesis

1.11.2.1 Mature embryos

Mature embryos are excised form ripe seeds and cultured mainly to avoid inhibition in the seed for germination. This type of culture is relatively easy as embryo requires simple nutrient medium containing mineral salts, sugar and agar for growth and development. The mature embryo is one of the best suited explants for advance biotechnological research. The use of mature embryos from dry seeds has several advantages: mature embryos are easy to handle, available throughout the year and their physiological state is less variable (Rostami et al. 2013). But regeneration response of mature embryos in barley is very low and highly genotype dependent. So there have the scope of re-evaluated on plant regeneration use as mature embryos in barley.

1.11.2.2 Immature embryo

This type of embryo culture is mainly used to grow immature embryos originating from unripe or hybrid seeds which fail to germinate. Excising such embryos is difficult and generally a complex nutrient medium is required to raise them to produce plants. Immature embryos are the most efficient tissue source to regenerate plants *in vitro* (Ozias-Akins and Vasil, 1982). However, it is usually difficult to obtain immature embryos throughout the year, and the suitable stage for their culture is also strictly limited. However, immature embryos are currently used as reliable and efficient target tissues or callus cultures for genetic transformation (Cho et al. 2002; Ahmadabadi et al. 2007).

1.12 Improvement of barley through androgenesis

Haploid production through anther or microspores culture is known as “Androgenesis”. Androgenetic embryos formed by male gametophyte (pollen grains/anthers (or) microspores). Anther and isolated microspore culture is a powerful

tool of *in vitro* plant breeding for haploid and doubled haploid plant production (Devaux and Pickering, 2005). During the last 15 years, doubled haploidy has been extensively used for the production of novel cultivars in cereals: 116 barley, 21 wheat, 8 rice and 3 triticale cultivars/lines originate from various techniques of haploid production (Forster, 2002).

Haploid plants (sporophytes) that contain a gametic chromosome number (n) and they can originate spontaneously in nature. Haploids produced from diploid species (2n = 2x), known as monoploids, contain only one set of chromosomes in the sporophytic phase (2n = x). Haploid production by wide crossing was reported in barley (Kasha and Kao, 1970). Barley is the most responsive species for doubled haploid production. Forster and Thomas (2003) reported that DHs techniques have been extensively used for the production of novel cultivars of cereals, e.g. barley, wheat, rice and triticale cultivars/lines during the last 20 years which originated from various techniques of haploid production. These procedures are based on chromosome elimination (bulbosum-method) and *in vitro* androgenesis. *Hordeium bulbosum* system has been applied to various hybrid genome constitutions as a substitute to genotype dependant anther culture (Eudes et al. 2009). Two-third of the doubled haploid barley cultivars have been produced using this technique (Forster and Thomas, 2003). In barley, many scientists have reported a spontaneous chromosome doubling capacity (Wan and Widholm, 1994; Chen et al. 2007).

1.13 Other ways for the productions of haploids

In barley, various approaches are employed in order to obtain haploid plants such as parthenogenesis, genome elimination, gynogenesis and androgenesis (Forster and Thomas, 2005; Germaná, 2011; Islam and Tuteja, 2012).

1.13.1 Parthenogenesis

Haploid regeneration through un-pollinated female gametophytes is an alternative process for haploid induction. This methodology normally described by the term gynogenesis, or haploid parthenogenesis. In plant terms, gynogenic haploid regeneration is widely used for all haploid induction methods, in which a female gametophyte is used as the origin of haploid cells, regardless of whether a pseudo-fertilization process is involved or not (Bohanec, 2009). Attempts have been made to

culture unfertilized ovules or ovaries in different plant species but growth of most of them stopped at the callus stages; only some crop species developed into haploid plants, e.g. *Hordeum vulgare* (San Noeum, 1976), *Triticum aestivum* (Zhu and Wu, 1979), *Oryza sativa* (Asslin de Beauville, 1980) and *Zea mays* (Ao et al. 1982). But the response through this process is very low, so practical application of parthenogenesis is rare in most of the species except *Solanum tuberosum.*

1.13.2 Genomic elimination

It is likely that the haploids were induced by a process of selective genome elimination of one of the parental species that follows certain inter-specific pollinations. This occurrence was first observed in barley with crosses between *Hordeum vulgare* and *H. bulbosum* (Kasha and Kao, 1970) and is now used routinely in wheat (Barclay, 1975; Moradi et al. 2009) and other cereals crop breeding programmes; haploids were induced in these species following pollination with maize (Sidhu et al. 2006) or other distantly related cereal species (Pratap et al. 2005; Komeda et al. 2007).

1.13.3 Gynogenesis

Gynogenesis is a process in which the embryos originate exclusively from female origin, following embryogenesis stimulation by a male gamete. Gynogenesis is another pathway for the production of haploid embryos. It can be achieved with the *in vitro* culture of various unpollinated flower parts, such as ovules, placenta attached ovules, ovaries or whole flower buds. Since the first report of gynogenesis in barley was reported by San Noeum (1976), then this approach has been successfully applied to raise haploid plants of *Helianthus annus*, *Oryza sativa*, *Triticum aestivum*, *Zea mays* and *Nicotiana tabacum* (Yang and Zhou, 1982).

1.14 Advantages of haploids/doubled haploids

The *in vitro* production of haploids through androgenesis is an efficient method for production of fully homozygous lines rapidly from heterozygous plants that are useful in genetics and breeding programme (Forster et al. 2007; Segui-Simarro, 2010; Redha and Islam, 2010). Traditionally, plant breeders can achieve homozygosity by using self-fertilization, a time consuming process, but in maximum cases plants are not fully

homozygous (Sopory and Munshi, 1997; Oleszczuk et al. 2011). Significant advantages of androgenesis systems are not only speed up the process of homozygosity, but also increase the selection efficiency (Germana, 2011). In many genotypes of barley, the efficiency of double haploid plants is quite low. Research of Lazaridou et al. (2011) conducted on a large set of barley genotypes showed average double haploid plants.

1.15 Limitations associated with haploid induction

For the production of haploids there are some limitations that are mentioned below:

1.15.1 Genotype

Success of anther culture is strongly genotype dependent and it was under genetic control reported by Bullock et al. (1982). The genotype dependency is a critical factor affecting the variability of response in plant tissue culture and androgenesis (Gosal et al. 1997; Islam et al. 2001). High androgenic ability could be considered, for a given genotype, as a marker of intragenetic diversity or instability that the anther culture technique might not be efficient in all genotypes (De Buyser et al. 1985). The physiological conditions of donor plants can drastically affect anther development and thus the number of embryogenic microspores induced (Olmedilla, 2010).

1.15.2 Mixoploidy

Another problem often encountered in androgenetic research is the emergence of plants of mixoploidy due to the fusion of haploid nuclei at the initial stages of androgenesis or endopolyploidy (Sangwan-Norreel, 1983). This phenomenon is inherent in haploid embryogenesis (Makowska and Oleszczuk, 2014).

1.15.3 Albinisms

The major problem of androgenesis in cereal crops is represented by the formation of high frequency regenerants devoid of chlorophyll in monocots, so called “albino plants” or albinism (Mùnoz-Amatriain et al. 2009). These plants cannot survive in nature and have no agronomic value. Albinism is often encountered by plant breeders when they create wide hybrids or when using tissue culture technology such as anther culture or isolated microspore culture. Torp and Andersen (2009) mentioned that combination of starvation and cold stresses applied simultaneously for shorter period

(3 - 4 days) have increased microspore survival rate which may cause to reduce the frequency of albinism. The development of albinos may also be due to mutations or expression of recessive genes (Wang et al. 1973).

Jacquard et al. (2006) obtained albinos during their study on barley, regardless of optimizing their anther culture protocol by adding mannitol and copper sulphate. This suggests that barley microspores and derived structures are particularly sensitive to albinism. There are many factors that can influence the regeneration of albino plants. Factors include genotype, environment, meiotic abnormalities, hormonal imbalances and various others. In barley anther culture, the frequency of albinism is dependent on genotype as established by Larsen et al. (1991). Incompatibility between the plastid- and nuclear genome is one of the major factors inhibiting chlorophyll formation (Yao et al. 2000). Green and albino plantlet regeneration depends on a number of factors that include donor plant growth conditions, culture temperature, cold pretreatment, sucrose concentration in combination of growth hormones and the development of the microspore (Chen et al. 2007).

1.15.4 Physical treatment

Some physical pretreatments such as heat, drought starvation, pH, heavy metal, osmotic stress, gamma radiation, hypertonic shock, atmospheric pressure etc of anthers can be used to enhance androgenetic responses (Shariatpanahi et al. 2006; Islam and Tuteja, 2012; Islam et al. 2013a). Recent year there are many reports on successful application of physical or abiotic stress pretreatments for crop improvement (Maraschin et al. 2005; Chauhan and Khurana, 2010; Islam, 2010; Roy et al. 2011). Various stress pre-treatments during development of pollen grains is known to promote the induction of androgenesis in several crop plants, including cereals. Shariatpanahi et al. (2006) found that there were multiple approaches in anther culture as well as microspore culture. Some of these pretreatments included chilling, heat application, high humidity, water stress, anaerobic treatment, centrifugation, sucrose starvation, nitrogen starvation, microtubule disruptive agents, electro-stimulation and high pH levels in medium. Datta (2005) mentioned that the type, duration and the time of application of stress pretreatments may vary with the species or even variety was found to improve anther and microspore culture in crops. Jacquard et al. (2009) stated that barley anthers are able to perceive abiotic stress

conditions early during treatment, and respond by triggering various aspects of stress-related physiology.

1.15.4.1 Cold pretreatment

The purpose of applying pre-treatment is to create the stress necessary to change the development pathway of microspores from gametophytic to sporophytic pathway. The most common pre-treatments used are cold treatment, where the donor material is kept at a low temperature. Temperature shock has been reported to be one of the most effective treatments. However, the optimum temperature and pre-treatment vary with the genotype. Huang and Sunderland (1982) tested different pretreatment methods for optimal callus and green plant production on the barley cultivar 'Sabarlis'. They found that there were greater callus yields at 4°C than 25°C, and that maximum yields were best produced at 4°C incubated over 3-5 weeks and 3-5 days for 25°C. Jähne and Lörz (1999) stated that cold shock has a duel effect by providing interruption of microspore mitosis in order to produce embryos, as well as providing ample time for microspores to be nurtured by the anther. In general it has been shown by numerous scientists (Oleszczuk et al. 2006; Shariatpanahi et al. 2006) that a cold pre-treatment is an effective stressor. However, the time period associated to it may vary depending on the plant species.

1.15.4.2 Drought pretreatment

Drought or heat pretreatment has been used to trigger embryogenesis of various crops. The heat pretreatment usually used at 26°C to 32°C for several hours or a few days (Shariatpanahi et al. 2006). Heat pretreatment of inoculated anthers or microspores at 30 - 35ºC was effective in induction of androgenesis in cereals, such as maize (Genovesi, 1990), wheat (Islam, 2010; Li et al. 1988) and rice (Reddy et al. 1985). Islam (2010) reported that in wheat drought stress (1-5 hours) significantly better compared to the control for anther culture. Shariatpanahi et al. (2006) further state that heat shock affects the appearance of pre-prophase bands and that structural changes occur. These structural changes include electron-dense deposits at the plasma membrane or cell wall interface, vesicle-like structures in the cell walls and organelle-free regions in the cytoplasm.

1.15.5 Chemical treatment

The application of chemical pretreatment is necessary for efficient induction of anther or microspore embryogenesis in cereals (Islam, 2010). Sugar starvation by placing anthers on a medium with mannitol as the carbohydrate source is one of the most commonly used stress pretreatment factors (Pulido et al. 2006; Soriano et al. 2007). This pretreatment has provided consistently high chromosome doubling rates in barley (Kasha et al. 2001). For chromosome doubling colchicine, pronamide, oryzalin and amiprophosmethyl (AMP) are used in different crops and colchicine is very effective chemical treatment (Soriano et al. 2007). The direct application of colchicine to the induction medium of anther culture using different concentrations and durations caused an increase in the frequency of fertile plants up to 76% in cereals crops (Obert and Barnabás, 2004; Redha et al. 1998). Hassawi and Liang (1990) applied different concentration of colchicine in wheat during mannitol stress pretreatment or during the first 48 h of culture at concentrations of 0, 150 and 300 mg/l. They observed a significant increase in chromosome doubling at 300 mg/l concentration of colchine but low androgenic response.

1.16 Objectives

The present investigation was conducted mainly in three parts: the first part deals with morphological study; the second part deals with somatic embryogenesis (using mature and immature embryos) and the third part deals with androgenesis (anther culture) in barley (*Hordeum vulgare* L.). Therefore, the main objectives of the present study was-

- ⟐ Comparison among local and European barley genotypes on growth and yield.
- ⟐ Screening of suitable genotypes of barley for somatic embryogenesis.
- ⟐ Effect of salt and heat pretreatment to improve *in vitro* regeneration system in barley.
- ⟐ The effects of copper sulphate and cobalt chloride to improve callus induction and regeneration in barley using mature embryos.
- ⟐ Optimization of media and plant growth regulators (PGRs) to improve callus induction and regeneration through somatic embryogenesis.

- Improvement of regeneration efficiency using carbon sources and growth regulators in barley.
- Standardization of silver nitrate and amino acids for high frequency plants regeneration.
- Improvement of regeneration efficiency using callus age and size in barley.
- Screening of suitable genotypes and optimization of media and other culture conditions for androgenetic (anther culture) study.
- Enhancement of anther culture responses using different physical stress factors (cold, drought etc).

Chapter II

Materials and Methods
(General)

2. Materials and Methods (General)

2.1 Plant materials

Plant materials consisted of seven local genotypes collected from Bangladesh Agricultural Research Institute (BARI), Gazipur, Bangladesh and eight European genotypes of barley (*Hordeum vulgare* L.) were collected from IPK, Gatersleben, Germany (**Table 6**). Morphology of local as well as European barley genotypes are presented in **Fig. 1 (A, B) & 2**. Experiments were carried out on the basis of morphological characteristics and yield, *in vitro* somatic embryogenesis through mature and immature embryos and androgenetic study (anther culture).

Table 6: Name, origin and source of the genotypes

Genotypes	Country of origin	Sources	Type
BARI Barley-1 (BB 1)	Bangladesh	BARI, Joydebpur	Hulled
BARI Barley-2 (BB 2)	Do	Do	Do
BARI Barley-3 (BB 3)	Do	Do	Hull-less
BARI Barley-4 (BB 4)	Do	Do	Hulled
BARI Barley-5 (BB 5)	Do	Do	Do
BARI Barley-6 (BB 6)	Do	Do	Hull-less
BHL-18	Do	Do	Do
Hor-291	Europe	IPK, Germany	Do
Hor-8903	Do	Do	Do
Hor-9465	Do	Do	Do
Hor-9580	Do	Do	Do
Hor-10874	Do	Do	Do
Hor-17016	Do	Do	Do
Hor-17277	Do	Do	Do
Hor-17287	Do	Do	Do

Fig. 1 A: Morphology of local barley seeds: (1) BARI barley-1, BARI barley-2, BARI barley-3, BARI barley-4, BARI barley-5 and BARI barley-6.

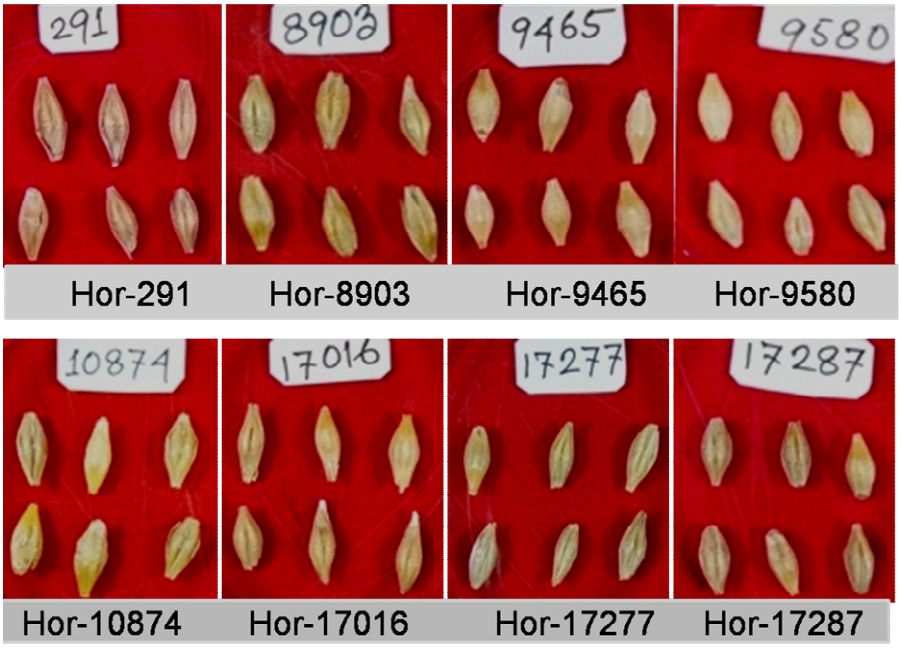

Fig. 1 B: Morphology of eight European barley seeds *viz.* Hor-291, Hor-8903, Hor-9465, Hor-9580, Hor-10874, Hor-17016, Hor-17277 and Hor-17287.

Fig. 2: Morphology of eight European barley genotypes: (I) **Growth stage** - A) Hor 17277, B) Hor 10874, C) Hor 291, D) Hor 9580, E) Hor 9465, F) Hor 8903, G) Hor 17016, H) Hor 17287; (II) **Booting stage**, (III) **Heading stage** and (IV) **Anthesis stage**.

2.2 Culture media

The culture media is one of the most important factors for the induction and subsequent development of new structure and plants. Several induction media *viz.* MS (Murashige and Skoog, 1962), B5 (Gamborg et al. 1968), N6 (Chu, 1975), FHG (Hunter, 1987) and AMS_3 (Islam, 2000) were used for callus and/or embryoid induction are shown in **Table 7**. Different carbon sources e.g. sucrose, sorbitol and maltose and plant growth regulators (PGRs) with various combination and concentrations were used under this study. MS and MSR (modified MS; Islam, 2000) were used for regeneration and for rooting half strength of MS and GM medium (modified MS; Islam, 2000) were used. The pH of all media was adjusted at 5.6 - 5.8 using 0.1N NaOH or 0.1N HCl.

Table 7: Media composition for induction, regeneration and rooting of barley

Components	Induction media (mg/l)					Regeneration medium (mg/l)	Rooting Medium (mg/l)
	MS	B5	N6	FHG	AMS_3	MSR	GM
Macronutrients							
$(NH_4)_2 SO_4$	-	134	463	-	250	200	-
$MgSO_4 .7H_2O$	370	500	185	370	200	-	71.5
$Ca(NO_3)_2 .4H_2O$	-	-	-	-	-	-	500
KCl	-	-	-	-	40	-	65.0
$CaC1_2 . 2H_2O$	440	150	166	440	100	200	-
KNO_3	1900	3000	2830	1900	950	1000	950
NH_4NO_3	1650	-	-	165	-	825	825
$NaH_2PO_4 .H_2O$	-	150	-	-	-		
KH_2PO_4	170	-	400	170	285	85	350
Micronutrients							
$MnSO_4 .4H_2O$	22.3	10.0	4.4	16.9	8.0	22.3	4.9
$ZnSO_4.7H_2O$	8.6	2.0	1.5	8.6	3.0	8.6	2.7
$CuSO_4 .5H_2O$	0.025	0.025	-	0.025	-	0.025	-
$CoC1_2 .6H_2O$	0.025	0.025	-	0.025	-	0.025	-
K1	0.83	0.75	0.8	0.83	0.5	0.83	0.75
H_3BO_3	6.2	3.0	1.6	6.2	3.0	6.2	1.6
$Na_2M_OO_4 .2H_2O$	0.25	0.25	-	0.25	-	0.25	-
Iron							
$FeSO_4 .7H_2O$	27.8	27.8	27.8	40.0	27.8	13.9	13.9
$Na_2 EDTA$	37.3	37.3	37.3	40.0	41.0	18.6	18.6
Vitamins							
Pyridoxine HCl	0.5	1.0	0.5	-	0.5	0.5	5.0
Nicotinic Acid	0.5	1.0	0.5	-	0.5	0.5	5.0
Thiamine HCl	1.0	1.0	1.0	0.4	1.0	0.1	1.0
Myo-Inositol	100	100	100	100	100	100	100
Hormones							
2, 4-D	-	-	-	2.0	2.0	-	-
Kinetin	-	-	-	0.5	0.5	1.0	-
IAA	-	-	-	-	1.0	-	1.0
Carbon sources							
Sucrose	30000	20000	30000	-	-	20000	20000
Maltose	-	-	-	62000	90000	-	
Amino acids							
L-glutamine	-	-	-	730	500	-	-
L-proline	-	-	-	-	200	-	-
L-asparagine	-	-	-	-	50	-	-
Casein hydrolysate	-	-	100	-	-	-	-
Glycine	2.0	-	2.0	-	2.0	2.0	2.0

2.3 Methods

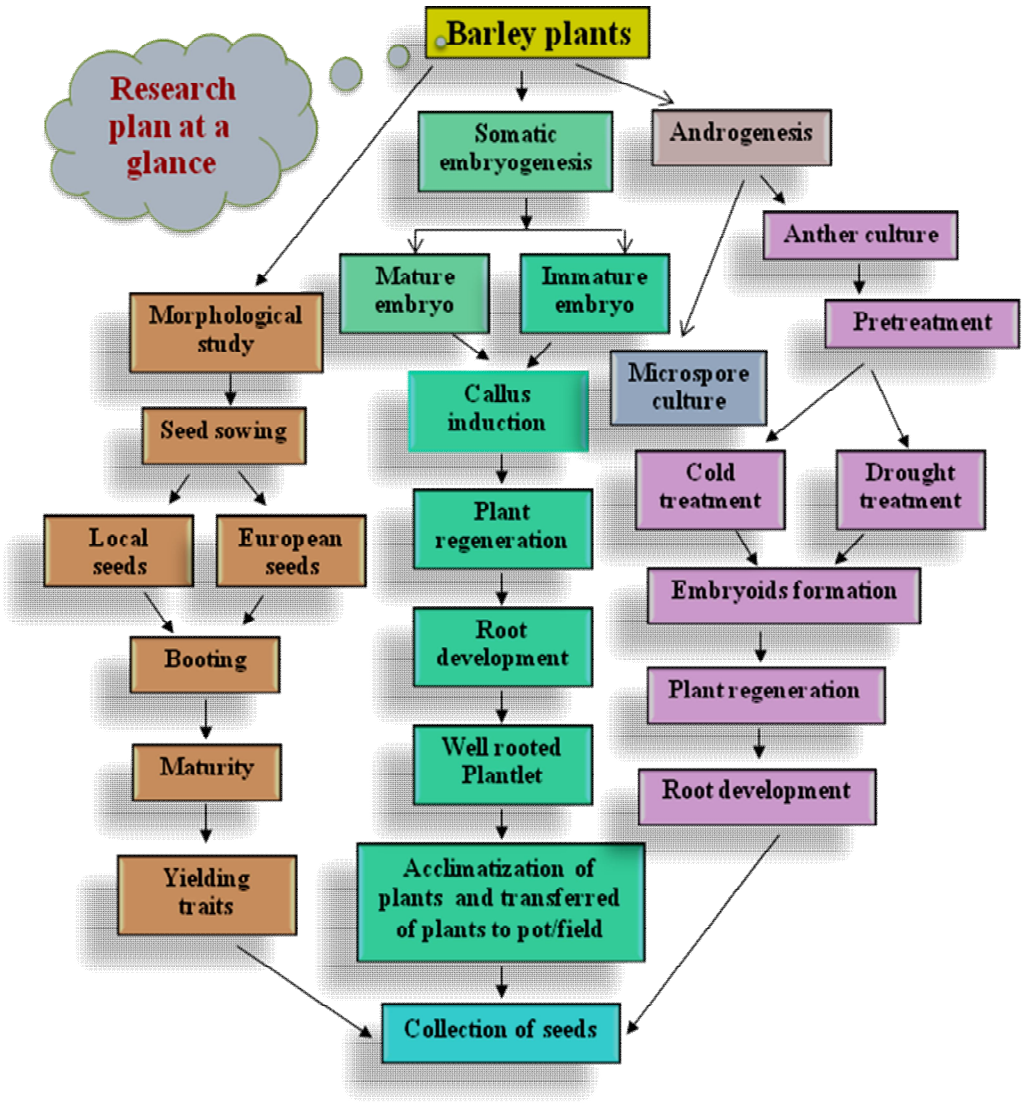

Fig 3: The flow chart is showing details research activities at a glance.

2.3.1 Mature embryo culture

2.3.1.1 Sterilization procedure

Dehusked seeds were surface sterilized by 70% ethanol for one minutes and rinsed 3 times with sterile distilled water. Then seeds were disinfected with 5% sodium hypochlorite (NaOCl) plus 0.1% Tween-20 for 30 minutes and rinsed them with sterile distilled water 3-5 times under the laminar air-flow cabinet. Afterwards seeds

were placed on sterilized filter paper for soaking excess water and approximately 8 - 10 seeds were inoculated for each petri dish.

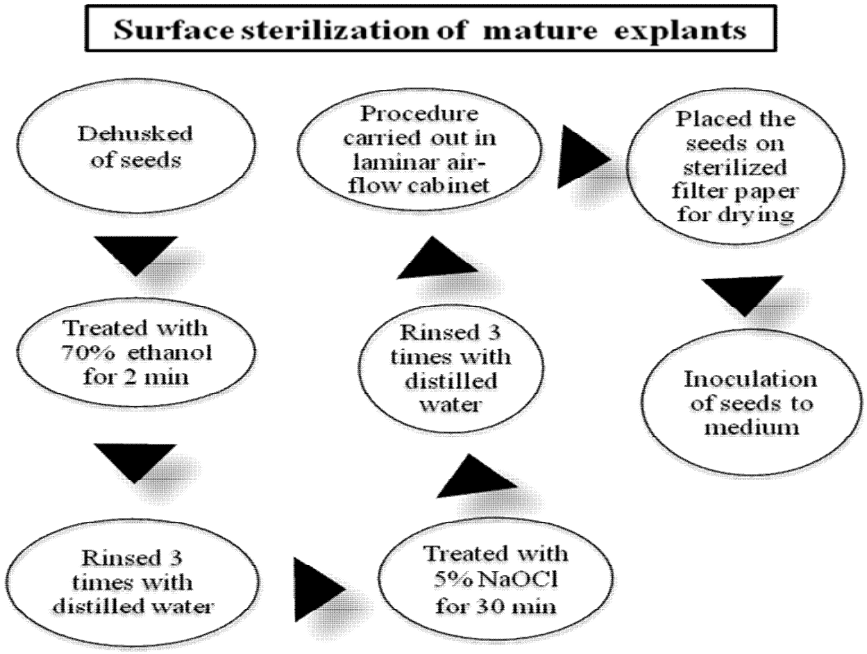

Fig. 4: Illustrates the process of sterilization of mature seeds.

2.3.1.2 Pretreatment

To evaluate the efficacy of 2,4-D for callus induction, barley seeds were sterilized and pre-treated by various concentration of 2,4-D (2.5, 3.5 and 6.0 mg/l) and incubated them at 4℃ for 1- 6 days prior to culture. Then pretreated seeds were rinsed 4-5 times and excess water was blotted onto a sterile Whatman No. 1 filter paper.

To observe the effect of salt pre-treatment, 3-4 weeks old calli were used and placed them on MS medium supplemented with various concentrations e.g. 0, 1.5, 2.5, 3.5, 4.5, 5.5 and 6.5 g/l of NaCl.

To evaluate the effect of heat stress, four weeks old calli (fresh weight 110-125 mg) were transferred onto sterile empty petri dishes. Then incubated transferred calli for 6 hours at 25℃ for Control. For three temperatures at 30℃, 35℃ and 40℃ were considered for treatment purpose respectively.

2.3.1.3 Inoculation of seeds

Surface sterilized seeds were inoculated using different media that supplemented with various concentrations and combinations of plant growth regulators (PGRs) and amino acids (**Table 8**). Inoculated petri dishes were sealed with paraflim and incubated at 25 ± 2ºC in dark for callus induction.

2.3.1.4 Embryogenic callus

After 4-6 weeks of culture initiation, primary callus was separated and transferred to targeted basal medium that supplemented with various concentrations and combinations of plant growth regulators (PGRs) for *in vitro* somatic embryos development.

2.3.1.5 Plant regeneration

Embryogenic callus (7-9 weeks old) were transferred to regeneration medium containing the basal medium supplemented with different concentrations of PGRs. Foe all cases, 2-3% (w/v) sucrose was used as carbon sources and cultured were incubated at 25°C under a 16/8h (light/dark) photoperiod.

2.3.1.6 Root induction

The regenerated shoots were transferred to root induction medium containing full/half-strength MS medium supplemented with 20 g/l sucrose and different concentrations of PGRs for rooting.

2.3.1.7 Acclimatization

Well developed plants with good shoots and roots were removed from the culture bottles, and their roots were thoroughly rinsed with running water to eliminate residual media. Subsequently, the plantlets were transferred into pots containing equal mixture of soil and peat moss (1:1). The plants were covered with transparent polyethylene bags and placed in a growth chamber at 25 ± 1°C for 2-3 days. Finally, plants were transferred to field under natural condition.

2.3.2 Immature embryo culture

2.3.2.1 Explants

In order to obtain immature embryos, seeds of BARI barley-1, BARI barley-3 and BARI barley-6 were grown in the experimental field of the Institute of Biological Sciences, University of Rajshahi, Bangladesh during the barley growing season of November to February 2014.

2.3.2.2 Collection and sterilization of immature seeds

Barley spikes were collected when the immature embryos are around 1.5-2 mm in diameter. Before cutting the spikes, a single immature seed from the middle of each spike was checked to make sure the size of the immature embryos is correct. Then the immature seeds were removed from the spike and the awns broken off without damaging the seed coat. Afterwards the immature seeds were sterilized by 70% ethanol for 1 minute followed by three washes in sterile distilled water.

2.3.2.3 Isolation of immature embryos

Immature embryos were aseptically dissected from the immature seeds with a sterile scalpel. The embryo is then plated the scutellem side up keeping on callus induction medium.

2.3.2.4 Callus induction

To induce callus, the dissected embryos were inoculated on MS medium that supplemented with different concentrations of 2,4-D. The effect of five doses of $AgNO_3$ used either singly or combination with L-proline, L-glutamine *(*amino acids). Size of embryos, callus ages and its fresh weight were also considered under this study to evaluate the regeneration efficiency. The inoculated petri dishes were sealed and cultured were incubated in dark chamber at 25 ± 2°C for callus induction.

2.3.2.5 Regeneration, rooting and acclimatization

Various age groups of calli were weighted and transferred to regeneration medium containing the MS basal medium supplemented with different concentrations of PGRs and cultured were incubated with low light conditions at 25 ± 2°C together with 14/10 hrs (light/dark) photoperiods. When the regenerated shoots length around 2-3 cm were

transferred to rooting medium of GM. Then well rooted plantlets were transferred to pot that contained with peat moss and soil (1:1) and after acclimatization transferred to field.

2.3.3 Anther culture

2.3.3.1 Steps of anther culture in barley

Plant growing condition	:	Donor plants were grown in the field of the Institute of Biological Sciences, University of Rajshahi.
Harvest of spikes	:	Spikes were harvested when the microspore at early to mid uni-nucleate stage (observed by 1% aceto-carmine under microscope).
Cold treatment	:	Harvested spikes were pre-treated by cold at 4-7°C for 3-10 days.
Sterilization of spikes	:	Treated spikes were sterilized with 70% ethanol in laminar airflow cabinet prior culture.
Inoculation of anthers	:	Anthers were removed from the middle zone of the sterilized spikes using a fine forcep and inoculated on anther culture medium. Then incubated at 28°C chamber for 4-6 weeks for embryo formation.
Transfer of embryos	:	Embryos were picked up from petri dishes and placed them to embryo differentiation medium for regeneration.
Plant transfer	:	Regenerated plantlets were transferred to GM for good shoot and root development.
Transfer of plants to soil	:	After acclimatization well rooted plants were transferred to soil for DHs seed collection.

2.3.3.2 Donor plants

For anther culture two barley genotypes *viz.* BARI barley-3 and BARI barley-6 were considered for this study. Plants were grown during the growing season of November 2013 to February 2014.

2.3.3.3 Harvesting stage

Spikes were harvested when main tillers of the donor plants approximately 14-16 days of post anthesis levels. The spikes were harvested when the microspores were at the mid to late uninucleate stages. This developmental phase is indicated when the distance between the flag leaf and the penultimate leaf is 3 to 6 cm and the awns are visible just above the flag leaf.

2.3.3.4 Surface sterilization and pretreatment of spikes

Harvested spikes were kept in water to prevent wilting surface and placed them into tissue paper with a few drops of sterile water, then wrapped with aluminum foil and stored in the refrigerator at 4-6℃ for 3-10 day. The spikes harvested or collected from the donor plants will be placed on some form of pretreatment to initiate the process of androgenesis. The pretreatment can be a cold treatment, heat shock or a combination of these. After pretreatment the excised anthers were placed on induction medium for embryos development. This will induce the generation of calli or embryos which in turn will regenerate plantlets.

2.3.3.5 Induction of anthers

After pretreatment, spikes were sterilized for 45 seconds to a maximum of 1 minute using 70% ethanol in a laminar air-flow cabinet. Anthers dissected from the central part of the spikes were cultured in different induction media (solid or liquid) for embryoids induction (**Table 7)**. Then petri dishes were sealed with parafilm and incubted at 26 ± 2℃ in the dark for up to 4-8 weeks.

2.3.3.6 Plant regeneration and rooting

Anther derived embryoids were transferred to MSR and FHG medium for regeneration that supplemented with various PGRs and 20 g/l sucrose. The petri dishes were maintained in the culture chamber at 25℃ with a 16/8 hours photoperiod for around 2-3 weeks for plant regeneration. To induce growth of a strong root system, well-developed plantlets (2-3 cm in length) were transferred to rooting medium. The components of GM medium are shown in **Table 7**.

Chapter III

Effect of Sowing Time

3. Effect of Sowing Time on Yield and Yield Contributing Characters in Comparison With Local and European Genotypes of Barley (*Hordeum vulgare* L.)

3.1 Introduction

Barley is one of the important cereals among others, best-adapted and an oldest domesticated crop that grown in a wide eco-geographic range around the world (Usubaliev et al. 2013; Jakob et al. 2014; Haque and Islam, 2015). It ranks fourth with respect to area and production among cereals after wheat, rice and maize (USDA, 2014). It has a comparably stable yield in spite of climatic variation within the growing season. In this respect wheat and other small grain cereals cannot compete with barley (Goyal and Ahmed, 2012). It can produce a high yield under ideal cultivation, such as moderate rainfall (400-800 mm), well-drained, loamy soil, irrigation and with moderate temperature (15-30°C) regimes (Ullrich, 2011).

However, appropriate sowing time is very important for ensuring better growth performances in barley. It depends on weather, topography and harvesting time of the preceding crops (Alam et al. 2006). In Bangladesh, barley is a minor crop but it has the potential to become one of the important crops that can play an important role for food security of the country and in drainage of foreign currency. The yield and quality of barley seed is known to be influenced by several factors such as time of sowing, high yielding or disease resistance variety, water and nutrient management, harvesting time and other agronomic practices (Bonari and Macchia, 1980; Conry and Hegarty, 1992; Yesmin et al. 2014). Sowing time of barley is a major limiting factor in Bangladesh. Early November is usually dry, warm and rich in soil moisture but the temperature decreases sharply to the lowest level in early January when the crop is in the vegetative stage (Alam et al. 2007). On the contrary, late-planted barley experiences low temperature at seedling emergence and decreases physiological process and cause a greater yield decrease (Korovin and Mamaev, 1983). Later on high temperature reduces number of tillers, grain growth and overall crop productivity (Savin et al. 1997; Wallwork et al. 1998 a,b). Therefore, sowing at an appropriate

time is necessary for ensuring maximum yield and that is why sowing time needs to be adjusted it will germinates well and utilize the soil moisture stored in the soil profile effectively (Mollah and Paul, 2008). Yield and yield contributing attributes like plant height, number of tillers, number of spikelets, spike length, grain size and yield, and other yield attributes and quality of seeds differ from one variety to another (Ferdous et al. 2010; Paul et al. 2014; Yesmin et al. 2014). Therefore, the present was undertake to find out a suitable time of sowing for better performance on yield and yield attributes as well as seed quality of local and European barley genotypes under field condition.

3.2 Materials and Methods

As plant material six local and eight European barley genotypes were used for this study that are briefly described in the previous section of 3.1, Table 6 (General materials and methods).

3.2.1 Harvesting stages

Plants were grown and data were recorded from the experimental field of the Institute of Biological Sciences, University of Rajshahi, Bangladesh during the growing season of November, 2014 to April, 2015. Different stages of harvesting of local barley genotypes are shown in **Fig. 5.** The European barley genotypes are shown in **Fig. 2** in the previous section 2.1 (general materials and methods).

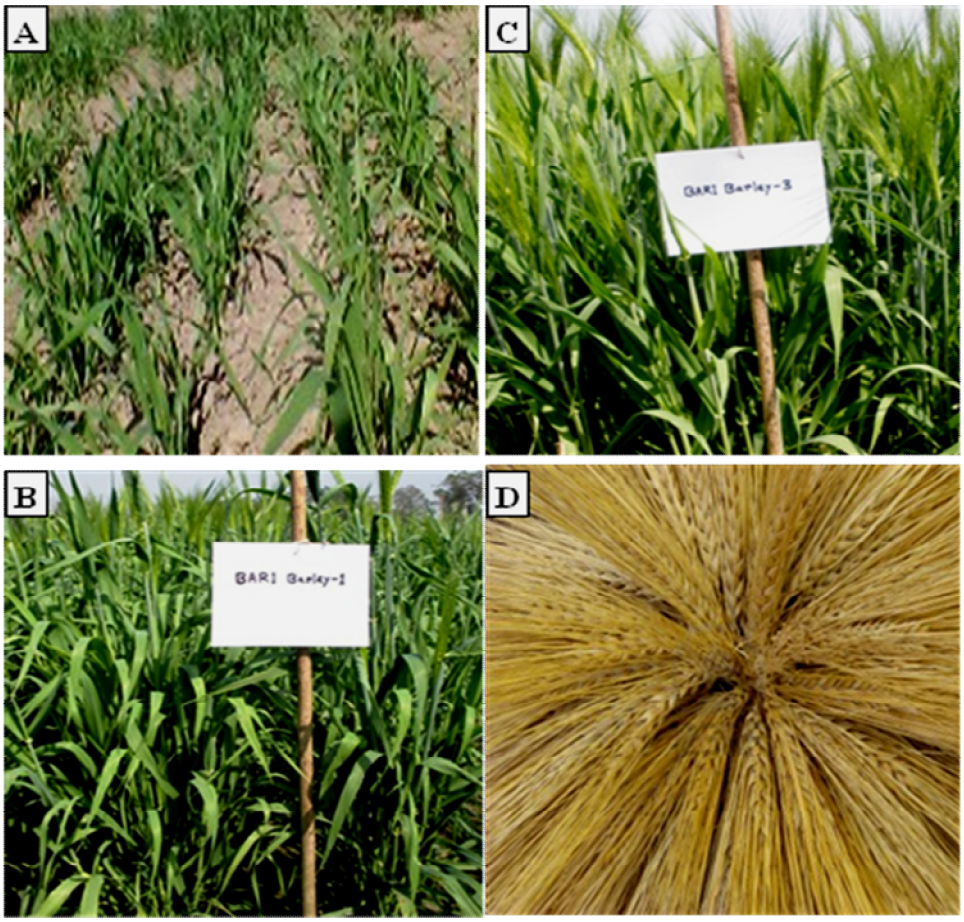

Fig. 5: Different harvesting stages of local barley genotypes: (A) early stage of growth, (B) booting stage, (C) pre- mature stage and (D) mature stage.

3.2.2 Climate conditions for growth of barley

Temperature were measured in the experimental field during advanced stages of crop developments and observed the variations of photoperiod in 2014-2015. During the growing season of crop, the average temperature was 21.6°C and average day length were recorded 10.38 hours that are suitable for barley production in Bangladesh (**Fig. 6)**.

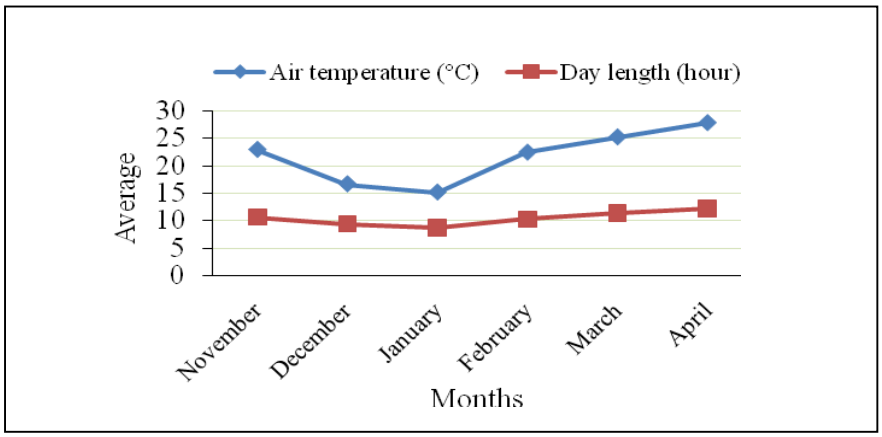

Fig. 6: Average temperature and day length from November 2014 to April 2015.

3.2.3 Field preparation and fertilizer uses

The experimental field was made ready for barley cultivation from mid October, 2014. All weeds and stubbles were removed from the field during the field preparation time. Chemical fertilizer such as urea (200 kg/ha), triple super phosphate (185 kg/ha) and potash (40 kg/ha) as well as cow dung (5000 kg/ha) were applied in the experimental field. Cow dung was applied at the beginning of land preparation. Two third of urea and entire quantity of TSP and MP were applied at the time of final land preparation. The rest one third of urea was applied after the first irrigation (25 days after sowing).

3.2.4 Experimental design and sowing time

The field was laid out in a randomized block design (RBD) with three replications. Each replication consisted of six plots (2.0 m long) with five rows. Seeds were sown in the field following three times of sowing in 2014 e.g S_1 = 1st November, S_2 = 21st November and S_3 = 11th December.

3.2.5 Observation of weeds

Different kinds of weeds were observed to infest the crop. Among them *Cyperus rotundus* (mutha) and *Chenopodium album* (bathua) were prominent in the field. The harmful weeds disposed from the experimental field.

3.2.6 Data recording and statistical analysis

Statistical analysis of the data was performed by SPSS software (version 16). Mean performance of the genotypes were tested by Duncan's multiple range tests (DMRT). Data were recorded on the basis of individual plant from 6 randomly selected lines of each genotype. Among the characters studied, days to booting, heading and maturity were recorded on the basis of row and height of plant from the field. Remaining data were recorded after harvesting. Parameters on data recording and the procedure are mentioned below:

(i) **Booting:** Booting time was recorded when at least 50% of plants from whole populations in the row showed boot formation.

(ii) **Maturity:** Observation of maturity was made when flag leaf, top internodes and spikes of 80% of the plants did not have any green tissue.

(iii) **Plant height:** Length of the main tiller at maturity was measured from the ground level to top of its terminal spikelet excluding owns.

(iv) **Spike length:** Length of the main spike without own was measured as average from the plants.

(v) **Spikelets per spike:** Total number of spikelets of the main spike of each plant was counted and average was taken.

(vi) **Grains per spike:** Total number of grains was counted from the main spike of each plant and then average was taken.

(vii) **1000 grain weight:** One thousand grains were counted randomly from each row and weighted in gram (g).

(viii) **Grain yield per plant:** Total grains per spike were weighted and averaged was taken in gram.

3.3 Results

3.3.1 Morphological traits

It was observed that the variation among the genotypes and sowing times were significantly higher for days to booting. The genotype BB-5 showed the earliest in booting (59.0 days) that sown on 11th December (S_3) and longest duration (82.0 days) was recorded in Hor-17016 that shown on 1st November (S_1), which was statistically different from the rest of the genotype. The range of means of this trait was 62.0-75.33 days. The coefficient of variation of BB-5 was 4.83% and 8.63% for the genotype Hor-17016; indicating the variation in booting (**Table 8**).

Table 8: Influence of different sowing times on booting in barley genotypes

Genotypes	Days to booting			Mean ± SE	CV%
	S_1	S_2	S_3		
BB-1	71	67	64	67.33 ± 2.02	5.21
BB-2	72	68	64	68.0 ± 2.30	5.88
BB-3	68	65	61	65.0 ± 2.08	5.54
BB-4	70	67	62	66.33 ± 2.33	6.09
BB-5	65	62	59	62.0 ± 1.73	4.83
BB-6	67	63	61	63.67 ± 2.02	5.51
Hor-291	75	71	66	70.67 ± 2.60	6.38
Hor-8903	76	71	65	70.67 ± 3.17	7.79
Hor-9465	80	73	70	74.33 ± 2.96	6.90
Hor-9580	79	74	69	74.00 ± 2.88	6.75
Hor-10874	77	73	67	72.33 ± 2.90	6.95
Hor-17016	82	75	69	75.33 ± 3.75	8.63
Hor-17277	81	74	70	75.00 ± 3.21	7.42
Hor-17287	80	75	69	74.67 ± 3.17	7.37

S_1 = 1st November, S_2 = 21st November, S_3 = 11th December

ANOVA showed that days to maturity were highly significant among the genotypes (**Table 10**). Minimum number of days to maturity (101days) was observed in S_3 (BB-5) and maximum 129 days in S_1 (Hor-9580). It was observed that out of nine four European barley genotypes (Hor-8903, Hor-17016, Hor-17277 and Hor-17287) didn't showed booting (**Table 9**). It was found that means of maturity ranged was 108 - 123.33 days and coefficient of variance ranged was 4.72 - 7.26%.

Table 9: Influence of different sowing times on maturity in barley genotypes

Genotypes	Days to maturity			Mean ± SE	CV%
	S_1	S_2	S_3		
BB-1	118	111	102	110.33 ± 4.63	7.26
BB-2	120	112	105	112.33 ± 4.33	6.68
BB-3	119	109	104	110.67 ± 4.40	6.90
BB-4	121	115	107	114.33 ± 4.05	6.14
BB-5	116	107	101	108.00 ± 4.35	6.99
BB-6	118	108	103	109.67 ± 4.40	6.96
Hor-291	128	126	112	122.00 ± 5.03	7.14
Hor-8903	-	-	-	-	-
Hor-9465	123	119	112	118.00 ± 3.21	4.72
Hor-9580	129	125	116	123.33 ± 3.84	5.39
Hor-10874	126	123	113	120.67 ± 3.92	5.64
Hor-17016	-	-	-	-	-
Hor-17277	-	-	-	-	-
Hor-17287	-	-	-	-	-

- Indicating no maturity

Table 10: Analysis of variance (ANOVA) subjected to morphological traits in barley genotypes

Subject of ANOVA (dependent variable)	Source	Sum of Squares	df	Mean Square	F. value
Days to booting	Genotype (G)	820.11	13	63.08	48.92**
	Sowing time (ST)	585.14	2	292.57	226.90**
	G × ST	33.52	26	1.28	
Days to maturity	Genotype (G)	855.2	9	95.02	30.87**
	Sowing time (ST)	1027.2	2	513.63	166.88**
	G × ST	55.40	18	3.07	

** = Significant at P <0.01, * = Significant at P <0.05, NS = Non-significant

3.3.2 Yield and yield contributing traits

It was observed that the mean performances for grain yield and different yield contributing characters were significantly varied among the genotypes and sowing times (**Table 11**). Among the genotype, Hor-9465 showed best plant height (117.0 cm), tillers per plant is 49.63 and fertile tillers 37.43, spike length (14.33 cm), spikelets per spike (15.67), grains per spike (89.0) and grain yield per plant (7.75 g). BARI barley-1 showed better weight of grain (34.10 g) than other genotypes. Most yield contributing traits were found in Hor-9465, BB-5, Hor-291, Hor-9580 and BB-6 promising with good yield potentials compared to other studied genotypes. Analysis of variance showed significant results on yield and yield components for Hor-9465, BB-5, Hor-291, Hor-9580 and BB-6 genotypes which indicating existence of real difference among the other genotypes as well as sowing times (**Table 12**).

Table 11: Influence of sowing times on yield and yield contributing traits in barley

Traits	Sowing times	Genotypes									
		BB 1	BB 2	BB 3	BB 4	BB 5	BB 6	Hor 291	Hor 9465	Hor 9580	Hor 10874
Plant height (cm)	S_1	101	100	98	99	102	101	110	114	111	109
	S_2	112	111	109	110	119	117	121	126	124	123
	S_3	100	99	93	94	105	104	107	111	109	108
	Mean	104.3	103.3	100.0	101.0	108.6	107.3	112.6	117.0	114.6	113.3
	± SE	± 3.8	± 3.8	± 4.7	± 4.7	± 5.2	± 4.9	± 4.2	± 4.5	± 4.7	± 4.8
Total tillers/ plant	S_1	8.1	6.9	9.6	6.5	8.3	33.8	41.8	50.3	40.7	37.4
	S_2	9.6	8.8	11.2	7.9	10.5	36.3	49..3	62.7	51.2	53.6
	S_3	6.3	5.7	8.4	5.6	6.8	21.5	32.4	35.9	26.1	28.3
	Mean	8.00	7.13	9.73	6.67	8.53	30.53	41.17	49.63	39.33	39.77
	± SE	± 0.9	± 0.9	± 0.8	± 0.6	± 1.0	± 4.5	± 4.8	± 7.7	± 7.2	± 7.3
Fertile tillers/ plant	S_1	5.3	5.8	8.2	5.2	7.1	24.1	32.5	38.6	33.4	21.9
	S_2	6.2	7.5	9.6	6.4	8.6	27.3	33.6	45.4	36.6	35.1
	S_3	4.8	4.6	7.1	4.3	5.9	15.8	24.1	28.3	21.6	23.7
	Mean	5.43	5.97	8.30	5.30	7.20	22.4	30.07	37.43	30.53	26.90
	± SE	± 0.4	± 0.8	± 0.7	± 0.6	± 0.7	± 3.4	± 3.0	± 4.9	± 4.5	± 4.1
Spike length (cm)	S_1	10.6	10.1	9.1	11.6	12.4	11.5	12.5	14.1	10.9	11.3
	S_2	12.3	11.8	10.3	12.5	14.9	13.2	14.2	15.3	12.6	13.5
	S_3	9.7	9.2	8.6	10.2	11.6	10.9	11.2	13.6	9.2	10.4
	Mean	10.87	10.37	9.33	11.43	12.97	11.87	12.63	14.33	10.90	11.73
	± SE	± 0.7	± 0.7	± 0.5	± 0.6	± 0.9	± 0.6	± 0.8	± 0.5	± 0.9	± 0.9
Spikelet/ Spike	S_1	12.1	11.5	10.8	12.4	13.5	14.6	13.7	15.8	12.2	13.6
	S_2	13.4	12.7	11.6	13.6	14.2	15.8	14.5	16.6	13.5	14.7
	S_3	11.5	10.2	9.6	10.7	12.4	13.1	12.3	14.6	11.4	12.3
	Mean	12.33	11.47	10.67	12.23	13.37	14.50	13.50	15.67	12.37	13.53
	± SE	± 0.5	± 0.7	± 0.5	± 0.8	± 0.5	± 0.7	± 0.6	± 0.5	± 0.6	± 0.7
Grains/ Spike	S_1	85	81	77	75	82	78	81	87	74	76
	S_2	92	97	82	89	105	101	100	108	97	99
	S_3	71	74	70	69	78	72	77	72	68	69
	Mean	82.67	84.00	76.33	77.67	88.33	83.67	86.00	89.00	79.67	81.33
	± SE	± 6.1	± 6.8	± 3.4	± 5.9	± 8.4	± 8.8	± 7.1	± 10	± 8.8	± 9.1
1000 grain wt. (g)	S_1	33.3	31.8	30.2	29.2	33.5	31.1	28.5	30.6	27.4	26.9
	S_2	39.2	38.5	36.6	33.4	38.6	34.3	33.6	35.4	32.6	31.1
	S_3	29.8	28.6	27.1	24.3	29.9	26.8	24.1	28.3	21.6	23.7
	Mean	34.10	32.97	31.30	28.97	34.00	30.73	28.73	31.43	27.20	27.23
	± SE	± 2.7	± 2.9	± 2.8	± 2.6	± 2.5	± 2.1	± 2.7	± 2.1	± 3.1	± 2.1
Grain yield (g) /plant	S_1	4.81	4.76	4.62	4.16	5.8	5.21	5.45	7.65	6.19	4.36
	S_2	5.79	5.45	5.21	4.59	6.06	5.94	6.19	9.28	8.22	5.54
	S_3	4.28	4.56	3.95	3.88	4.9	4. 86	4.82	6.32	5.6	3.71
	Mean	4.96	4.92	4.59	4.21	5.58	5.34	5.48	7.75	6.67	4.53
	± SE	± 0.4	± 0.2	± 0.3	± 0.2	± 0.3	± 0.3	± 0.4	± 0.8	± 0.8	± 0.5

S_1 = 1st November, S_2 = 21st November, S_3 = 11th December (2014).

Table 12: Analysis of variance (ANOVA) for yield and yield contributing traits

Subjects	Sources	Df	Sum of Squares	Mean of square	F. value
Plant height (cm)	ST	2	972.70	108.07	42.85**
	G	9	1217.26	608.63	241.30**
	Error	18	45.40	2.52	
Total tillers/plant	ST	2	8285.41	920.60	31.40**
	G	9	771.30	385.65	13.158**
	Error	18	527.57	29.31	
Fertile tillers/plant	ST	2	4360.90	484.54	38.98**
	G	9	290.54	145.27	11.68**
	Error	18	223.74	12.43	
Spike length (cm)	ST	2	54.57	6.06	48.22**
	G	9	34.61	17.30	137.65**
	Error	18	2.26	0.12	
Spikelet/spike	ST	2	57.73	6.41	132.32**
	G	9	25.36	12.68	261.55**
	Error	18	0.87	0.04	
Grains/spike	ST	2	484.80	53.86	3.07 ^{NS}
	G	9	3285.06	1642.53	93.68**
	Error	18	315.60	17.53	
1000 grain wt. (g)	ST	2	178.84	19.87	32.00**
	G	9	399.54	199.77	321.77**
	Error	18	11.17	0.62	
Grain yield (g)/plant	ST	2	31.24	3.47	22.01**
	G	9	12.00	6.00	38.06**
	Error	18	2.83	0.15	

Showing time = ST, Genotype = G, NS = non significant, ** = significant at 1% level of probability respectively.

3.4 Discussion

Plant height is an important morphological character that directly linked with grain yield (Alam et al. 2007). It was observed that plant height significantly affects due to delay of sowing time. Yesmin et al. (2014) and Kozlowska- Ptaszynska (1993) observed that similar results in barley. In this case found that Hor-9465 showed long heighted plant (117.0 cm) than Hor-9580 (114.6 cm), Hor-10874 (113.3 cm), Hor-291 (112.6 cm) and BB-5 (108.6 cm) in comparison with local and European studied genotypes.

It was observed that late sowing reduces number of tillers. Among the genotypes, Hor-9465 produced the highest number (49.63) and fertile tillers (37.43) per plant. These results corroborated the findings of Petr et al. (1979), Makki and Habib (1979), Noworolnik and Leszczynska (1997), Chun et al. (2000) and Alam et al. (2007). Late sowing reduces the spike length also which agreed with the findings of Abdel-Raouf et al. (1983) and Alam et al. (2007). Alam et al. (2007) reported that reduction of panicle length at the later sowing dates might be due to low temperature at the vegetative stage and scarce soil moisture as well. The longest spike was found in Hor-9465 (14.33 cm) followed by BB-5 (12.97 cm) and Hor-291(12.63 cm). Hor-9465, BB-6 and Hor-10874 exhibited higher spikelet per spike (15.67) compared to other genotypes.

Grain weight is an important yield component as declared by Petr et al. (1979). Hor-9465 produced higher number of grains (89.0) per spike followed by BB-5 (88.33) and Hor-291 (86.0). Higher grain weight (34.10 g) was recorded in BB-1 than other genotypes BB-5 (34.0), BB-2 (32.97) and Hor-9465 (31.43). The genotype of Hor-9465 showed best performance on grain yield (7.75). It was observed that per plant Hor-9580 (6.67), BB-5 (5.58), Hor-291(5.48) and BB-6 (5.34) showed high yielding than others. The time of sowing point S_2 produced higher grain than S_1 and S_3. In this case found that late sowing reduced grain yield and similar results were found in barley by Kozlowska-Ptaszynska (1993), Ekeberg (1994), Noworolnik & Leszczynska (1997) and Alam et al. (2007). Ekeberg (1994) reported that, on average, cereal grain yield decreased by 21 kg/ha with delay of sowing. Nass et al. (1975) reported that yield reduction for late sowing due to shorter growing period in the vegetative phase and steep rise in temperature at the grain filling stage. High temperatures speed up spike development from floral initiation to anthesis, and also have been found to reduce the number of spikelets formed per spike (Dawson and Wardlaw, 1989). Frank et al. (1992) reported that the number of fertile spikelet decreased significantly in barley as temperature increased from 16℃ to 18°C. Miralles et al. (2000) reported that increased duration of stem elongation period in wheat and barley increased floret fertility and grain number. On the other hand,

shorter photoperiods reduced the rate of floret development. Green et al. (1985) found linear decrease in maximum grain yield due to delayed sowing. They also found that early sowing induced faster production of tillers and consequently gave higher maximum tillers and tended to accumulate dry matter at a faster rate and provided the highest grain yield led by optimum temperature during the growth period. High temperature at the later stages of growth also reduced grain yield affects on growth (particularly in grain filling period) reduced kernel weight, which resulted lower grain yield. Similar results were reported by Alam et al. (2007), Wallwork et al. (1998 a,b) and Savin and Nicolas (1999).

Chapter IV

Somatic Embryogenesis

4. Somatic Embryogenesis (Mature and Immature Embryos)

4.1 *In vitro* Screening of Some Efficient Barley Genotypes Through Somatic Embryogenesis in Bangladesh

4.1.1 Introduction

Somatic embryogenesis is a development process of cells, which resembles morphologically zygotic embryogenesis. It is an important pathway for regeneration of plants from cell culture system and a method commonly used in a large scale production of plants and synthetic seeds. In most of the important crops, tissue culture is well established for plant regeneration via somatic embryogenesis (Mamun et al. 2002; Neto et al. 2003; De Silva et al. 2009; Paul et al. 2013; Morshed et al. 2014; Rahman et al. 2015). Many workers have emphasized somatic embryogenesis as a preferred method for genetic improvement and multiplication of valuable germplasm of a number of woody perennials (Gupta and Durzan, 1987; Bhansali, 1990; Islam and Bhattacharjee, 2015). Since somatic embryo cultures often originate from a single cell, it is an ideal system for induction of mutations as it helps in preventing chimeras. The rate of somatic embryo germination is very poor, which has become major hurdle for large-scale plant multiplication of desirable induced mutants (Dahleen and Bregitzer, 2002; Lazaridou et al. 2011). The multiplication of true type plants through somatic embryogenesis will help in propagating elite and new genotypes in shorter periods of time.

Maximum barley cultivars are often considered as less responsive to tissue culture due to poor callus induction, low frequency of embryogenesis and lesser percentage of plant regeneration (Chauhan and Kothari, 2004). For barley plant regeneration has been highly genotype dependent reported by Castillo et al. (1998) and Han et al. (2011). Therefore, screening for highly responsive *in vitro* genotypes is very important for advance biotechnological work in barley. The process of somatic embryogenesis is not only important for the production of plants and secondary products, but also for the transgenic plants and somatic cell genetics. It plays an

important role in clonal propagation also. When integrated with conventional breeding programs and molecular and cell biological techniques, somatic embryogenesis provides a valuable tool to enhance the pace of genetic improvement of commercial crop species (Stasolla and Yeung, 2003). The present investigation was undertaken to improve somatic embryogenesis as well as screening of suitable barley genotypes in Bangladesh using mature embryos.

4.1.2 Plant materials, sterilization and inoculation

Mature and dry seeds of six barley genotypes namely BB-1, BB-2, BB-3, BB-4, BB-5 and BB-6 were used for this study. Sterilization procedures are described in the section of **2.3.1.1** (general materials and methods)**.** Sterilized seeds were kept on filter paper for excess water soaking and approximately 8 -10 seeds were inoculated for each petri dish.

4.1.3 Callus induction

For primary callus induction, MS medium used that supplemented with different concentrations and combination of 2,4-D (2,4 dichlorophenoxyacetic acid), BAP (benzyl aminopurine), L-proline, casein hydrolysate and 3% sucrose (w/v) are shown in **Table 13**. Under this study two barley genotypes of BB-1 and BB-6 and twelve (12) media combination were used. The culture petri dishes were incubated in the dark at 25±2°C. After four weeks of culture initiation data were recorded on the basis of number of mature embryos induction.

$$\text{Percentage of callus formation} = \frac{\text{Mature embryos formed callus}}{\text{Total number of embryos cultured}} \times 100$$

Friable and compact calli were assumed as potentially embryogenic (considered as effective callus) and were selected for maintenance and regeneration.

Table 13: Different combinations of media for callus induction

Medium	2,4-D (mg/l)	BAP (mg/l)	L-Proline (mg/l	Casein hydrolysate (mg/l)
MS + CIM_1	1.0	-	100	150
MS + CIM_2	1.0	-	200	300
MS + CIM_3	1.0	-	300	450
MS + CIM_4	2.5	0.1	100	150
MS + CIM_5	2.5	0.1	200	300
MS + CIM_6	2.5	0.1	300	450
MS + CIM_7	4.0	-	100	150
MS + CIM_8	4.0	-	200	300
MS + CIM_9	4.0	-	300	450
MS + CIM_{10}	5.5	0.2	100	150
MS + CIM_{11}	5.5	0.2	200	300
MS + CIM_{12}	5.5	0.2	300	450

4.1.4 Embryogenic callus formation

After 4 weeks of culture, primary callus was separated from explants and transferred them to CIM_4 (MS + 2.5 mg/l 2,4-D + 0.1 mg/l BAP + 100 mg/l L-proline + 150 mg/l casein). Data were recorded after 3 weeks of incubation and percentage of embryogenic calli (EC) were evaluated.

4.1.5 Plant regeneration

Under this study nine different concentration and combinations of MS media *viz.* RM_1 (MS + 0.5 mg/l BAP + 20 g/l sucrose), RM_2 (MS + 1.0 mg/l BAP + 20 g/l sucrose), RM_3 (MS + 1.5 mg/l BAP + 20 g/l sucrose), RM_4 (MS + 2.0 mg/l BAP + 20 g/l sucrose), RM_5 (MS + 0.5 mg/l BAP + 30 g/l sucrose), RM_6 (MS + 1.0 mg/l BAP + 30 g/l sucrose), RM_7 (MS + 1.5 mg/l BAP + 30 g/l sucrose), RM_8 (MS + 2.0 mg/l BAP + 30 g/l sucrose) and RM_9 (MS + 1.5 mg/l BAP + 40 g/l sucrose) were used and evaluted their regeneration efficiency. In all cases, 2-3% (w/v) sucrose was used as carbon sources. Cultured were maintained at 25°C with 16/8h (light/dark) and

regeneration frequency was evaluated after 4 weeks of incubation in regeneration medium. Regenerated shoots were transferred to half-strength of MS medium that supplemented with 20 g/l sucrose and 2.0 mg/l NAA for rooting. The well-rooted plants were transferred to pots and acclimatized according the section of 2.3.1.5 (general materials and methods).

4.1.6 Data recording and statistical analysis

Each treatment contained three replications and the whole experiment was repeated three times. Statistical analysis was performed using SPSS software (version 16). Data were evaluated on the basis of primary callus induction and regeneration among the studied genotypes was evaluated by one-way analysis of variance (ANOVA). The significance of differences and comparisons between the mean values were determined by least significant difference (LSD) formulation at 5% level.

4.1.7 Results

4.1.7.1 Optimization of media

For primary callus induction two barley genotypes of BB-1 and BB-6 were cultured and evaluated their efficiency using twelve different combinations of MS medium. The induction frequency of primary callus ranged was recorded from 4.72% to 38.17% (**Table 14**). Among the mentioned formulations CIM_8 (MS + 4.0 mg/l 2, 4-D +200 mg/l L-proline + 300 mg/l casein) showed the best performance for callus induction for both genotypes (24.04% in BB-1 and 38.17% in BB-6). While the lowest (4.72% in BB-1 and 6.94% in BB-6) callus was observed in CIM_{12} (MS +5.5 mg/l 2, 4-D + 0.2 mg/l BAP + 300 mg/l proline + 450 mg/l casein) medium. Analysis of variance showed significantly higher results between the genotypes and culture combinations (**Table 16**). From these findings it may be concluded that the media combination of CIM_8 is the best for the primary callus induction using mature embryos in barley.

Table 14: Effects of different media combinations on callus induction from mature embryos in BB-1 and BB-6

Medium	No. of inoculated seeds	% of callus induction (Mean ± SE)	
		BB-1	BB-6
CIM_1	180	5.55 ± 0.55^{ab}	8.33 ± 0.96^{ab}
CIM_2	360	12.22 ± 1.0^{d}	16.38 ± 0.73^{cd}
CIM_3	360	10.83 ± 0.48^{cd}	13.61 ± 1.21^{c}
CIM_4	360	8.61 ± 0.27^{bc}	10.27 ± 1.21^{b}
CIM_5	360	9.72 ± 0.73^{c}	12.50 ± 0.96^{bc}
CIM_6	450	7.33 ± 0.38^{b}	9.55 ± 1.35^{b}
CIM_7	450	18.66 ± 2.03^{e}	25.77 ± 1.55^{d}
CIM_8	495	24.04 ± 1.06^{f}	38.17 ± 2.09^{e}
CIM_9	450	16.22 ± 1.55^{e}	23.55 ± 1.93^{d}
CIM_{10}	360	6.66 ± 0.48^{b}	10.00 ± 0.48^{b}
CIM_{11}	360	7.50 ± 0.96^{b}	11.11 ± 1.21^{b}
CIM_{12}	360	4.72 ± 0.55^{a}	6.94 ± 0.73^{a}

Different superscripts of mean values in the same column indicate significant differences within culture combinations (LSD test, $p < 0.005$).

4.1.7.2 Genotypic difference in primary callus induction

To detect the genotypic difference, mature embryos of six barley genotypes were inoculated in CIM_8 medium (MS + 200 mg/l L-proline + 300 mg/l casein + 4.0 mg/l 2,4-D). The results indicated that BB-6 showed highest (38.17%) percentage of callus followed by BB-3 (30.11%) and BB-1 (24.04%). Whereas, BB-2 showed very low (7.65%) and BB-4 and BB-5 showed no callus induction (**Fig. 7**). The compact and friable calli were transferred to regeneration medium and evaluated its regeneration efficiency (**Fig. 8**).

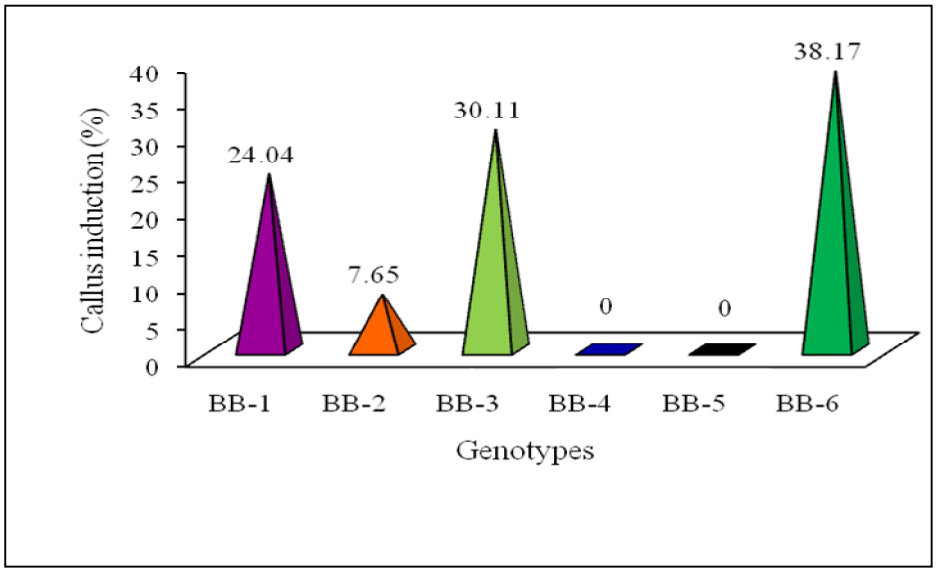

Fig. 7: Frequencies of primary callus induction responses of mature embryos in six barley genotypes.

4.1.7.3 Embryogenic callus

MS medium (CIM_4) containing 2.5 mg 2,4-D, 0.1 mg BAP, 100 mg/l L-proline and 150 mg/l casein hydrolysate was found to be efficient for embryogenic callus induction. The percentage of embryogenic callus was 27.49%, 20.66% and 14.89% in BB-6, BB-3 and BB-1, respectively (**Fig. 9**). The genotype of BB-6 showed the highest frequency of embryogenic callus compared with BB-3 and BB-1. In this case nodular and heart-shaped embryogenic calli gave better green plants than others (**Fig. 8**).

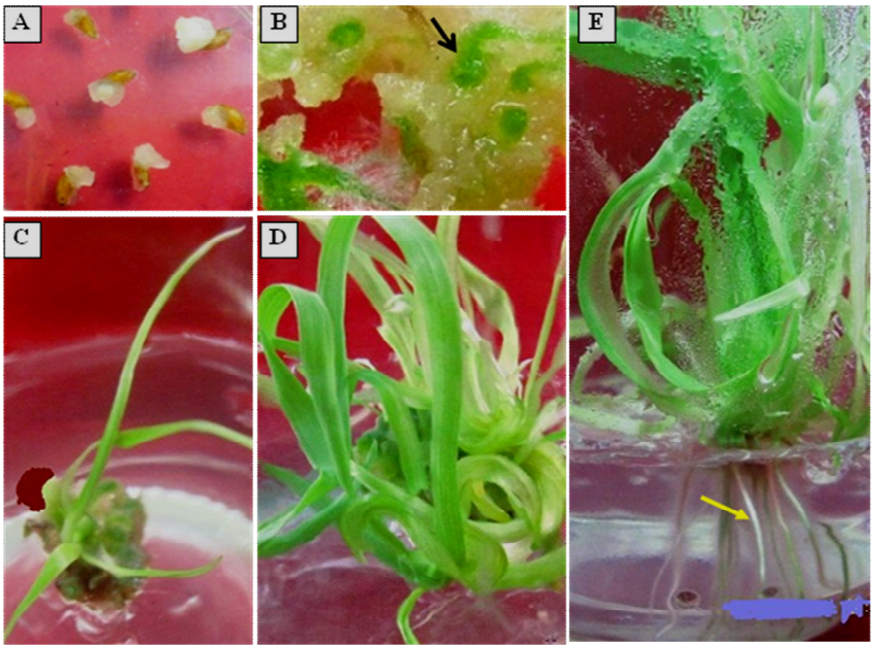

Fig. 8: Somatic embryogenesis and plant regeneration in barley; (A) Callus derived from seeds after 1 week of culture initiation, (B) Embryogenic callus after 5 weeks, (C) Initiation of shoots after 7 weeks of culture, (D) Shoot developments after 9 weeks of culture, (E) Regenerated plants with good roots and shoots after 11 weeks of culture.

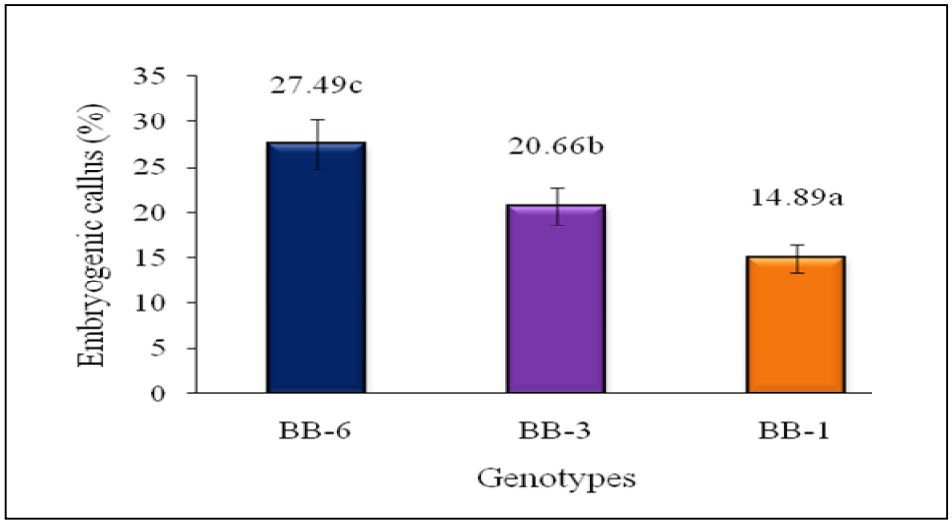

Fig. 9: Frequencies of embryogenic calli derived from mature embryos of three barley genotypes.

4.1.7.4 Plant regeneration

The media formulation of RM_7 (MS + 1.5 mg/l BAP + 30 g/l sucrose) showed significantly better results for regeneration than others. The highest regeneration percentages were recorded for BB-6 (19.25%), BB-3 (13.33%) and BB-1 (9.72%) in RM_7 mdium (**Table 15**). On the other hand, RM_1 (MS + 0.5 mg/l BAP + 20 g/l sucrose) showed lowest regeneration (4.48% for BB-6 and 3.80% for BB-3). The RM_7 proved to be the most effective medium for regeneration compared with others. It was observed that the genotype BB-6 showed good regeneration (9.26%) in terms of average number of plants per somatic embryos. Analysis of variance (ANOVA) showed highly significant differences among the media and the genotypes (**Table 16**). Regenerated plants were transferred to rooting medium. Then after acclimatization well-rooted plants were transferred to the soil (**Fig. 8**).

Table 15: Effect of nine different RM medium to evaluate their regeneration efficiency using three barley genotypes

Genotypes	Medium	Embryogenic calli	Regenerated calli	% of regeneration (Mean ± SE)
BB-6	RM_1	156	7	4.48 ± 0.64a
	RM_2	135	13	9.62 ± 1.95bc
	RM_3	135	14	10.37 ± 1.48c
	RM_4	135	10	7.40 ± 1.95b
	RM_5	165	10	6.06 ± 1.60b
	RM_6	126	17	13.49 ± 2.09d
	RM_7	135	26	19.25 ± 1.95e
	RM_8	120	9	7.5 ± 1.44b
	RM_9	135	7	5.18 ± 0.74ab
BB-3	RM_1	105	4	3.80 ± 0.95a
	RM_2	75	5	6.66 ± 1.33b
	RM_3	84	7	8.33 ± 1.19bc
	RM_4	96	5	5.20 ± 1.04ab
	RM_5	99	4	4.04 ± 1.01a
	RM_6	78	8	10.25 ± 1.28c
	RM_7	60	8	13.33 ± 1.66d
	RM_8	66	4	6.06 ± 1.51ab
	RM_9	102	5	4.90 ± 0.98ab
BB-1	RM_1	24	0	0
	RM_2	66	2	3.03 ± 1.51ab
	RM_3	84	4	4.76 ± 1.19b
	RM_4	60	2	3.33 ± 1.66ab
	RM_5	78	2	2.56 ± 1.28ab
	RM_6	61	4	6.34 ± 1.58bc
	RM_7	72	7	9.72 ± 1.38c
	RM_8	102	4	3.92 ± 0.98ab
	RM_9	126	2	1.58 ± 0.79a

Different letters of mean values in the same column indicate significant differences within culture combinations (LSD test, $p < 0.005$).

Table 16: Analysis of variance (ANOVA) subjected to primary callus induction for two genotypes and plant regeneration for three barley genotypes

Variable	Source of variation	df	Mean sum of square	F. value
Callus Induction	Genotype	1	113.13	18.14**
	Culture combination	11	122.04	19.57**
	Genotype × Culture combination	11	6.23	
Plant regeneration	Genotype	2	64.69	49.02**
	Media formulation	8	37.28	28.26**
	Genotype × Media formulation	16	1.31	

** = significant at 1% level of probability.

4.1.8 Discussion

Plants regeneration is very essential for establishing a successful tissue culture system. In case of tissue culture for all crops do not show regeneration easily. Sometimes it is difficult to culture and regenerate agronomically important crops (Puhan and Siddiq, 2013). Somatic embryogenesis is a multi-step regeneration process starting with formation of pro-embryogenic masses, followed by somatic embryo formation, maturation and plant regeneration (Arnold et al. 2002; Sharmin et al. 2014). The difference in the composition of culture medium and the concentrations of hormones affect the callus induction and regeneration ability of barley and other plant genotypes (Haque and Islam, 2014; Tariq et al. 2008). As auxin 2,4-D is very important and required for the production of somatic embryogenesis in cereal crops (Nasircilar et al. 2006; Armstrong et al. 1987). Somatic embryos are formed on nutrient medium with a reduced 2,4-D concentration is reported by Delporte et al. (2001). Furthermore, genotype variation also plays a vital role in callus initiation, proliferation and even regeneration in barley (Gubišová et al. 2012) and rice (Khanna and Raina, 1998).

Twelve different concentration and combinations of PGRs were used with MS medium and found that significant differences between media components. It was observed that 4.0 mg/l 2,4-D + 200 mg/l L-proline and 300 mg/l casein hydrolysate promoted callus induction in barley. However, increasing amount of 2,4-D from 1 mg/l to 4.0 mg/l showed significantly highest percentages of callus induction. The culture combination of CIM_8 contained MS + 4.0 mg/l 2, 4-D +200 mg/l L-proline + 300 mg/l casein hydrolysate gave best primary callus induction when used mature embryos as explants. Chernobrovkina et al. (2004) studied with addition of L-proline (160 mg/l) showed negative impact on the *in vitro* embryo culture in barley. Aguado-Santacruz et al. (2011) demonstrated that 2 mg/l of 2,4-D showed better callus induction. Whereas, in addition of amino acid with higher concentrations (230 mg/l) did not show any callus induction.

Till several protocols for *in vitro* callus culture of barley and other crops have been successfully developed by Lupotto (1984), Bregitzer (1992), Castillo et al. (2000), Chauhan and Kothari (2004), Asakavičiūtė and Pašakinskienė (2006), Gubišová et al. (2012), Haque and Islam (2014), Mrízová et al. (2014), Haque and Islam (2015). However, only a few barley genotypes have been identified that possesses good regeneration capacity (Lemaux et al. 1999; Aguado-Santacruz et al. 2011). Suitable barley response to *in vitro* culture with highest regeneration potential and correct explants as well as their proper developmental stage to be used as genetic transformation (Chang et al. 2003; Kasha, 2007).

It was reported that mature (Sharma et al. 2005; Yadav et al. 2011) and immature embryos (Walmsley et al. 1995; Haque and Islam, 2015) were suitable explants for somatic embryogenesis in barley and other cereal crops. Under this study, successfully induced callus from mature embryos of some barley genotypes using MS medium. The medium was supplemented with different concentrations of 2,4-D, L-proline and casein hydrolysate. The results obtained in this study are quite similar with the report of Ganeshan et al. (2003), who successfully induced callus from mature embryo of barley. They used similar components in the medium (2,4-D, L-proline and casein hydrolysate) but the concentration was different with the present findings.

Various concentration and combination of auxins, cytokinins play an important role for embryogenic callus induction in barley reported by Serhantova et al. (2004). The present results showed that MS + 2.5 mg/l 2,4-D + 0.1 mg/l BAP + 100 mg/l L-proline + 150 mg/l casein hydrolysate was adequate for embryogenic callus formation. Walmsley et al. (1995) stated that 2.0 mg/l of 2,4-D was suitable for initiation of embryogenic callus in barley. Bregitzer et al. (1998) reported that the formation of embryogenic callus in various barley genotypes was depended on 2,4-D concentration, and 2-3 mg/l was adequate in most of the cases. Amali et al. (2014) demonstrated that the addition of L-proline considerably improved the somatic embryo formation in MS medium containing 2.5 mg/l 2,4-D and 500 mg/l casein hydrolysate in sorghum. Casein hydrolysate can be used as a relatively cheap source of a mixture of amino acids (Slater et al. 2003). In addition of amino acids in the medium serve as a source of reduced nitrogen required for plant metabolism and growth. The present study revealed that the addition of casein hydrolysate to the medium enhanced embryogenic callus formation in barley.

In this study plant regeneration efficiency were tested and for that nine regeneration media were used that contained MS basal medium with different concentrations of BAP and sucrose. The present results demonstrated that regeneration formulation of RM_7 (MS + 1.5 mg/l BAP + 30 g/l sucrose) functioned better than others. This result agreed well with previous works where the MS medium and BAP were successfully used in barley (Aguado-Santacruz et al. 2011). There are some reports about the effect of sucrose was scrutinized on induction of plant (Shah et al. 2014). A similar type of result was found by Lee et al. (2012). They reported that sucrose has been commonly used at the concentration of 20 and 30 g/l as a carbon source in tissue culture medium. But they have not found any combined effect of different concentrations of BAP and sucrose for regeneration purpose. In this case BB-6 showed particularly high stability in callus induction over the different culture combination and higher regeneration compared to BB-3 and BB-1. However, this results proven that embryogenic callus formation and plant regeneration ability are depends using a suitable genotype.

4.2 Effect of Salt and Heat Pretreatment Factors to Enhance *In vitro* Regeneration Efficiency in Barley

4.2.1 Introduction

Plant tissue culture technologies have the prerequisite of efficient and reproducible protocol for regeneration. Efficient plant regeneration in barley has been reported to be highly genotype dependent and limited to model cultivars (Castillo et al. 1998; Haque and Islam, 2014). In the past few decades there has been a renewed interest in food uses of barley due to its nutritional advantages. Now world agriculture is facing a lot of challenges like producing 70% more food for an additional 2.3 billion people by 2050 (FAO 2009). Plant tissue culture techniques provide a promising and feasible approach to develop salt tolerant plants. In spite of many abiotic stresses are played negative role on cereal production (Islam and Tuteja, 2013). However, the productivity of crops is not increasing in parallel with the food demand.

Till there are many reports using different explants such as immature embryos (Chang et al. 2003), immature inflorescence (Havrlentova et al. 2001), coleoptile (Sahrawat and Chand, 2004), mature embryo (Abumhadi et al. 2005; He and Jia, 2008) and seedling explants (Sharma et al. 2004) for callus induction and plant regeneration in barley. Suitable plant regeneration based on mature embryos in other cereal crops such as rice (Kyungsoon et al. 2002), maize (Huang and Wei, 2004; Morshed et al. 2014) and wheat (Zale et al. 2004; Saha et al. 2015) has been reported also. Akula et al. (1999) have reported regeneration response of mature embryos in barley with low regeneration frequencies. Recent reports on plant regeneration from mature embryos have re-evaluated the scope of their use as explants in barley tissue culture (Gurel et al. 2009). Mature embryos are readily available throughout the year and their physiological state is less variable and using mature embryos reduces requirement of greenhouse making their *in vitro* use as more cost-effective.

In recent years tissue culture techniques are using by plant scientist as a useful tool to elucidate the mechanism involved in salt tolerance. Using Bangladeshi barley genotypes till there are no report on successful regeneration using salts and evaluation of their stress tolerance level. Therefore, the present study has been undertaken to

determine the effect of heat treatment and by NaCl to enhance somatic embryogenesis and their subsequent regeneration using three barley genotypes.

4.2.2 Plant materials, sterilization and inoculation

For this study three barley genotypes of BB-3, BB-6 and BHL-18 were considered and the process of brief sterilization procedure has been described in the previous section of **2.3.1.1**. In this case mature seeds were cultured for callus induction on MS medium supplemented with 4.0 mg/l 2,4-D, 200 mg/l L-Proline, 300 mg/l casein hydrolysate and 30 g/l sucrose. The pH was adjusted 5.8 before adding 6 g/l agar. Inoculated petri dishes were sealed with paraflim and incubated at 25 ± 2ºC in dark for 2-3 weeks.

4.2.3 Salt treatment

After three weeks of incubation, the induced calli (300-360 calli for each treatment) were individually weighted and placed on MS medium supplemented with various concentrations of NaCl (0, 1.5, 2.5, 3.5, 4.5, 5.5 and 6.5 g/l). Data were recorded after four weeks of culture initiation in salt stress condition. Through visual observation, the viable calli were counted and the efficiency of callus viability was measured by the following formula.

$$\text{Percentage of callus viability (CV)} = \frac{\text{Viable callus}}{\text{Total number of embryos tested}} \times 100.$$

After the salt pretreatment the viable calli (39-75 calli for each treatment) were transferred to regeneration medium (MS + 1.0 mg/l BAP + 0.5 mg/l NAA) and kept for 3 weeks at 25 ± 1°C with 16/8 hr (light/dark) conditions. Then after three weeks the calli with green spot were counted and the regeneration efficiency was measured by the following formula.

$$\text{Percentage of regeneration (PR)} = \frac{\text{No of callus with green spot}}{\text{Total number of callus}} \times 100.$$

Uniform size (approximately 75-100 mg) and three weeks old calli were placed on the medium MS with different concentration of NaCl (0, 2.5, 4.5 and 6.5 g/l) and in addition with different concentration of plant growth regulators (PGRs). For each treatment calli were weighted individually which was known as initial fresh weight

(FWi) and culture into vessel singly. After four weeks calli were rinsed with sterile distilled water 4-5 times and moisture was removed by blotting paper and final fresh weight (FWf) was taken.

Relative growth rate (RGR) of callus was determined on a fresh weight (FW) using the standard formula (FWf − FWi) / FWi followed by Smith and McComb (1981). FWi is the initial weight of callus in each treatment and FWf is the final weight of the callus after being moved onto NaCl-containing media. To compare genotype-related responses to stress conditions, tolerance index (TI), based on RGR was calculated according to formula TI = $RGR_{treatment}$ / $RGR_{control}$ as follows Soheilikhah et al. (2013). The moisture was removed by blotting and fresh mass was taken.

4.2.4 Heat treatment

To observe the effect of heat stress, four weeks old calli (fresh weight 110-125 mg) were transferred onto sterile empty petri dishes that contained with 2-3 pieces of sterile filter paper. The petri dishes were sealed with parafilm and kept at 25°C (Cont.) for 6 hrs. For heat treatment at 30, 35 and 40°C considered respectively for calli incubation to 6 hours. After heat treatment, callus mass was determined before and after heat treatment. The desiccation percentage was calculated by the following formula.

$$\text{Desiccation percentage} = \frac{\text{Fresh callus weight} - \text{heat treated callus weight}}{\text{Fresh callus weight}} \times 100.$$

The heat treated calli (72-108 calli for each treatment with similar size and shape) were transferred to the regeneration medium containing MS + 1.0 mg/l BAP + 0.5 mg/l NAA and incubated at 25 ± 1°C with a 16/8 hr (light/dark) photoperiod. After seven weeks of incubation the regeneration frequency was calculated by the following formula.

$$\text{Regeneration frequency (\%)} = \frac{\text{No of callus with green spot}}{\text{Total number of callus}} \times 100.$$

After 3-4 weeks, the plantlets were transferred to tubes containing water for hardening and kept at room temperature for a week. The plants were finally transferred to the pots.

4.2.5 Statistical analysis

The values were recorded by three replicates. Post hoc Duncan multiple range test (DMRT) were done using SPSS16.0 software.

4.2.6 Results

4.2.6.1 Effect of salt on callus viability and regeneration

It was observed that the responding genotypes showed well embryogenic response to induce callus (**Fig. 10 A, B**). Nevertheless, significant differences were observed among salt levels. The results indicated that salinity has preventive effects on callus viability as well as regeneration percentage in the three genotypes. Control treatments showed the maximum callus viability and regeneration frequency. In fact, the more salt used the less number of calluses as well as regeneration was recorded. Callus viability and regeneration rate of salt treated callus were greatly influenced by the genotype. Among the genotypes, BB-6 showed highest viability of callus (14.72%) and regeneration (7.69%) at high concentrations of NaCl (6.5 g/l). Data analysis showed a callus viability rate of 14.72%, 11.66% and 10.83%, respectively for BB-6, BHL-18 and BB-3 genotypes at high concentrations (6.5 g/l) of NaCl (**Table 17**). Though BB-6 and BHL-18 showed but BB-3 did not show regeneration at high concentrations (6.5 g/l) of NaCl. All of the treatments (T_1-T_6) showed significantly decreased callus viability as well as regeneration in comparison to the control (**Table 18**). Moreover, frequencies in most of the treatments were significantly different at 0.01 probability levels for callus viability and regeneration from the control except in the frequency of regeneration in T_1.

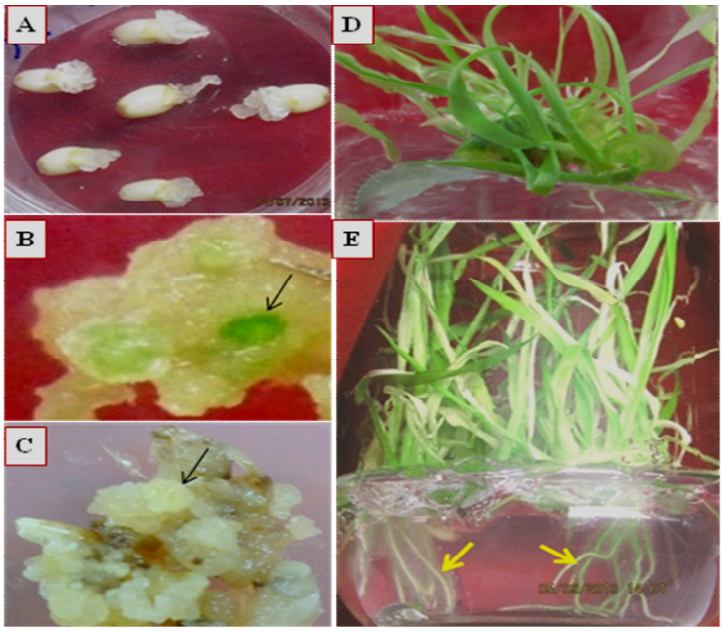

Fig. 10: *In vitro* development of callus and its subsequent regeneration of barley. A: callus initiation. B: embryogenic callus, C: callus after 4 weeks of incubation, D: regenerated plantlet, E: regenerated plants with good shoot and roots.

Table 17: Callus viability and regeneration frequency of seed derived callus exposed to different salt treatments in three barley genotypes (Mean of percentage ± SE)

Salt treatment (g/l)	Genotypes					
	BB-3		BB-6		BHL-18	
	CV	PR	CV	PR	CV	PR
Cont.	30.33 ± 2.02	13.33 ± 3.52	38.66 ± 2.60	20.00 ± 2.30	33.66 ± 1.20	14.66 ± 1.33
T_1	25.66 ± 0.88	12.00 ± 2.30	34.33 ± 1.76	17.33 ± 1.33	28.33 ± 2.40	13.33 ± 266
T_2	21.81 ± 1.38	11.11 ± 3.20	29.69 ± 1.32	16.66 ± 3.20	23.63 ± 1.04	12.96 ± 1.85
T_3	17.87 ± 1.09	9.25 ± 1.85	25.15 ± 1.09	14.81± 1.85	19.69 ± 1.32	11.11 ± 3.20
T_4	15.27 ± 0.73	7.69 ± 4.44	20.00 ± 0.96	12.82 ± 5.12	15.27 ± 1.54	10.25 ± 2.56
T_5	13.33 ± 0.48	5.12 ± 2.56	16.94 ± 0.73	10.25 ± 2.56	13.05 ± 0.73	7.69 ± 4.44
T_6	10.83 ± 0.96	00	14.72 ± 0.55	7.69 ± 4.44	11.66 ± 0.48	5.12 ± 2.56

Cont. = without salt pretreatment, T_1 = 1.5, T_2 = 2.5, T_3 = 3.5, T_4 = 4.5, T_5 = 5.5, T_6 = 6.5 g/l salt pretreatment. CV = Callus viability, PR = Plant regeneration.

Table 18: Effect of salt treatment on callus viability and its regeneration efficiency for barley embryo culture (Mean of percentage ± SE)

Salt treatment (g/l)	Callus viability		Regeneration	
	Mean	Difference	Mean	Difference
T_1	29.44	4.77*	14.22	1.77^{NS}
T_2	25.04	9.17*	13.57	2.42*
T_3	20.90	13.31*	11.72	4.27*
T_4	16.84	17.37*	10.25	5.74*
T_5	14.44	19.77*	7.68	8.31*
T_6	12.40	21.81*	4.27	11.72*
Control	34.21	-	15.99	-

*Indicating the mean difference is significant at the 0.01 level (LSD test). NS = Non-significant and T= Treatment.

4.2.6.2 Relative growth rate (RGR) to NaCl stress

Relative growth rate and tolerance index (TI) were determined and shown in **Fig. 11 A and B**. In both cases, the genotypes differed significantly. However, 0.91, 0.49 and 0.48 RGR values were recorded at 6.5 g/l salt stress in BB-6, BHL-18 and BB-3 respectively. On comparison to the controls, RGR values were decreased at 57.67, 74.87 and 72.41% for respective genotypes. BB-6 showed the highest capability in the top most level of NaCl stress (6.5 g/l). The same genotype carried the highest TI (0.42) number which expressed the high capability to grow in salt stress condition developed by NaCl. Comparatively lower TI numbers were investigated in other two genotypes BHL-18 (0.25) and BB-3 (0.28).

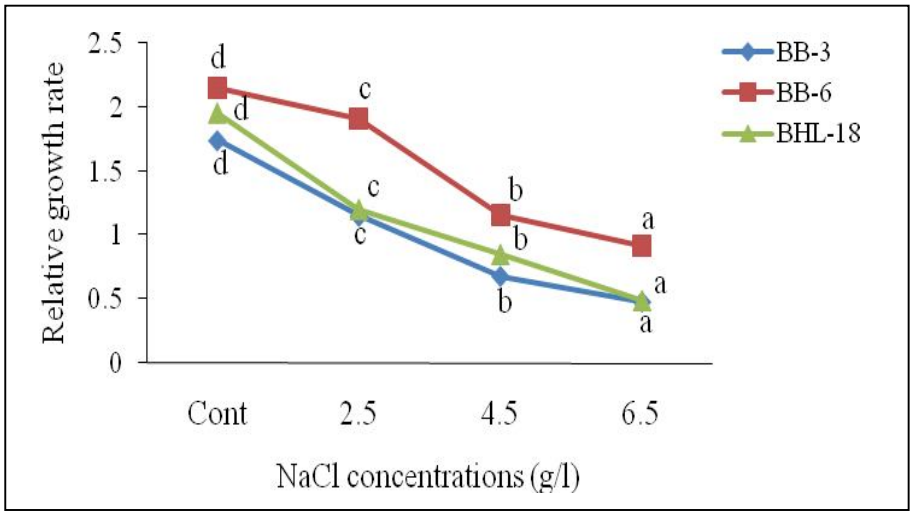

Fig. 11 (A): Effect of different concentrations (0, 2.5, 4.5 and 6.5 g/l) of NaCl subjected to relative growth rate (PGR). Different letters are significantly different at $P < 0.01$ according to Duncan's multiple range test (DMRT).

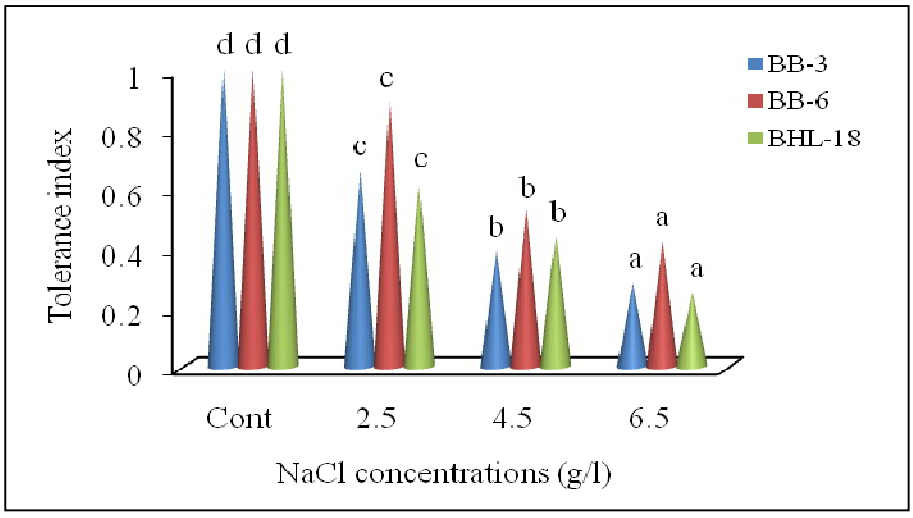

Fig. 11 (B): Effect of different concentrations (0, 2.5, 4.5 and 6.5 g/l) of NaCl subjected to tolerance index (TI). Different letters are significantly different at $P < 0.01$ according to Duncan's multiple range test (DMRT).

4.2.6.3 Effect of heat on desiccation and regeneration

Four weeks old calli (**Fig. 10C**) of three genotypes were subjected at 25℃ (Cont), 30, 35 and 40℃ temperature, respectively. It was observed that the term of desiccation percentage to indicate the percentage loss in callus fresh weight before and after heat treatment as well as regeneration percentage of heat treated calli. There were significant differences were found within the genotypes on regeneration and frequency of desiccation at 0.05 probability levels (**Table 19**). It was observed that calli of BHL-18 performed highest desiccation (59.70%) among the genotypes when it was heated at 40℃ temperature. The performance was around 3 folds higher than the control (19.71%). However, we showed that BB-6 gave the best regeneration (41.66%) among the genotypes when the calli heated at 35℃ temperature that was around 2.14 folds higher than the control (19.44%). All heat treatments induced significantly more plant regeneration than control, with 35℃ of temperature being better treatment (**Table 19**). This treatment induced 25.0–41.66% regeneration over the three genotypes, representing a 1.8–2.14 fold increase compared to control (13.88 -19.44%).

Table 19: Desiccation and regeneration frequency of seed derived callus exposed to different heat treatments in three barley genotypes (Mean of percentage ± SE)

Temp. (℃)	Mean of percentage ± SE					
	Desiccation			Plant regeneration		
	BB-3	BB-6	BHL-18	BB-3	BB-6	BHL-18
Cont. (25)	21.07 ± 0.70a	18.48 ± 2.12a	19.71 ± 1.04a	13.88 ± 1.38a	19.44 ± 1.60a	15.62 ± 1.80a
30	35.47 ± 1.16b	32.42 ± 0.80b	37.97 ± 0.77b	18.05 ± 2.77ab	28.70 ± 2.44a	23.95 ± 2.08bc
35	42.40 ± 0.92c	39.70 ± 1.09c	43.49 ± 1.50c	25.00 ± 2.40b	41.66 ± 3.20b	30.20 ± 2.75c
40	58.67 ± 1.41d	55.75 ± 0.80d	59.70 ± 2.09d	15.27 ± 1.38a	24.07 ± 2.44a	19.79 ± 1.04ab

Cont. = Control, Temp. = Temperature. The values (Mean of percentage) followed by different letters in a column are significantly different at P <0.05 according to Duncan's multiple range test (DMRT).

Heat treated calli (60 calli for each treatment) also performed with increased regeneration in NaCl stress condition (**Fig. 12**). Four weeks old calli of BB-6 were pretreated at 35℃ temperature and transferred to regeneration medium (RM) supplemented with different NaCl concentrations (0, 1.5, 2.5, 3.5, 4.5, 5.5 and 6.5 g/l). BB-6 performed the highest regeneration (15.0%) at the top level of NaCl induced stress (6.5 g/l) from heat treated calli; whereas 8.33% regeneration was recorded from untreated calli. Remarkably, increased regeneration frequencies were appeared, in which calli cultured on salt stress medium after heat pretreatment.

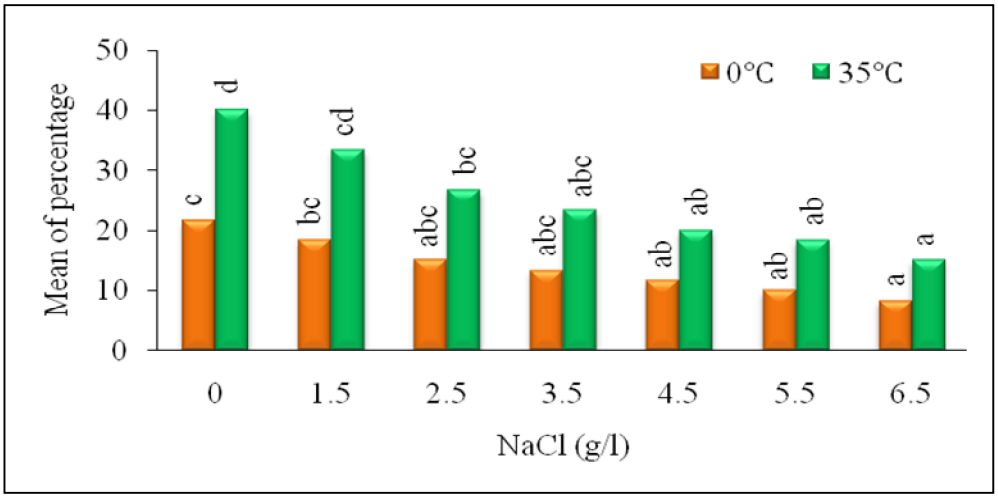

Fig 12: Effect of heat and salt pretreatment on regeneration in BARI barley-6. Different letters are significantly different at $P < 0.01$ according to Duncan's multiple range test (DMRT).

4.2.7 Discussion

4.2.7.1 Viability and regeneration of callus in salt treatment

NaCl has been used to simulate salt stress either *in-vivo* or *in vitro* in barley (Ye et al. 1987). Application of NaCl during the callus formation and/or regeneration processes constitutes a convenient way to study the effect of salinity and selective pressure can be applied (Saleem et al. 2005). In the present investigation significant differences were found among the barley genotypes on viability of calli in NaCl stress. The calli of BB-6 exhibited highest viability of calli (14.72%) with higher doses (6.5 g/l) of NaCl out of six concentrations. Other two genotypes BHL-18 and BB-3 survived with

11.66% and 10.83% existing calli when the calli were exposed in 6.5 g/l for four weeks. The phenomenon was happened due to presence of necrotic cells in the calli. A huge number of necrotic cells turned the calli blackish or deep brown in color and survival disability was appeared. Through visual observation, it was shown that higher frequency of necrosis was appeared in higher level salt concentration than the lower one. It might be happened due to lesser osmotic potentiality of the cells and genotypic effect which created the variability of cell viability among the genotypes. At the end of four weeks cultured periods most calli of the genotypes were died except BB-6, which partially adapted to the NaCl stress and showed highest viability in all tested levels of salt concentration. The results are in agreement with the previous finding that Giza 123 genotye can tolerate 5 g/l salt stress (Metwali et al. 2013). Siddique et al. (2014) reported that callus viability decreased in rice on comparison to the controls, when they were cultured in 200 mM NaCl level. The present experimental findings showed that salt treatment could decrease the shoot regeneration of treated calluses derived from the tested genotypes. All of the treatments (T_1-T_6) showed significantly decreased regeneration in comparison to the control (**Table 18**). BB-6 and BHL-18 showed shoot regeneration at high concentrations (6.5 g/l) of NaCl. Therefore, the present investigations are in agreement with the previous report where 47- 64% decreased cell viability in NaCl induced stress for Safflower (*Carthamus tinctorius* L.) varieties (Soheilikhah et al. 2013) and callus growth as well as shoot length both decreased with increasing NaCl concentration in the medium. Motohashi et al. (2010) improved various salt tolerance rice cultivars by a catalase gene, katE which could grow in 200 mM NaCl.

4.2.7.2 Growth rate and tolerance index

Significant differences were found among the genotypes examined on relative growth rate (RGR) and tolerance index (TI). In the top most level of NaCl stress (6.5 g/l) recorded RGR values were 0.91, 0.49 and 0.48; TI were 0.42, 0.25 and 0.28 for the genotypes BB-6, BHL-18 and BB-3, respectively. Among three genotypes BB-6 exhibited the highest potentiality to survive in NaCl induced abiotic stress with

maximum RGR (0.91) and TI (0.42). The recorded parameters expressed the higher survival capability against the NaCl stress conducting the physiological activities of BB-6. On the other hand stress sensitivity was found in BHL-18 and BB-3 considering lower value of the parameters. RGR and TI values were decreased when the calli were cultured in higher NaCl level. These phenomena might be happened due to reduction of water availability and lose of turgor pressure (TP) in the cells of the calli. Such physiological causes were reported in previous investigation for *Oryza sativa* (Siddique et al. 2014), *Carthamus tinctorius* (Soheilikhah et al. 2013), *Triticum eastivum* (Fazeli-nasab et al. 2012) and *Saccharum* sp. (Errabi et al. 2007). Errabi et al. (2007) mentioned that due to interference of Na+ and Cl- ions to uptake and translocation processes. As a result nutritional imbalance might be created and growth of callus is declined. However, NaCl treated calli of BB-6 was least affected by the highest dose of salt stress and exhibited high ability in terms of both cellular viability and growth of calli.

4.2.7.3 Heat treatment to desiccation and regeneration

Temperatures and drought represent stress factors associated with plant cell dehydration. Dehydration stress factors induce profound cellular response aimed at an elimination of water loss (Kosová et al. 2014). The experimental results showed that desiccation could increase the shoot regeneration of treated calluses derived from the tested barley genotypes, but the increasing efficiency was crucially dependent on the degree of temperature. It was observed that calli of BHL-18 performed highest desiccation (59.70%) among the genotypes when it was heated for 40℃ temperature. The performance was around 3 folds higher than the control (19.71%). However, the results showed that BB-6 gave the best regeneration (41.66%) among the genotypes when the calli heated at 35℃ temperature that was around 2.14 folds higher than the control (19.44%). The water losses of calluses of three genotypes were different with same degree of temperature. This implied that enhancement of shoot regeneration by desiccation is not only dependent on the amount of water loss, but is also related to the degree of temperature. Heat treatment for 35℃ led to greater regeneration

percentage compared to control temperature. Our findings agree with those of Benderradji et al. (2012) who found significant effects of thermal stresses on callus induction and shoot regeneration in wheat.

Surprisingly, we found increased regeneration of those calli which were cultured on salt stress medium after heat pretreatment. BB-6 performed the highest regeneration (15.0%) at the top level of NaCl induced stress (6.5 g/l) from heat treated calli; whereas 8.33% regeneration was recorded from untreated calli. It could be possible when the cells of calli acquired higher osmotic potential (OP). Due to reduction of water at a suitable level, OP might be increased, and so that the heat treated calli survived and was been able to perform higher regeneration. Siddique et al. (2014) observed approximately similar results in rice and reported that, in the salt induced abiotic stress condition desiccated calli gave higher regeneration than the controls.

4.3 An Efficient Callus Induction and Regeneration by Copper Sulphate and Cobalt Chloride in Barley

4.3.1 Introduction

It has become evident that several heavy metals like cobalt, iron, copper, manganese, and zinc are essential for plant development that plays an important role to the regeneration of plant tissue cultures (Al-Mayahi, 2014). Although, macronutrient and micronutrient levels for plant tissue culture system has been optimized by Murashige and Skoog (1962). But no clear optimal levels were apparent and the specific effect of copper sulfate and cobalt chloride on tissue culture has not been described (Litvay et al. 1985). Hussein et al. (2010) demonstrated that heavy metals are required in a very small or trace amounts and become toxic at higher concentrations. Several reports suggest that the positive influence of copper during *in vitro* culture of various explants in barley (Nuutila et al. 2000) and other cereals (Guo and Pulli, 2000). To increase the copper sulfate in the culture medium influences plant regeneration from callus cultures has been reported by Dahleen (1995). In barley, the optimal concentration of copper sulfate needs to be adjusted according to the type of explants. It was reported that the best results (100 fold higher than the control) were obtained when 10 mM copper sulfate was added in the culture medium (Wojnarowiez et al. 2002).

There are some reports about the effect of cobalt chloride that showed a powerful inhibitor in ethylene biogenesis and encourages somatic embryo development at higher concentrations and stimulates growth and developments in plants (Ascough et al. 2007; Shah et al. 2013). The appreciative use of cobalt chloride improved the *in vitro* shoot regeneration in tomato (Osman and Khalafalla, 2010). So far as I know till there is no report about the effect using those chemicals in barley and other cereal crops in Bangladesh through *in vitro* culture. So why this research programme has been undertaken to evaluate the effect of copper sulphate and cobalt chloride in addition to the medium and evaluated their effects on callus induction and subsequent plant regeneration using mature embryos in barley.

4.3.2 Methods

Mature seeds of BARI barley-3 and BARI barley-6 were considered for their good performance in tissue culture system previously reported (Section 4.1). Seeds were sterilized following the standard protocol described in the section of 2.3.1.1 (general materials and methods). Around 10 -12 seeds were inoculated in each petri dish.

4.3.2.1 Application of copper sulphate and cobalt chloride for callus induction

The callus induction medium consisted of MS salts supplemented with various concentrations of copper sulphate and cobalt chloride (2.5, 5.0, 7.5 and 10.0 g/l). For control no copper sulphate or cobalt chloride were used. For each genotypes the effect of copper sulphate or cobalt chloride experiments were set up separately. Three replication of each treatment were considered for each case. The pH was adjusted to 5.8 and medium was solidified with 0.6% (w/v) agar (Sigma) before autoclaving at 120°C for 15 min.

The cultures were incubated in dark at 25 ± 1°C for 3 weeks for callus induction. After 1 week of culture initiation the germinated portions of the mature embryos were separated by a sterile surgical blade and callus was placed on the same medium again. Percentage of callus formation was assessed after 3 weeks of culture initiation and data were recorded by the following formula mentioned below.

$$\text{Percentage of callus formation} = \frac{\text{Mature embryos formed callus}}{\text{Total number of embryos cultured}} \times 100$$

4.3.2.2 Application of copper sulphate and cobalt chloride for regeneration

Responding calli was transferred to regeneration medium that contained 1.5 mg/l BAP and 20.0 g/l sucrose. The basal components and different concentrations of copper sulphate or cobalt chloride or both additives of the regeneration medium were identical to those of induction medium. Cultures were incubated at 25 ± 1°C under 16/8 h photoperiod. Calli derived from each mature embryo were maintained separately on regeneration medium. Percentage of regeneration was evaluated after 4 weeks of culture on regeneration medium. Well developed shoots were transferred on

to ½MS medium (0.2% w/v Phytagel) for rooting. Then well rooted plants were transferred in the mixture of peat moss and soil (1:1).

$$\text{Percentage of regeneration} = \frac{\text{The number of explants regenerated shoots}}{\text{Number of embryos cultured on the induction medium}} \times 100$$

4.3.3 Results

4.3.3.1 Effect of copper sulphate and cobalt chloride on callus induction

To observe the effect of copper sulphate and cobalt chloride on callus induction were tested for BARI brley-3 and BARI barley-6. According to the results obtained, both additives of copper sulphate and cobalt chloride at 2.5 to 7.5 mg/l (T_9 to T_{11}) were found suitable for the production of callus in both genotypes. However, the rate of callus formation was decreased when the concentration of copper sulphate or cobalt chloride or both additives was 10 mg/l in the media. It was observed that compact embryogenic callus, creamy in colour were produced in the media that contained 2.5 and 5.0 mg/l of copper sulphate and cobalt chloride (**Fig. 13**). Comparing copper sulphate and cobalt chloride, copper sulphate showed better performance for callus formation. The rate of callus formation was increased in the media containing cobalt chloride at 2.5 and 5.0 mg/l (T_5 to T_6). However, statistical analysis did not show significant differences between same concentrations of copper sulphate (T_1 to T_2) and control on callus formation at 0.05 levels for both genotypes (**Table 20, 21**). The best callus formation was significantly higher in T_{10} (5.0 mg/l $CuSO_4$ and 5.0 mg/l $CoCl_2$), 54.66% in BARI barley-3 and 75.49% in BARI barley-6 than other treatments (**Table 21**). The lowest percentage of calli were recorded 5.76% and 6.42% respectively in BARI barley-3 and BARI barley-6 in T_4 (10.0 mg/l copper sulphate). Moreover, comparing the genotypes BARI barley-6 was better than BARI barley-3 (**Fig. 14**). Among the doses highest callus induction (75.49%) was recorded for BARI barley 6 in T_{10} (5.0 mg/l $CuSO_4$ and 5.0 mg/l $CoCl_2$) which was around 2 fold higher than (38.32%) control.

Table 20: Effect of different concentrations of copper sulphate and cobalt chloride on callus induction in BARI barley-3

Chemicals (mg/l)	Treatments	No. of embryos	Responded to callus induction	% of callus formation (Mean ± S.E)
$CuSO_4$				
2.5	T_1	180	63	35.00 ± 1.92fg
5.0	T_2	180	67	37.22 ± 2.00gh
7.5	T_3	180	25	13.88 ± 1.46c
10.0	T_4	156	9	5.76 ± 1.11a
$CoCl_2$				
2.5	T_5	195	61	31.28 ± 1.35ef
5.0	T_6	195	66	33.84 ± 1.77efg
7.5	T_7	195	21	10.76 ± 0.88bc
10.0	T_8	195	14	7.17 ± 0.51ab
$CuSO_4$ + $CoCl_2$				
2.5 + 2.5	T_9	225	89	39.55 ± 1.17hi
5.0 + 5.0	T_{10}	225	123	54.66 ± 1.53j
7.5 + 7.5	T_{11}	225	97	43.11 ± 1.17i
10.0 + 10.0	T_{12}	225	55	24.44 ± 0.88d
Control -	-	180	55	30.55 ± 1.11e

Control = Normal MS level of $CuSO_4$ and $CoCl_2$. Values (Mean of percentage ± SE) followed by different letters in a column are significantly different at $P<0.05$ according to Duncan's multiple range test.

Table 21: Effect of different concentrations of copper sulphate and cobalt chloride on callus induction in BARI barley-6

Chemicals (mg/l)	Treatments	No. of embryos	Responded to callus induction	% of callus formation (Mean ± S.E)
$CuSO_4$				
2.5	T_1	180	77	42.77 ± 2.93ef
5.0	T_2	180	82	45.55 ± 2.42f
7.5	T_3	180	32	17.77 ± 1.46c
10.0	T_4	156	10	6.42 ± 1.69a
$CoCl_2$				
2.5	T_5	195	79	40.51 ± 2.85ef
5.0	T_6	195	84	43.07 ± 1.77ef
7.5	T_7	195	29	14.87 ± 1.84bc
10.0	T_8	195	18	9.23 ± 0.88ab
$CuSO_4$ + $CoCl_2$				
2.5 + 2.5	T_9	225	106	47.11 ± 1.17fg
5.0 + 5.0	T_{10}	225	170	75.55 ± 3.11h
7.5 + 7.5	T_{11}	225	119	52.88 ± 2.35g
10.0 + 10.0	T_{12}	225	70	31.11 ± 1.60d
Control -		180	69	38.32 ± 1.92e

Control = Normal MS level of $CuSO_4$ and $CoCl_2$. Values (Mean of percentage ± SE) followed by different letters in a column are significantly different at $P<0.05$ according to Duncan's multiple range test.

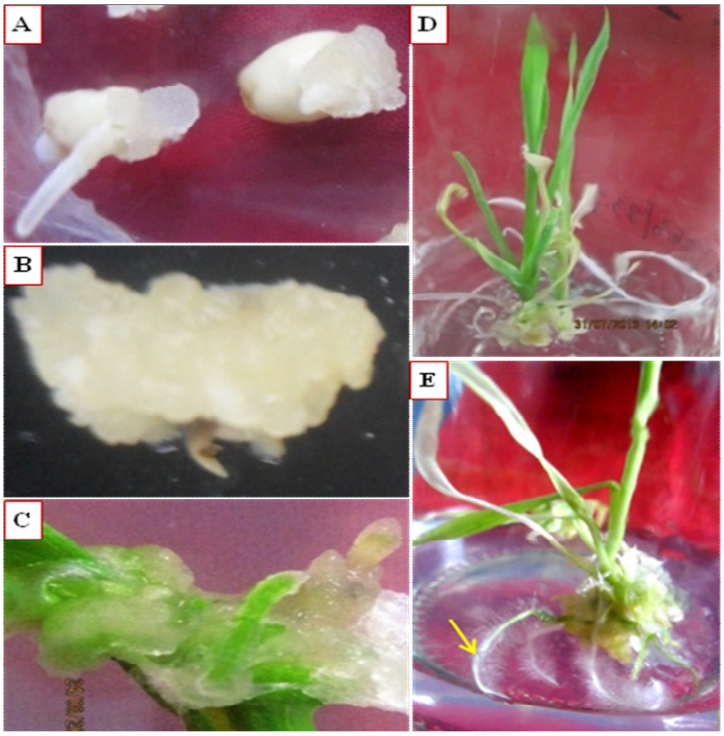

Fig. 13: Mature embryo derived callus and its subsequent regeneration of barley that containing copper sulphate and cobalt chloride: A) callus formation, B) embryogenic callus, C) greenish callus, D) regenerated plantlet, E) well rooted plantlet.

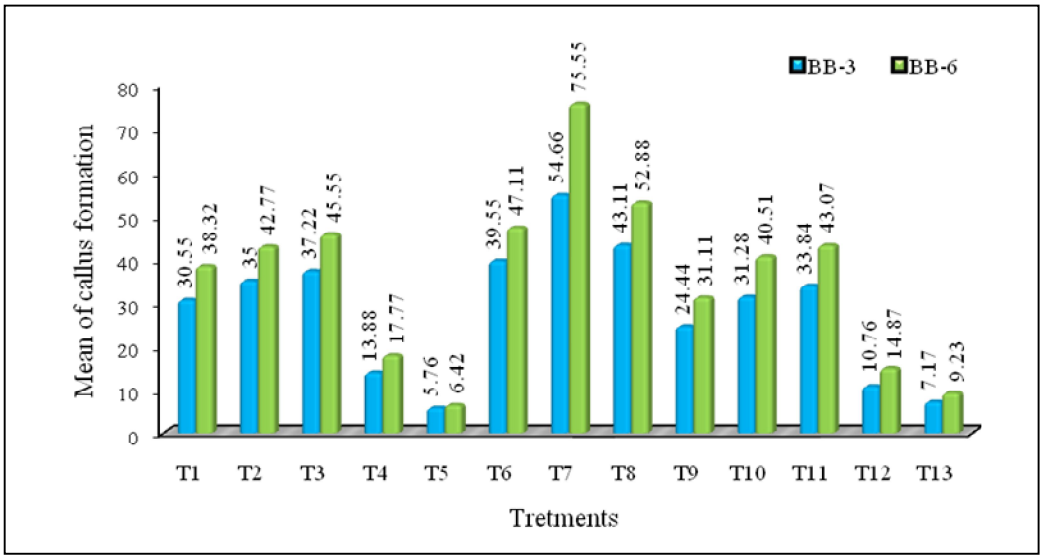

Fig. 14: The effect of additional copper sulphate and cobalt chloride on the growth of callus derived from mature embryos in BARI barley-3 and BARI barley-6.

4.3.3.2 Effect of copper sulphate and cobalt chloride on regeneration

It was observed that the optimal concentration for plant regeneration was 5.0 mg/l of $CuSO_4$ + 5.0 mg/l $CoCl_2$. Higher percentage of regeneration was showed in T_{10} (5.0 mg/l $CuSO_4$ and 5.0 mg/l $CoCl_2$) than other treatments. Combination of copper sulphate and cobalt chloride at 5.0 mg/l significantly enhanced the plant regeneration (34.22% and 53.33%) in BARI barley-3 and BARI barley-6 respectively (**Table 22**). The minimum value of plant regeneration was found with the frequency of 2.05% and 2.54% respectively in BARI barley-3 and BARI barley-6 when 10.0 mg/l cobalt chloride used singly (T_8). In BARI barley-6, the treatment T_{10} (5.0 mg/l copper sulphate and cobalt chloride) showed the maximum plant regeneration (53.25%) that was around 3 fold higher than control (19.44%). Hence, the increased number of green plants produced in the media containing both copper sulphate and cobalt chloride (**Fig. 14**). It also observed that BARI barley-6 was better than BARI barley-3 for regeneration.

Table 22: Effect of different concentrations of copper sulphate and cobalt chloride on regeneration of callus derived from mature embryos in two barley genotypes

Chemicals (mg/l)	Treatments	Genotypes			
		BB-3		BB-6	
		Responded embryos to regeneration	% of regeneration	Responded embryos to regeneration	% of regeneration
$CuSO_4$					
2.5	T_1	29	16.11 ± 2.42	47	26.11 ± 2.00
5.0	T_2	33	18.33 ± 2.54	53	29.44 ± 2.93
7.5	T_3	13	7.22 ± 0.55	25	13.88 ± 1.46
10.0	T_4	06	3.84 ± 1.11	08	5.12 ± 0.64
$CoCl_2$					
2.5	T_5	29	14.87 ± 1.35	42	21.53 ± 0.88
5.0	T_6	27	13.84 ± 0.88	40	20.51 ± 1.35
7.5	T_7	08	4.10 ± 1.02	18	9.23 ± 1.53
10.0	T_8	04	2.05 ± 0.51	05	2.56 ± 0.51
$CuSO_4$ + $CoCl_2$					
2.5 + 2.5	T_9	46	20.44 ± 1.93	71	31.55 ± 1.93
5.0 + 5.0	T_{10}	77	34.22 ± 2.70	120	53.33 ± 2.30
7.5 + 7.5	T_{11}	48	21.33 ± 2.03	91	40.44 ± 2.70
10.0 + 10.0	T_{12}	26	11.55 ± 1.17	44	19.55 ± 1.60
Control	-	25	13.88 ± 1.11	35	19.44 ± 1.46

Control = Normal MS level of $CuSO_4$ and $CoCl_2$.

4.3.4 Discussion

The adjustment of copper and cobalt chloride concentration appears to be a key point in promoting plant regeneration but it depends upon the type of *in vitro* culture. It was observed that in addition of both chemicals such as copper sulphate and cobalt chloride at 2.5 to 7.5 mg/l (T_9 to T_{11}) were suitable on callus induction. In the case of plant regeneration, the treatment T_{10} (5.0 mg/l copper sulphate and cobalt chloride) showed the maximum plant regeneration (53.25%) that was around 3 fold higher than control.

Some recent reports suggested that the positive influence of copper during *in vitro* culture of various explants in barley (Nuutila et al. 2000; Wojnarowiez et al. 2002; Bartlett et al. 2008; Yadav et al. 2011) and other cereals (Tahiliani and Kothari, 2004). In barley, the optimal concentration of copper sulfate needs to be adjusted according to the type of explant and the cultivar used. Dahleen (1995) studied that the effect of different concentrations of $CuSO_4$ on callus culture and found that medium containing 50 µM copper regenerated significantly more plants. Szafrańska et al. (2011) tested the responses of the plant material regenerated from the androgenic embryos obtained from the anther culture of carrot cultivated in the presence of different Cu ion concentrations. Ghaemi et al. (1994) reported that the addition of 40 µM CuSO4 to the medium significantly increased the embryos production from wheat anther cultures. The Copper (Cu) is require to supply within the culture because it is essential for plant cell and tissue growth of some monocotyledons (Schenk and Hildebrandt, 1972). Callus proliferation is an energy consuming activity. Therefore, the rate of respiration in cells is normally higher during callus proliferation and cell division to produce required energy. Micronutrients in plant cells, specially copper, iron, manganese and zinc are important in the process of respiration where iron and copper are the functional parts of some oxidative enzymes contained in plant tissues (Sumner and Somers, 1953). On the other hand, cobalt is a transition element which is an essential factor in many enzymes and co-enzymes. It affects the growth and

metabolism of plants in various degrees depending on the concentration of cobalt in the surrounding medium (Palit et al. 1994; Shah et al. 2013). The rate of callus proliferation may have been improved with synergistic effects when copper and cobalt are together in the medium (Al-Mayahi, 2014). Amarasinghe (2009) carried out research on *in vitro* performance of nine indica rice varieties and concluded that the rate of calli production was significantly higher in all the tested varieties on MS basal medium supplemented with 5-10 mg/l cobalt chloride.

4.4 Effect of Carbon Sources and Plant Growth Regulators on Efficient Callus Induction and Regeneration in Barley (*Hordeum vulgare* L.)

4.4.1 Introduction

Barley (*Hordeum vulgare* L.) is regarded as an inferior staple food crop compared to wheat, rice and maize and considered as poor people's bread (Elsayed, 2013). For barley and other cereals improvement it is important to develop an efficient cultures systems enormous potential for various biotechnological applications such as *in vitro* selection, doubled haploids technology and genetic transformation (Cistué et al. 2004; Islam, 2007; Kahrizi et al. 2011; Islam and Tuteja, 2012; Haque et al. 2014). However, an efficient plant regeneration system using mature embryos is still lacking and till there is no enough report on barley in Bangladesh. Delporte et al. (2014) obtained high regeneration system using mature embryo (ME) and developed a regeneration procedure based on fragmented mature embryo through *in vitro* culture in wheat. The mature embryo is arguably one of the best explants for advance biotechnological research because of its unlimited availability and lack of growth season restriction (Rostami et al. 2013). Gürel et al. (2009) standardized a suitable protocol on callus induction and its subsequent regeneration systems in barley. On this regard they have screened some suitable barley genotypes and optimized related other culture conditions also. Recently successful plant regeneration based on mature embryos has been reported in different cereal crops e.g rice (Siddique et al. 2014), wheat (Hakam et al. 2014) and maize (Morshed et al. 2014).

Carbohydrates in the nutrient medium perform important functions as the main source of energy in plant tissues and provide the proper osmo-regulation (Yaseen et al. 2013). The composition of the culture media including carbohydrates and growth regulators play an important role for callus induction and regeneration in *Wedelia trilobata*, *Neoglaziovia variegata* and wheat and other crops (Keerthiga et al. 2012; Silveira et al. 2013; Asif et al. 2014). Sucrose is often assumed to be the best choice of carbon source in tissue culture media, because it is the main sugar translocated in the phloem (Hossan et al. 2009; Gauchan, 2012; Petrova et al. 2015). However, in some cases sucrose may not suitable for callus induction and plant regeneration and there are a number of plants that can grow using various carbohydrates. Several

authors have reported that maltose is more effective than sucrose for embryogenesis in barley (Blanc et al. 2002; Sharma et al. 2005). Al-Kaaby et al. (2013) reported that sorbitol and 2,4-D in combination showed a significant effect on callus fresh weight and enhanced somatic embryos. Using sorbitol instead of sucrose in the regeneration medium had a greater regenerative capacity than sucrose in maize (Swedlund and Locy, 1993). Hassan et al. (2009) reported that using sorbitol in induction medium showed significant results on callus induction and regeneration and they also found that sorbitol has given more strength to regenerated plant.

Plant growth regulators are fundamental to produce embryogenic calli with improving regenerative capacity through *in vitro* culture (Aguado-Santacruz et al. 2011; Bhattacharjee and Islam, 2014, 2015). Using optimal auxins and cytokinins in the callus induction and sub-culturing media improves its quality and regeneration efficiency in rice and other cereals (Islam et al. 2001; Khatun et al. 2012; Lakshmi et al. 2013). Pretreatment of the plant material play an important role in cereal crops (Sayar et al. 1999). So far as I know, there is no sufficient work on *in vitro* culture of barley in Bangladesh. So why this study has been undertaken to improve an efficient callus induction and its subsequent regeneration system using different carbon sources and plant growth regulators in barley.

4.4.2 Seed sterilization and pretreatment by 2,4-D

For this study BARI barley-6 has been considered for its better performance on *in vitro* regeneration system and agronomical performance (**Section 2.1**). Brief sterilization procedures are described in the section of **2.3.1.1.** To evaluate the efficacy of 2,4-D on callus formation, sterilized seeds were deep in sterile water that contained with 2.5, 3.5 and 6.0 mg/l 2,4-D solution and keep them at 4°C for 1-6 days prior to culture. Then pretreated seeds were rinsed 4-5 times and excess water was blotted onto a sterile Whatman No. 1 filter paper. Then pretreated seeds were inoculated in MS medium supplemented with 200 mg/l L-proline, 300 mg/l casein hydrolysate, 1.5 mg/l 2,4-D and 30 mg/l sucrose for callus induction (**Fig. 15**). The pH of all media adjusted 5.8. After 2 weeks small sized calli derived from pre-treated seeds were removed and sub-cultured in every week to the same medium for callus development.

4.4.3 Optimization of carbohydrates

To optimize a suitable carbon sources various amount of carbons were added in different media *viz.* Murashige and Skoog 1962 (MS), Gamborg et al. 1968 (B5) and Chu, 1975 (N6). All media were supplemented with 1.5 mg/l 2,4-D, 200 mg/l L-proline, 300 mg/l casein hydrolysate and as solidifying agent 6.0 g/l agar was used. Different concentrations of sucrose, maltose and D-sorbitol were used either single or in combination [T_1: sucrose (30 g/l), T_2: sucrose (60 g/l), T_3: maltose (30 g/l), T_4: maltose (60 g/l), T_5: D-sorbitol (30 g/l), T_6: D-sorbitol (60 g/l), T_7: sucrose (15 g/l) + maltose (15 g/l), T_8: sucrose (15 g/l) + D-sorbitol (15 g/l) and T_9: maltose (15 g/l) + D-sorbitol (15 g/l)]. Around 200 seeds were inoculeted for each treatment (T). For callus proliferation, the cultures were incubated at 25±1°C in dark and mature embryos were transferred separately to regeneration medium.

4.4.4 Embryogenic callus induction and regeneration

Embryogenic calli (3-4 weeks old) were transferred to MS medium for secondary embryogenesis and incubated them at 25 ± 1°C in the dark for 1 week for 16/8 h (light/dark) photoperiod. Then the calli were transferred onto fresh medium for its proliferation. To determine the effect of plant growth regulators on embryogenic callus formation, various concentrations of 2,4-D, BAP, NAA and Kinetin were used either single or in combination. In order to determine a suitable concentration of plant growth regulators (PGRs) for plant regeneration four weeks old embryogenic calli were cultured in MS basal medium where 30g/l sucrose were added. Then cultures were incubated at 25 ± 1°C with a 16/8 hr (light/dark) photoperiod for 3-4 weeks.

4.4.5 Rooting and acclimatization

Well developed shoots were transferred to three rooting media e.g MS, ½MS and GM (modified from MS; Islam, 2000) supplemented with 1.0 mg/l of NAA, IAA and IBA phytohormones + 20 g/l sucrose. For rooting as gelling agent 3 g/l phytagel were used. Then well rooted plants were transferred to pot that contained with peat moss and soil (1:1). To evaluate the root formation, average number of roots per plants were recorded and calculated.

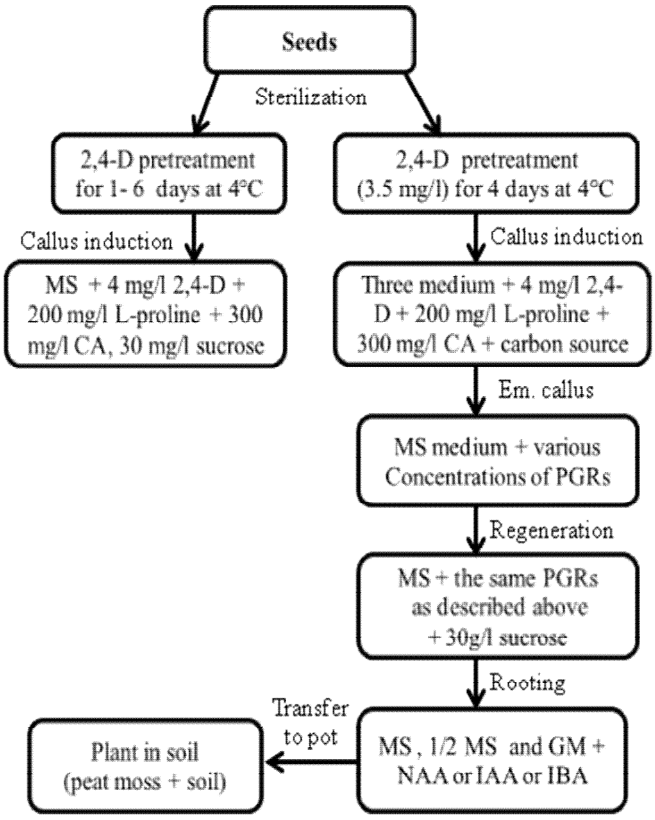

Fig. 15: A schematic representation on plant development using mature embryos of barley.

4.4.6 Data recording and statistical analysis

For each treatment, three replications were evaluated and each experiment was repeated three times. Statistical analysis of the data was performed by SPSS software (version 16). Within the treatment groups, the differences among means were compared by Duncan's multiple range tests (DMRT).

4.4.7 Results

4.4.7.1 Optimization of 2,4-D concentration as pretreatment factors on callus induction

To enhance callus induction seeds were pretreated by 2,4-D with different concentrations and durations. The result showed that calli development was strongly affected by pretreated seeds, and the frequency was increased when it was soaked by 3.5 mg/l 2,4-D for 4 days (**Table 23**). Highest frequency of callus 71.38% was in 3.5

mg/l followed by 65.27%, in 2.5 mg/l 2,4-D soaked with same duration. The lowest callus (15.55%) induction was recorded when the seeds were not pretreated by 2,4-D (Control). Furthermore, it was observed that when higher concentration of 2,4-D (more than 3.5 mg/l) used in the medium the calli were reduced.

Table 23: Effect of 2,4-D pretreatment period and concentration prior to culture of inoculated seeds for callus induction in BARI barley 6

Duration (days)	No. of inoculated seeds	Pretreatments			
		Control	Concentration of 2, 4-D (mg/l)		
			2.5	3.5	6.0
1	60	15.55 ± 1.46a	32.77 ± 2.42bc	38.33 ± 1.92c	29.44 ± 2.00b
2	120	23.61 ± 1.68a	40.55 ± 2.64b	51.94 ± 2.46c	36.11 ± 1.94b
3	120	31.11 ± 2.16a	46.66 ± 2.20b	58.61 ± 3.13c	38.33 ± 1.73a
4	120	37.77 ± 2.42a	65.27 ± 3.37c	71.38 ± 3.41c	50.55 ± 1.68b
5	120	13.61 ± 1.21a	44.16 ± 2.40b	56.94 ± 2.27c	37.77 ± 2.42b
6	120	10.83 ± 1.44a	21.38 ± 1.46b	38.05 ± 1.94c	15.27 ± 1.21a

The mean values (percentage ± SE) having same letter(s) in a row are statistically identical.

4.4.7.2 Effects of carbohydrates on medium

Different carbohydrates were added in the targeted medium and found that 60 g/l D-sorbitol (T_6) showed highest callus induction (89.16%) followed by 60 g/l maltose (76.83%). Sucrose (30 g/l) gave 71.16% callusing in MS medium but 15 g/l maltose with 15 g/l D-sorbitol (T_9) showed low callus induction (11.33%) in B5. Mature embryos that pretreated by 2,4-D in all of three induction media (B5, N6 and MS) showed significantly higher number of callus induction. Out of three media, maximum callus induction was recorded on MS and minimum with B5 medium (**Table 24**). It was observed that significantly differences among carbohydrates and the tested three media *viz*. MS, N6 and B5 on callus induction at $P<0.05$ levels.

Compact and yellowish-white callus was observed when mature embryos were cultured on MS and N6 media (**Fig. 16**). Therefore, MS medium would be efficient for embryogenic callus induction as well as regeneration.

Table 24: Effect of carbohydrates for callus induction in different media

Media	Amount of carbohydrates (g/l)								
	Single application						Combined uses		
	Sucrose		Maltose		D-Sorbitol		Sucrose and Maltose	Sucrose and D-Sorbitol	Maltose and D-Sorbitol
	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9
B5	27.67 ± 1.76cd	15.83 ± 1.30ab	18.46 ± 2.35b	31.79 ± 1.84d	20.51 ± 3.11b	33.84 ± 2.66d	22.05 ± 1.35bc	16.41 ± 2.71ab	11.33 ± 1.01a
N6	35.38 ± 2.66e	21.02 ± 1.84ab	26.15 ± 1.77bcd	47.18 ± 3.11f	28.20 ± 1.35cd	52.83 ± 2.31f	30.76 ± 2.35de	23.07 ± 0.88abc	17.43 ± 1.35a
MS	71.16 ± 3.17d	27.69 ± 1.77ab	33.33 ± 2.71bc	76.83 ± 3.32d	35.89 ± 2.23bc	89.16 ± 4.33e	36.92 ± 2.35c	32.30 ± 2.66abc	24.10 ± 1.84a

The mean values (percentage ± SE) having same letter(s) in a row are statistically identical. MS = Murashige and Skoog (1962); B5 = Gamborg et al. (1968); N6 = Chu (1978); T_1: Sucrose (30 g/l), T_2: Sucrose (60 g/l), T_3: Maltose (30 g/l), T_4: Maltose (60 g/l), T_5: D-Sorbitol (30 g/l), T_6: D-Sorbitol (60 g/l), T_7: Sucrose (15 g/l) + Maltose (15 g/l), T_8: Sucrose (15 g/l) + D-Sorbitol (15 g/l) and T_9: Maltose (15 g/l) + D-Sorbitol (15 g/l).

4.4.7.3 Effect of growth regulators on embryogenic calli and regeneration

Embryogenic callus formation and its regeneration frequency was measured (weight) on the basis of percentage of calli (each 100 mg) produced and found that its frequency was varied when PGRs and its concentrations was different in the medium. In case of embryogenic callus formation 2,4-D and BAP or NAA and BAP combined uses had a positive effect in barley (**Table 25**). The best embryogenic callus (70.0%) observed with 2.0 mg/l 2,4-D and 0.5 mg/l BAP. On the other hand, combined uses of NAA, BAP and Kinetin showed very low frequency of embryogenic callus formation. Green plantlets were recorded within three weeks of culture (**Fig. 16C and D**). Under this study different concentration (single and combined) of 2,4-D, BAP, Kinetin and NAA were tested and the maximum percentage of regeneration (47.40%) was

recorded in MS medium supplemented with 0.5 mg/l NAA and 1.0 mg/l BAP (**Table 25**). It was observed that the combined used of 2,4-D with BAP had no significant effect on regeneration, although highest frequency of embryogenic callus was observed when 2.0 mg/l 2,4-D with 0.5 mg/l BAP were used. It was observed that NAA and BAP had a positive effect on embryogenic callus formation and on plant regeneration (**Table 25**). Furthermore, combination of a low level of auxin with a high level of cytokinin for plant regeneration showed better results, which showed significant results compared to the effects of a low level of cytokinin. Medium with or without cytokinin + 2,4-D did not show any effect on plant regeneration.

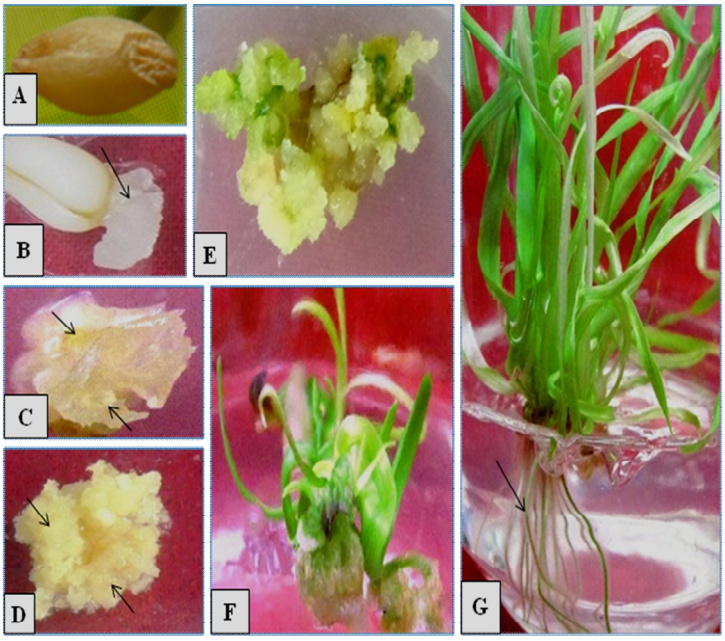

Fig. 16: Callus induction and plant regeneration of *Hordeum vulgare*. A) Pretreated mature embryo (seed) on induction medium, B) Callus induction after 10 days of culture initiation, C-D) Proliferated callus on the induction medium after sub-cultured.; E-F) Embryogenic callus regeneration, G) Well developed plants with shoot and roots.

Table 25: Effects of different concentration and combination of PGRs on embryogenic callus induction and plant regeneration

PGRs and amount (mg/l)	No. of callus	Mean of percentage ± SE	
		Embryogenic callus	Plant regeneration
2,4-D			
0.5	12	11.11 ± 2.77b	-
1.0	12	16.66 ± 4.81cd	-
2.0	12	19.44 ± 2.77d	-
3.0	12	13.88 ± 5.55bc	-
BAP			
0.1	15	15.55 ± 2.22c	4.44 ± 2.22a
0.5	15	24.44 ± 4.44e	6.66 ± 3.84ab
1.0	15	37.77 ± 2.22fg	15.55 ± 2.22c
1.5	15	20.00 ± 3.84d	8.88 ± 2.22b
2,4-D + BAP			
2.0 + 0.1	20	53.33 ± 4.40gh	3.33 ± 1.66a
2.0 + 0.5	20	70.00 ± 2.88h	8.33 ± 3.33ab
2.0 + 1.0	20	48.33 ± 3.33gh	10.0 ± 2.88b
2.0 + 1.5	20	26.66 ± 1.66ef	6.66 ± 1.66ab
NAA + BAP			
0.1 + 0.5	45	22.96 ± 1.95de	10.37 ± 2.67b
0.2 + 0.5	45	34.07 ± 3.22f	18.51 ± 1.95cd
0.5 + 1.0	45	65.18 ± 4.50h	47.40 ± 4.12f
1.0 + 1.5	45	46.66 ± 3.39g	24.44 ± 2.56de
BAP + KIN			
0.1 + 0.1	15	13.33 ± 3.84bc	8.88 ± 2.22b
0.5 + 0.2	15	24.44 ± 2.22e	13.33 ± 3.84bc
1.0 + 0.5	15	37.77 ± 4.44fg	28.88 ± 5.87e
1.5 + 1.0	15	8.88 ± 2.22ab	4.44 ± 2.22a
NAA + BAP + KIN			
0.1 + 1.0 + 0.1	25	12.00 ± 2.30 b	10.66 ± 2.66b
0.2 + 1.0 + 0.2	25	10.66 ± 1.33b	21.33 ± 3.52d
0.5 + 1.0 + 0.5	25	8.00 ± 2.30ab	6.66 ± 2.66ab
1.0 + 1.0 + 1.0	25	5.33 ± 1.33a	5.33 ± 1.33a

The mean values having same letter(s) in a column are statistically identical. PGRs = Plant growth regulators, 2,4-D = 2,4-Dichlorophenoxyacetic acid, BAP = 6-Benzylaminopurine, NAA = 1-naphthalene acetic acid, KIN = Kinetin, - = No response/ results.

4.4.7.4 Effect of carbons and PGRs on rooting

Different strengths of full and ½MS (Murashige and Skoog, 1962) and GM medium were used for rooting in barley. In this case the maximum number of roots (11.33) per plant was recorded in GM medium that contained 1.0 mg/l IAA and minimum roots (0.67) were found in MS + 1.0 mg/l NAA (**Fig. 16**). Moreover, the highest root length (17 cm) were recorded when plantlets were grown on GM medium (**Fig. 17**). On the other hand, less number of roots produced when it was grown in ½MS medium. Furthermore, in this study three auxins *viz*. NAA, IAA and IBA were tested for rooting.

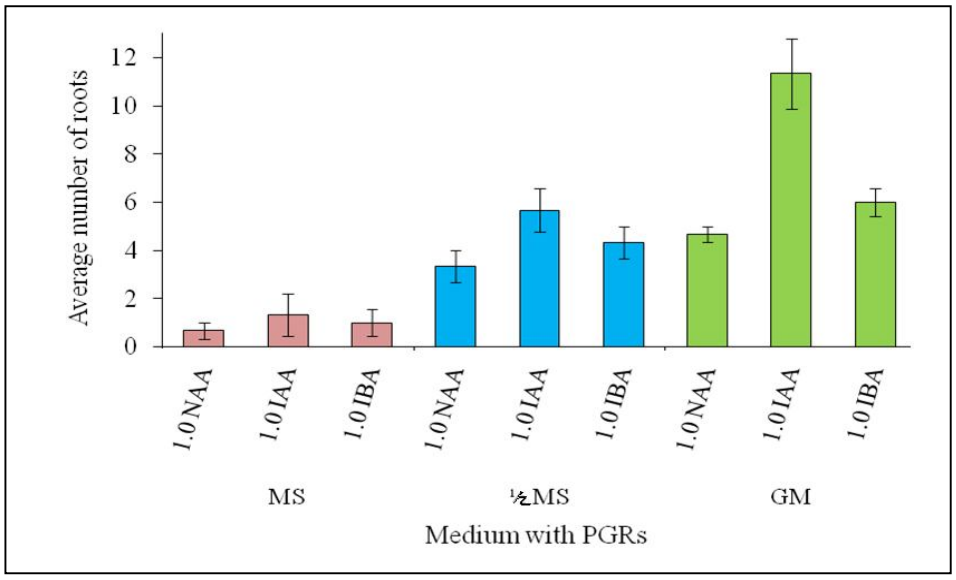

Fig. 17: Effects of media and PGRs on root formation.

4.4.8 Discussion

Several authors examined that the period of pretreatment with 2,4-D played an important role on callus induction and plant regeneration in sugarcane (Franklin et al. 2006). Results obtained from this findings indicated that the mature seeds pretreatment by 2,4-D (3.5 mg/l) that deep in sterile water and incubated them at 26ºC for 4 days showed very effective results on increasing callus induction. Sharma et al. (2005) pre-treated barley mature embryos (ME) using 3.0 or 6.0 mg/l of 2,4-D in addition with water and found that slightly higher primary callus induction (approximately 11% higher than control), but they did not observed the pretreatment

duration. On the other hand 2,4-D pretreatment for eight days was optimum for suitable regeneration in sugarcane was reported by Franklin et al. (2006). Other research on embryo culture concluded that 2,4-D solution pretreatment was necessary for efficient callus formation in wheat (Sayar et al. 1999). Fitch and Moore (1990) reported that after 10 days of auxin pretreatment, sugarcane explants produced callus in high frequency on medium fortified with BAP, indicating that they lose their amenability towards BAP after the optimum period. Yadav et al. (2011) demonstrated that the soaking of seeds in water containing picloram at 8.0 mg/l resulted in better primary callus induction response compared to soaking in plain water.

Various carbohydrates and concentration have been playing an important role in different stages of the tissue culture processes are reported by Hakam et al. (2014), Amiri and Kazemitabar (2011). In this case, among different types and concentrations (single or combined) of carbohydrates e.g. sucrose, maltose and D-sorbitol showed maximum frequency of callus induction (89.16%) when 60 g/l D-sorbitol added in MS medium followed by 60 g/l maltose (76.83%) and 30 g/l sucrose (71.16%). The present study showed that the performance of callus induction of barley was affected by both the type and concentration of carbohydrates. In general, D-sorbitol was better for callus induction than other carbon sources. Hassan et al. (2009) observed that in addition of sorbitol (20 g/l) in culture medium can increase the efficiency of callus induction in wheat. They also demonstrated that sorbitol was not only improves the quantity but enhanced the quality of callus induction. Al-Kaaby et al. (2013) focused on the positive effect of sorbitol on callus induction and somatic embryos formation in wheat. They reported that in MS medium + 20 g/l sorbitol + 30 g/l sucrose improved the callus production. There are some reports where maltose showed better results than other carbohydrates (Sharma et al. 2005; Han et al. 2011). Aguado-Santacruz et al. (2011) reported that maltose apparently interacted positively with MS, favoring the embryogenic response of barley. There are some reports about the effect of sucrose and sorbitol was scrutinized on induction of plant (Shah et al. 2014). But they have not found any combined effect of different carbohydrates. In this case, the results demonstrated that MS functioned better than N6 and B5 on regeneration. This

observation agreed well with previous works where MS medium successfully used for *in vitro* regeneration of barley (Aguado-Santacruz et al. 2011). Using 2,4-D (2.0 mg/l) and BAP (0.5 mg/l) in combination showed better embryogenic callus (70.0%) compared with 1.0 mg/l NAA + 1.0 mg/l BAP + 1.0 mg/l Kinetin (5.33%). Similar type of results obtained in maize by Jia et al. (2008). They observed that in addition of BAP was essential for embryogenic callus induction for all levels of 2,4-D. In this case, calli were sub-cultured to MS medium containing cytokinin (BAP) with low concentrations of auxin (NAA) and found 0.5 mg/l NAA and 1.0 mg/l BAP was adequate for regeneration in BARI barley-6. It was observed that 0.5 mg/l NAA + 1.0 mg/l BAP produced highest frequency of regeneration. Similarly, Nawaz et al. (2013) indicated that in addition of 0.5 mg/l NAA + 2.5 mg/l BAP produced maximum number of shoots in sugarcane. Morshed et al. (2014) reported that 1.0 mg/l BAP and 0.5 mg/l IAA were suitable for regeneration in maize.

Rooting media also play a significant role for successful plant regeneration and its subsequent development. In this case maximum roots were found in GM medium that contained 1.0 mg/l IAA. It was also observed that maximum root length (17 cm) were recorded when plants were grown in GM medium. Similar results were found in barley anther culture (Haque and Islam, 2014). On the contrary, the best growth of hairy root achieved on MS medium containing 3% or 5% sucrose in *Arnica montana* (Petrova et al. 2015). Sharmin et al. (2014) demonstrated that higher concentration of NAA suppressed root formation but lower concentration of NAA delayed on root formation.

4.5 Effect of Silver Nitrate and Amino Acids on High Frequency Plants Regeneration in Barley (*Hordeum vulgare* L.)

4.5.1 Introduction

Barley (*Hordeum vulgare* L.) is an ancient, widely distributed cereal crop in the world and also in Bangladesh. It is used for malt, feed, food and many commercially industrial foods manufacture. A reliable improvement of barley genotypes is needed to develop through *in vitro* culture that advantageous over traditional methods of propagation. It provides a source of standardized plant material for the analysis of plant metabolism and other cellular processes and responses (Taji et al. 1992). Orton (1979) reported that growth and development of barley in tissue culture, depends on media used in each phase, i.e. callus initiation, callus maintenance, and differentiation of callus into shoots and roots.

The efficiency of callus formation and plant regeneration depends on the donor plant material i.e. species or cultivars (Bregitzer, 1992). Moreover, callus quality varied considerably among genotypes (Vasil and Vasil, 1987; Islam, 2010) and most barley varieties initiate friable and translucent callus (Hanzel et al. 1985; Ward and Jordan, 2001; Chauhan and Kothari, 2004). Bregitzer et al. (1998) also reported that the poor regeneration potential of modern cultivars is one current limitation of barley transformation. Besides, it is an essential approach in biotechnology to improve plant genotypes. Indian barley cultivars are often considered as less responsive to tissue culture due to poor callus induction, low frequency of embryogenesis and lesser percentage of plant regeneration (Chauhan and Kothari, 2004). However, regeneration ability is strongly affected by several factors such as genotypes, developmental stages and composition of culture medium and type of explants in barley (Gubišová et al. 2012; Haque and Islam, 2014) and rice (Siddique et al. 2014). Different explants are used to induce callus and subsequent regeneration, such as immature embryos (Chang et al. 2003), immature inflorescence (Havrlentova et al. 2001; mature embryo (Abumhadi et al. 2005; He and Jia, 2008), coleoptile (Sahrawat and Chand, 2004) and seedling parts (Sharma et al. 2004). Klčová et al. (2004) reported that donor plant quality and environmental conditions are also influenced biotechnological research.

However, callus induction and plant regeneration potential is highly dependent upon the genotype, culture media and growth regulators in barley and other cereal crops (Goldstein and Kronstad, 1986; Bhaskaran and Smith, 1990; Islam et al. 2001; Chauhan and Kothari, 2004; Gürel et al. 2009; Khatun et al. 2010). The medium containing BAP in combination with 2,4-D showed the positive effect on regeneration in barley anther culture (Cho et al. 1998).

Recently, some reports have been mentioned that use of silver nitrate ($AgNO_3$) and/ or amino acids in media, plays an important role to improve callus induction, shoot and root formation in various plants; such as wheat (Wu et al. 2006; Bouiamrine et al. 2012), maize (El-Itriby et al. 2003), sorghum (Pola et al. 2009), pearl millet (Oldach et al. 2001) and Naga chilli (Sharma et al. 2008; Bora et al. 2014). Some authors also reported that regeneration can be improved by $AgNO_3$ in both dicots and monocots (Kumar et al. 2009; Kabir et al. 2013). Hussein et al. (2004) examined that $AgNO_3$ promotes callus formation and regeneration in barley. So far as we know that there is not enough work has been done on Bangladeshi barley cultivars using biotechnological approach. Hence, this study has been conducted to provide a suitable and efficient protocol for callus induction and regeneration using $AgNO_3$ and amino acids through immature embryos of barley in Bangladesh.

4.5.2 Sterilization and callus induction

Three barley genotypes *viz.* BARI barley-1 (BB-1), BARI barley-3 (BB-3) and BARI barley-6 (BB-6) were used for this study. The spikes of milky phase, containing immature embryos were collected and used as explants. Collection and sterilization procedures are described in the section of 2.3.2.2 (materials and methods, general).

To induce callus the embryos were aseptically dissected from the seeds, and inoculated on callus induction medium CIM containing MS (Murashige and Skoog, 1962) supplemented with different concentrations of 2,4-D (1.0, 1.5, 2.0, 2.5 and 3.0 gm/l). The inoculated petri dishes were sealed and cultured were incubated in dark chamber at 25 ± 2°C for callus induction.

4.5.3 Regeneration and rooting

When the calli ages were around three weeks old were transferred them to regeneration medium (RM = MS + 1.0 mg/l BAP) and cultured under low light conditions at 25 ± 2°C together with 14/10 hrs (light/dark) photoperiods. The regenerated shoots length around 2-3 cm were transferred to root formation medium (RFM) that was GM (modified from MS; Islam, 2000) + 1.0 mg/l IAA for better roots. The well developed rooted plantlets were transferred to pots, and acclimatized plants were transferred to field's growing up to maturity for seed collection.

4.5.4 Application of $AgNO_3$ and amino acids

To observe the effect of $AgNO_3$ and amino acids (AA) on callus induction (CI), plant regeneration (PR) and root formation (RF), five different concentrations of $AgNO_3$ singly and combinations with L-proline and/or L-glutamine were added to related media as shown in **Table 26** (CIM) and **Table 27** (RM and RFM). The combinations were added to CIM for callusing, RM for regeneration and RFM for rooting. The media without $AgNO_3$ and amino acids considered as control. Sucrose (30%), gelling agent agar (7%) were added to medium and pH adjusted 5.8.

4.5.5 Data recording and statistical analysis

The average or mean values were computed from five replicates with standard error (SE) and each experiment was repeated thrice. Analysis of variance (ANOVA) and post hoc Duncan multiple range test (DMRT) were done using SPSS16.0 software.

4.5.6 Results

4.5.6.1 Effect of 2,4-D on callus induction

Five different concentrations of 2,4-D were examined in three studied genotypes, and the results showed that BARI barley-6 performed with highest frequency of callus induction (13.86%) in MS with 2.0 mg/l 2,-4-D; followed by BRRI barley-3 (11.73%). On the other hand the lowest vale was recorded for the genotype of BARI barley-1 (**Fig. 18**). No callusing was found in control (without 2,4-D) for studied genotypes. The effect of 2,4-D levels on the genotypes showed significant difference at $P < 0.05$.

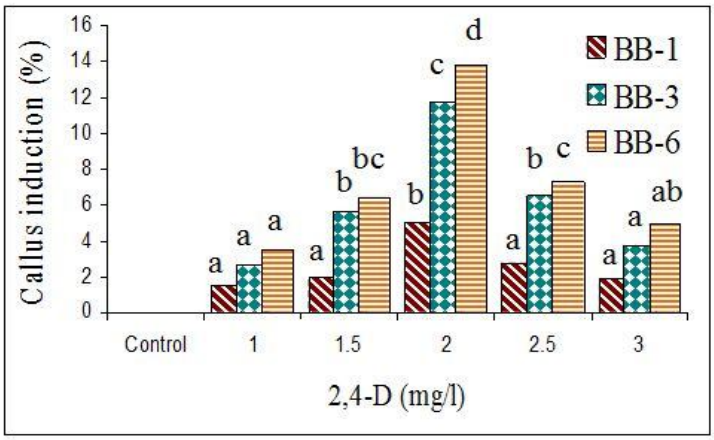

Fig. 18: Comparison of callus induction using different concentration of 2,4-D ($P < 0.05$).

4.5.6.2 Effect of $AgNO_3$ and amino acids on callus induction

Five different concentrations of $AgNO_3$ singly and/or combined with five levels of L-proline or L-glutamine were tested, and the highest frequency of callusing was recorded in 2.0 mg/l $AgNO_3$+ 200 mg/l L-proline for BARI barley-6 (49.20%); followed by BARI barley-3 (32.66%) in the same $AgNO_3$ and amino acid concentration (**Fig. 19**). The lowest value was recorded in BARI barley-3 (9.86%) when the explants were cultured on 2.5 mg/l $AgNO_3$ + 125 mg/l L-proline + 125 mg/l L-glutamine. Whereas, the frequencies of callus induction were 11.20% and 13.24% for BARI barley-3 and BARI barley-6 in control medium respectively. The recorded highest values were around 4 fold higher than the control of BARI barley-6 and 3 fold for BARI barley-3 (**Table 26**).

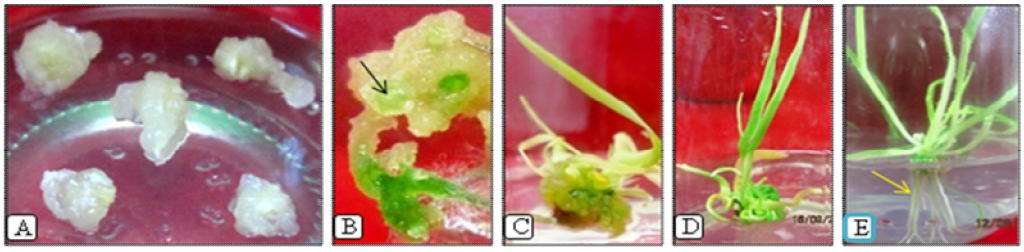

Fig. 19: Callus induction and plant regeneration of immature embryos of *Hordeum vulgare*. A) Callus induction after three weeks in CIM, B) Embryogenic callus and green spots, C) Developments of shoots, D) Well developed plantlet, E) Regenerated plants with good root and shoots.

Table 26: Effects of different concentration and combination of $AgNO_3$ and amino acids on callus induction in immature embryos of two barley genotypes (% ± SE)

Supplements (mg/l)	NIE	BARI barley-3	BARI barley-6
Control	100	11.20 ± 0.58a	13.24 ± 0.81a
$AgNO_3$			
1.0	150	12.40 ± 0.91c	14.13 ± 0.99bc
2.0	150	13.06 ± 1.02cd	15.86 ± 1.01cd
3.0	150	15.33 ± 1.22e	19.73 ± 1.20de
4.0	150	14.26 ± 1.10de	17.06 ± 1.10cd
5.0	150	13.86 ± 0.77de	16.40 ± 1.14cd
$AgNO_3$ + L-proline			
0.5 + 50	300	15. 93 ± 1.40e	17.33 ± 0.89cd
1.0 + 100	300	16.80 ± 1.07ef	20.66 ± 1.47e
1.5 + 150	300	18.46 ± 1.10g	23.86 ± 1.83f
2.0 + 200	300	17.60 ± 0.83fg	21.06 ± 1.21ef
2.5 + 250	300	16.86 ± 0.67f	18.40 ± 1.13d
$AgNO_3$ + L-glutamine			
0.5 + 50	300	20.06 ± 1.06h	21.53 ± 3.31ef
1.0 + 100	300	21.13 ± 1.09i	36.53 ± 2.28h
1.5 + 150	300	23.20 ± 1.79j	40.06 ± 2.97i
2.0 + 200	300	32.66 ± 2.13k	49.20 ± 3.11j
2.5 + 250	300	19.13 ± 0.95gh	24.66 ± 1.64g
$AgNO_3$ + L-proline + L-glutamine			
0.5 + 25 + 25	150	14.80 ± 0.82de	17.73 ± 0.80cd
1.0 + 50 + 50	150	16.53 ± 1.18ef	20.53 ± 1.76e
1.5 + 75 + 75	150	13.46 ± 0.61d	16.26 ± 1.43cd
2.0 + 100 + 100	150	12.13 ± 1.12bc	15.06 ± 0.83c
2.5 + 125 + 125	150	9.86 ± 0.71b	12.13 ± 0.71b

NIE: Number of inoculated embryos. The values followed by different letters in a column are significantly different at $P < 0.05$ according to Duncan's multiple range test (DMRT).

4.5.6.3 Effect of $AgNO_3$ and amino acids on regeneration

Different combinations of silver nitrate, L-proline and L-glutamine as mentioned earlier were tested, and maximum 37.20% plant regeneration was found in 1.5 mg/l $AgNO_3$ and150 mg/l L-glutamine for BARI barley-6. The variety BARI barley-3 performed with the frequency of 16.12% in the same level of silver nitrate and amino acids (**Table 27**). However, it was found that presence of silver nitrate or/and amino acids affected plant regeneration and enhanced the performance of studied genotypes significantly.

4.5.6.4 Effect of $AgNO_3$ and amino acids on rooting

Concentrations and combinations of $AgNO_3$ and amino acids as mentioned previously were also examined for root induction; and the maximum frequency of rooting (10.53%) was obtained in 3.0 mg/l $AgNO_3$ for BARI barley-3 and 15.73% for BARI barley-6 in the same concentration of $AgNO_3$ (**Table 27)**. No significant effect was observed in rooting comparison to the controls when amino acids were added with $AgNO_3$. Moreover, in some cases, especially in higher concentrations of $AgNO_3$ and amino acids no root induction was found in both genotypes.

Table 27: Regeneration response of immature embryos prior to culture with $AgNO_3$ and amino acids using two barley genotypes (% ± SE)

Supplements	Plant regeneration		Rooting	
(mg/l)	BB-3	BB-6	BB-3	BB-6
Control	0. 80 ± 0.20a	1.40 ± 0.24a	2.46 ± 0.35cd	3.40 ± 0.35bc
$AgNO_3$				
1.0	5. 46 ± 0.71c	7.33 ± 0.47c	3.46 ± 0.38d	5.86 ± 0.757d
2.0	6.93 ± 0.90d	10.66 ± 1.01d	6.13 ± 0.64f	9.60 ± 0.95e
3.0	8.13 ± 0.85de	13.06 ± 1.49e	10.53 ± 1.10g	15.73 ± 1.69f
4.0	7.06 ± 0.80d	11.73 ± 1.14de	4.26 ± 0.61e	6.40 ± 0.65d
5.0	6.26 ± 0.68d	8.40 ± 0.71cd	2.40 ± 0.33cd	3.86 ± 0.57c
$AgNO_3$ + L-proline				
0.5 + 50	1.60 ± 0.24ab	2.66 ± 0.43ab	0.60 ± 0.12a	0.93 ± 0.19a
1.0 + 100	2.13 ± 0.42ab	2.93 ± 0.46ab	0.93 ± 0.19ab	1.53 ± 0.22ab
1.5 + 150	3.06 ± 0.53ab	4.13 ± 0.77b	1.33 ± 0.23b	2.06 ± 0.28b
2.0 + 200	2.26 ± 0.37ab	3.86 ± 0.53b	0	0
2.5 + 250	1.33 ± 0.29ab	2.13 ± 0.34ab	0	0
$AgNO_3$ + L-glutamine				
0.5 + 50	9.46 ± 1.42ef	15.26 ± 1.28f	0.73 ± 0.19a	1.33 ± 0.23a
1.0 + 100	12.06 ± 1.09g	24.93 ± 2.46g	1.06 ± 0.28ab	2.46 ± 0.47b
1.5 + 150	16.13 ± 1.34h	37.20 ± 2.19i	2.13 ± 0.50c	3.40 ± 0.35bc
2.0 + 200	10.66 ± 1.01f	28.46 ± 2.76h	0.66 ± 0.18a	1.06 ± 0.19a
2.5 + 250	8.40 ± 0.92e	12.53 ± 1.25e	0	0
$AgNO_3$ + L-proline + L-glutamine				
0.5 + 25 + 25	3.73 ± 0.54bc	5.46 ± 0.77bc	1.73 ± 0.33bc	2.93 ± 0.33b
1.0 + 50 + 50	5.86 ± 0.57cd	6.13 ± 0.99bc	0.93 ± 0.16ab	1.46 ± 0.24ab
1.5 + 75 + 75	3.33 ± 0.47b	4.13 ± 0.57b	0	0
2.0 + 100 + 100	2.26 ± 0.33ab	3.06 ± 0.33ab	0	0
2.5 + 125 + 125	1.73 ± 0.45ab	2.93 ± 0.49ab	0	0

The values followed by different letters in a column are significantly different at $P < 0.05$ according to Duncan's multiple range test (DMRT).

4.5.7 Discussion

Castillo et al. (1998) successfully regenerated plants at earlier through *in vitro* system in barley. Zapata et al. (2004) optimized 2,4-D levels as 2.0 mg/l and reported approximately similar results on callus induction using mature embryos of barley. The same level of 2,4-D (2.0 mg/l) gave the best response in maize (Jakubeková et al. 2011), wheat (Pourmohammad, 2013) and chickpea (Zaman et al. 2010) which were also similar to our findings. Some studies have shown that 2,4-D is an important factor for callus initiation and proliferation of primary and embryogenic callus from immature embryos in maize (Carvalho et al. 1997; Manivannan et al. 2010). However, in the present investigation, 1.46 to 13.86% callus induction was recorded. The wide range of variability might be occurred due to genotypic effect along with the different levels of 2, 4-D. It has been reported that the frequency of callus induction, friability of embryogenic calli and regeneration are influenced by genotype, culture media and genotype × culture media interaction in barley (Bregitzer et al. 1998; Manoharan and Dahleen 2002), maize (Zhu, 2011; Jakubeková et al. 2011), wheat (Farshadfar, 2014; Islam et al. 2001), rice (Siddique et al. 2014; Sah and Kaur, 2013), citrus (Gholami et al. 2013), sorghum (Indra and Krishnaveni, 2009) and pearl millet (Jha et al. 2009). However, it could be suggested that among the 2,4-D levels (1.0 - 3.0 mg/l) tested, middle concentrations are comparatively better than lower and higher ones for callus induction in barley.

Fernandez et al. (1999) recorded enhanced frequency from immature embryo culture in durum wheat at large scale using $AgNO_3$. Chauhan and Kothari (2004) reported that Indian barley cultivars are often considered as less responsive to tissue culture due to poor callus induction, low frequency of embryogenesis and lesser percentage of plant regeneration. In our investigation, Bangladeshi barley cultivars also showed poor callusing in control conditions which can be overcome by addition of $AgNO_3$, L-proline and L-glutamine to callus induction medium at recommended above levels. Furthermore, different levels and combinations showed significantly different effects to studied barley cultivars. In previous report, it was suggested that concentrations of $AgNO_3$ is strongly depended on species and genotypes (Cristea et al. 2012; Al-Khayri and Al-Bahrany, 2004). L-glutamine concentration had a noticeable effect in *Eragrostis tef* (Gugsa and Kumlehn, 2011). However, most of the authors reported

similar positive effect of $AgNO_3$, proline and glutamine in various plants species; such as glutamine (Hunter, 1987; Mordhorst and Lörz, 1993), L-glutamine, casein hydrolysate and L-proline (Ganeshan et al. 2006), casein hydrolysate and L-proline (Chauhan et al. 2007), glutamine, L-asparagine and CH (Bi and Wang, 2008), aspartic acid, glutamine, proline, tryptophan and casein hydrolysate (Yu et al. 2008) proline, glutamine and asparagine (Islam and Tuteja, 2012; Haque and Islam, 2014). On the contrary, an inhibitory effect of glutamine has been observed in the production of barley pollen callus by Xu and Sunderland (1981).

Purnhauser et al. (1987) first reported that $AgNO_3$ (10.0 mg/l) effectively promoted regeneration in wheat. In barley cultivar Morex, almost doubled regeneration was recorded using $AgNO_3$, while 1.5 folds for Golden Promise (Jha et al. 2007). Moreover, addition of $AgNO_3$ increased the frequency of plant regeneration significantly in various plants species such as Pearl millet (Oldach et al. 2001), Naga chilli (Bora et al. 2014; Sharma et al. 2008), wheat (Bouiamrine et al. 2012; Wu et al. 2006), maize (El-Itriby et al. 2003; Huang and Wei 2004), sorghum (Pola et al. 2009). In our investigation around 27 folds higher regeneration was recorded in 1.5 mg/l $AgNO_3$ and 150 mg/l L-glutamine for BARI barley-6. Anantasaran and Kamnoon (2008) found almost similar results from *Zinnia* cultivars by adding 2.0 mg/l $AgNO_3$. Gubišová et al. (2012) obtained improved regeneration and were observed significant differences in Slovak spring barley cultivars. On the contrary, no positive effect of $AgNO_3$ on plant regeneration was found using immature embryos culture in barley by Hussein et al., (2004). Pua et al. (1999) suggested that Ag^+ might interfere with polyamines. Our observations argues with their reports and suggested that $AgNO_3$ clearly affect the regeneration positively and addition of L-glutamine gives more better results especially at 1.5 mg/l $AgNO_3$ and 150 mg/l L-glutamine.

Among the concentrations of $AgNO_3$ individually added, maximum 13.06% regeneration was found in BARI barley-6 at 3.0 g/l level which was around 10 fold higher than the control (1.40%). It was also observed that higher and lower concentrations than 3.0 g/l of $AgNO_3$, gave lower frequencies in both genotypes BARI barley-3 and BARI barley-6. Furthermore, addition of L-glutamine with $AgNO_3$ gave remarkable higher results in comparison with control. By using glutamine as a nontoxic nitrogen source, higher frequency of green plants was

obtained in barley genotypes (Olsen, 1987). Islam (2000) and Islam and Tuteja (2012) found that 0.5 g/l L-glutamine significantly promoted shoot regeneration in wheat androgenetic research. Glutamine and and/or L-proline promotes plant regeneration in microspore cultures of rice (Cho and Zapata, 1988; Ogawa et al. 1995). In barley, addition of complex amino acid mixture, improved the rate of androgenic plants production in barley (Ouédraogo et al. 1998).

Efficient regeneration of plants from embryogenic barley callus often is limited to specific genotypes that exhibit vigorous plant regeneration (Bregitzer et al. 1995; Przetakiewicz et al. 2003). In our study it was observed that addition of $AgNO_3$ gave better performance than control ($AgNO_3$ > Cont.). As such the influence of examined combinations could be recommended as ascending order i.e. ($AgNO_3$ and L-glutamine) > $AgNO_3$ > ($AgNO_3$, L-proline and L-glutamine) > (L-glutamine and L-proline) > Control, for plant regeneration through *in vitro* culture in barley.

No significant effect was observed in rooting comparison to the controls when amino acids were added with $AgNO_3$. Our findings agreed well with the reports as noticed in *Rotula aquatica* (Lour) where 2.67 mg/l of silver nitrate gave the improved frequency of rooting (Sunandakumari et al. 2004). Kumar et al. (2009) and Reddy et al. (2001) also observed that the positive effect of $AgNO_3$ for root formation in barley genotypes. They obtained higher values of rooting frequency using 9.12 mg/l of $AgNO_3$ in which elongation of roots were also increased. In my study, 2-5 folds increased rooting was recorded when $AgNO_3$ was individually added in various concentrations to rooting medium. On the contrary, the efficiency of $AgNO_3$ to influence the root induction was reduced when any amino acids were combined with $AgNO_3$. Silva et al. (2011) mentioned that although the effect of $AgNO_3$ is not well understood, it is supposed that the silver ion binds a possible ethylene receptor at the plasma membrane, thus inhibiting the binding of ethylene to this receptor and consequently triggering the specific action of the hormone.

4.6 Considering Callus Age and Size in Barley (*Hordeum vulgare* L.) Improves Regeneration Efficiency

4.6.1 Introduction

Barley is regarded as an inferior staple compared to wheat and is considered as the poor people's bread. Economically, is a major commodity in many European and North African countries (Elsayed, 2013). In recent years, advances in plant biotechnology have opened new avenues for crop improvement. The success of *in vitro* development of plants relies on several factors which include an efficient tissue culture system, for regeneration of plants from cultured cells and tissues (Khatun et al. 2012; Kumar et al. 2009). Successful callus induction and regeneration has been dependent of using efficient explants and different pre-treatments factors that are also influencing somatic and gametic embryogenesis in many cereal crops (Rakshit et al. 2010; Islam and Tuteja, 2012; Farshadfar et al. 2014). However, regeneration ability is strongly affected by several factors such as genotypes, developmental stages and composition of culture medium and type of explants (Gubišová et al. 2012; Haque and Islam, 2014; Siddique et al. 2014). Different explants are used for efficient callus induction and its subsequent regeneration e.g. immature embryos (Chang et al. 2003), immature inflorescence (Havrlentova et al. 2001), mature embryo (Abumhadi et al. 2005). Age of callus and embryo size is also playing an important role on regeneration as reported by Saad et al. (2004), Senarath (2007) and Islam (2010).

So far as I know there is no report on plant regeneration using age groups of callus and immature embryo sizes for developing *in vitro* somatic embryogenesis on barley. Under this study, using embryos size derived immature seeds, callus age and their weight have been considered for improving regeneration systems in barley, for advance biotechnological research.

4.6.2 Sterilization and callus induction

In order to obtain immature embryos, seeds of BARI barley-6 were grown in the experimental field for this study. Collection and sterilization procedures of spikes are described in the section of 2.3.2.2 (materials and methods, general).

4.6.3 Embryo size effects on callus induction and regeneration

The effect of the size of embryos: (A) 0.6-1.0 mm, (B) 1.1-1.5 mm, (C) 1.6-2.0 mm and (D) 2.1-2.5 mm, on callus induction and plant regeneration was studied for immature embryos derived from seeds. The embryos of each size were cultured on MS (Murashige and Skoog, 1962) medium supplemented with 2.0 mg/l 2,4-D, 200 mg/l L-glutamine and incubated in dark chamber at 25 ± 2°C. The pH of the medium was adjusted at 5.6-5.8. When the calli ages were around four weeks old, they were transferred to regeneration medium (MS + 1.0 mg/l BAP + 150 mg/l L-glutamine) and cultured under low light conditions at 25 ± 2°C together with 14/10 hrs (light/dark) photoperiods. Data on callusing was recorded after four weeks of immature seeds inoculation and regeneration frequency was recorded after eight weeks of culture, respectively.

4.6.4 Effects of callus age and weight on regeneration

To observe regeneration efficiency of callus age and its fresh weight calli were divided into three age groups: early (1-3 weeks), medium (4-6 weeks) and prolonged duration (7-12 weeks). After callus initiation cultured them to the same medium for regeneration (MS + 1.0 mg/l BAP + 150 mg/l L-glutamine). When the calli ages were around five weeks old, the calli were individually weighted and grouped into four categories: I (50-100 mg), II (101-150 mg), III (151-200 mg) and IV (>201 mg). Each callus were weighted individually and for each group around 25-75 numbers of callus were taken. Five weeks old calli were considered for callus weight. Then cultures were incubated around twelve weeks at 25 ± 1°C with a 16/8 hr (light/dark) photoperiod, for callus induction.

4.6.5 Rooting and acclimatization

Regenerated shoots were transferred to GM (Islam, 2000) medium supplemented with 1.0 mg/l IAA and 20 g/l sucrose. For rooting, 3 g/l phytagel were used as gelling agent. To evaluate the root formation, average number of roots per plants were recorded and calculated. Well rooted plantlets were transferred to pots that contained peat moss and soil (1:1) and acclimatized.

4.6.6 Data recording and statistical analysis

For each treatment, three replications were evaluated and each experiment was repeated three times. Statistical analysis of the data was performed by SPSS software (version 16). A one-way analysis of variance (ANOVA) was done to evaluate the effect of callus age and weight on regeneration. Within the treatment groups, the differences among means were compared by Duncan's multiple range tests (DMRT).

4.6.7 Results

In this study, the effects of callus age, fresh weight of callus and their interactions on regeneration and rooting traits were tested. Stages of callus development and their subsequent regeneration are illustrated in **Fig. 20**.

4.6.7.1 Effects of embryo size on callus induction and regeneration

To observe the effect of the size of embryos to regeneration, immature embryos derived from seeds were classified into four groups and recorded data were shown in **Fig. 21.** It was observed that the size of 1.6-2.0 mm (C) showed significantly the highest percentage of callus induction as well as plant regeneration. The highest frequencies were recorded as 56.83% and 42.31% for callus induction and plant regeneration respectively, when 1.6-2.0 mm (C) embryo size was used. Whereas 0.6-1.0 mm in length showed less (2.85 ± 0.56) callus induction and no regeneration was found. It was observed that when the embryo size was 2.1-2.5 mm regeneration decreased.

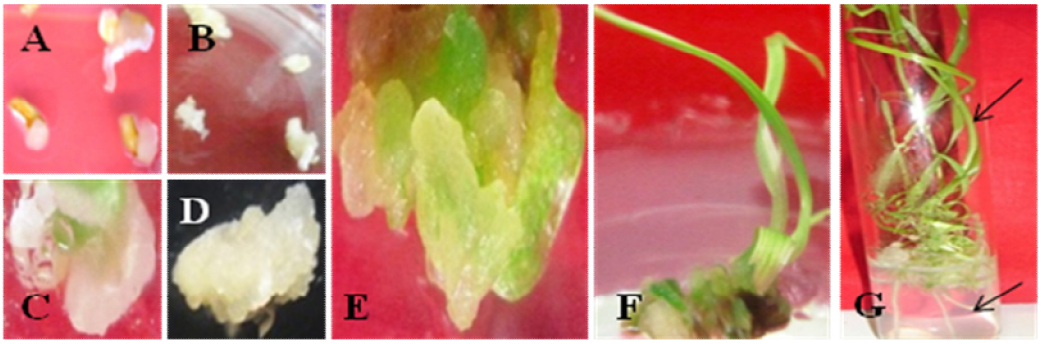

Fig. 20: Stages of callus development and regeneration from immature embryos in barley. (A) Callus initiation after 3 weeks of seed inoculation, (B-D) Callus development after 1-3, 4-6 and 7-12 weeks of inoculation, (E) Callus with green structures, (F) Development of shoots, (G) Regenerated plants with good root and shoots.

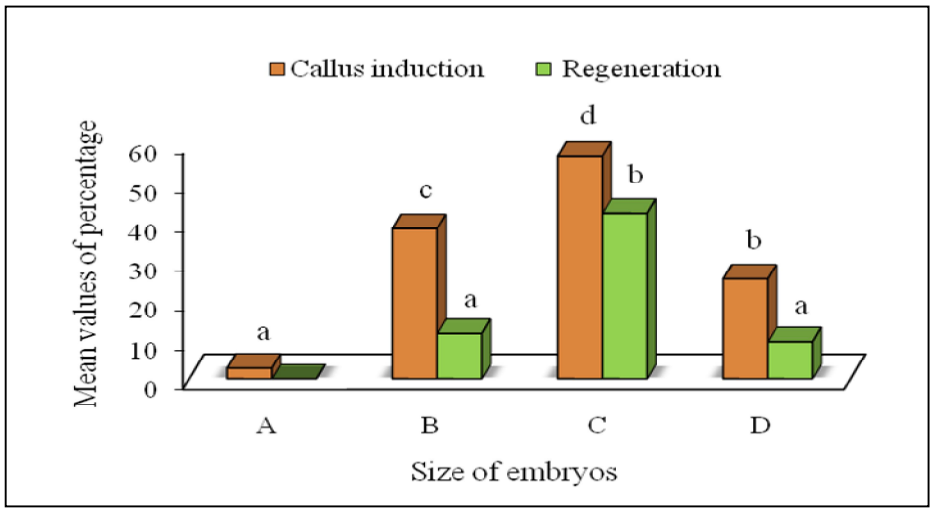

Fig. 21: Comparison of callus induction and regeneration among the different sizes of immature embryos ($P < 0.05$); A = 0.6-1.0 mm, B = 1.1-1.5 mm, C = 1.6-2.0, D = 2.1-2.5 mm size of embryos.

4.6.7.2 Effect of age and fresh weight of callus on shoots and roots development

In the current experiment it was observed that the fresh weight 151-200 mg (III) of callus showed significantly the highest percentage of green plantlets and roots (**Table 28**). Whereas small 50–100 mg (I) and large (>201 mg) (IV) weight showed less regeneration and roots. Moreover, when the calli were transferred to regeneration medium within 4-6 weeks (medium age) showed significantly a higher percentage of green plantlets along with good shoots and roots. But when early (1-3 weeks) and prolonged aged (7-12 weeks) calli were transferred to regeneration medium, plant regeneration decreased. Among the different callus ages and fresh weights, the callus weight of 151– 200 mg (III) and the callus age of 4-6 weeks (medium age) showed better regeneration (62.66 %) and rooting (53.77%).

The effects of callus age, fresh weight of callus and their interactions on regeneration and rooting were tested at $P<0.01$ level of significance by F-test (**Table 29**) and were found to be highly significant. It was also found that the fresh weight of callus influenced the regeneration efficiency. Callus weight of 151– 200 mg (III) yielded more plantlets than other fresh weights of the same age calli. Furthermore, the roots of

the plants were strong and healthy when 151-200 mg (III) weight and the 4-6 weeks (medium age) old callus was cultured. Therefore, the registered data proved that callus age and fresh weight are important factors for the production of increased plantlets and roots in barley.

Table 28: Effect of age and fresh weight of callus derived from immature embryos on plant regeneration and rooting

Fresh weight of callus (mg)	Age of callus (weeks)	No. of callus	Mean of percentage ± S.E	
			Regeneration	Rooting
Group-I (50-100)	1-3	25	5.33 ± 1.33	4.0 ± 2.30
	4-6	25	22.66 ± 3.52	18.66 ± 3.52
	7-12	25	9.33 ± 2.66	6.66 ± 1.33
Group-II (101-150)	1-3	75	10.22 ± 1.60	8.00 ± 1.53
	4-6	75	32.88 ± 2.70	24.44 ± 2.70
	7-12	75	13.77 ± 1.93	10.22 ± 1.60
Group-III (151-200)	1-3	75	21.77 ± 2.47	17.33 ± 2.30
	4-6	75	62.66 ± 4.28	53.77 ± 3.20
	7-12	75	34.22 ± 3.47	23.55 ± 3.11
Group-IV (201>)	1-3	50	15.33 ± 1.76	11.33 ± 0.66
	4-6	50	45.33 ± 4.05	34.0 ± 3.05
	7-12	50	18.0 ± 2.30	14.0 ± 1.15

Table 29: ANOVA for the effect of age and fresh weight of callus on plant regeneration and rooting

Source of Variation	df	Regeneration		Rooting	
		MS	F. value	MS	F. value
Callus weight	3	1205.84	65.31**	797.597	48.503**
Callus age	2	2571.07	139.26**	1795.33	109.176**
Callus age × weight	6	88.40	4.78**	83.624	5.085**
Error	24	18.46		16.444	

** indicating significant at P < 0.01.

4.6.8 Discussion

The present results on the influence of embryo size on callus induction and regeneration agreed well with the findings of Senarath (2007) and Jakubeková et al. (2011). Senarath (2007) mentioned that embryos of 1.6-2.0 mm showed the highest ability to produce callus capable of regenerating green plants. The percentage of primary and embryogenic callus formed from immature embryos of 3-4 mm was lower than that formed from 1-2 mm long embryos in maize (Jakubeková et al. 2011). This was probably due to a reduction in the meristematic activity of cells once with ageing, suggesting that the physiological and developmental state of immature embryos is important in determining callus initiation response. Bohorova et al. (1995) reported that maize embryo less than 0.5 mm in length did not showed any plant regeneration. Similar types of results found by Lu et al. (1983) when they were used embryo size less than 1 mm in maize. Whereas, barley embryos (0.5 mm to 2.0 mm) and (0.7 mm to 1.77 mm) in length produced rapidly growing callus with high frequency of plant regeneration (Dale and Deambrogio, 1979; Hanzel et al. 1985).

However, it differs from the observation of Islam (2010), where more green plantlets were produced from large (>2.0 - 3.0 mm) embryos in the case of wheat anther

culture, which is different than the findings within the present results. Indra and Krishnaveni (2009) observed that 0.8 - 1.4 mm size of embryos yielded more embryogenic calli in sorghum. Gugsa and Kumlehn (2011) demonstrated intermediate sized (0.2–0.35 mm) embryos of tef (*Eragrostis tef)* produced significantly more root and shoots than the small (0.1 - 0.2 mm) or large (0.35 - 0.75 mm) ones.

Generally, calli with earlier ages have more totipotency as compared to old ages calli, as reported by Rashid et al. (1994). Quainoo (2011) demonstrated that somatic embryos induced from callus tissues aged between 4-8 weeks showed no viral infection in cocoa shoots, but the virus infected the somatic embryos induced from older callus tissues. In this study, it was observed that the best age group for regeneration as well as rooting was that of 3-4 weeks. One to two weeks old calli was either too small or fragile that they cannot survive, so their regeneration and rooting frequency is lesser (Raja et al. 2009). On the other hand, 5-6 weeks old callus has lost their regeneration and rooting ability, possible due to repeated cell divisions. Therefore, it was clearly demonstrated that medium age of callus transferred into regeneration medium within 4-6 weeks was more efficient for plant regeneration in comparison with earlier ages (1-3 weeks) or prolonged culture (7-12 weeks). Similar type of result was reported for wheat by Raja et al. (2009), who observed that the best age for regeneration was between 22 to 30 days old calli. Similarly, Islam (2010) reported that an early transfer of embryos into the regeneration medium, within three - five weeks, was more efficient for regeneration of green plantlets in comparison to prolonged culture (6-8 weeks) in wheat anther culture.

Chapter V

Androgenesis (Anther culture)

5. Androgenesis (Anther culture)

5.1 Optimization of Media and Cold Pretreatment Duration to Enhance Anther Culture Response in Two Barley Genotypes

5.1.1 Introduction

Barley (*Hordeum vulgare* L.) is the fourth grain crop in the world and in Bangladesh ranks third after rice and wheat and used as supplementary food and fodder crops (FAOSTAT, 2010). Recently barley cultivation in Bangladesh is increasing due to climate change and for late harvesting of preceding crops. For barley improvement, modern biotechnology allows the androgenetic process to accelerate breeding specially for self-pollinating cereal crops. Use of unconventional techniques for doubled haploid production through anther and microspore culture is one of the most widely used methods (Khush and Virmani, 1996; Maraschin et al. 2005; Islam et al. 2013). Anther culture allows a rapid production of appropriate genotypes for breeding purposes in an effort to identify promising homozygous lines within shortening of time (Redha et al. 2000; Lazaridou et al. 2011; Islam et al. 2013b).

For successful development of doubled haploids various approaches are important e.g. i) microspores become embryogenic by changing from the programmed gametophytic pathway to sporophytic pathway, and ii) induction of embryogenesis and plant regeneration (Obert et al. 2009; Tadesse et al. 2012). Maheshwari et al. (1980) stated that certain physical and chemical treatments given to flower buds or anthers prior to culture can be highly inductive for the development of microspores into embryos. Recent success on microspore development and embryogenesis that might be enhanced by different stress pretreatment factors are reported in some cereal crops (Żur et al. 2009; Islam, 2010; Thakur et al. 2010; Islam and Tuteja, 2012; Islam and Tuteja, 2013; Rukmini et al. 2013). Induction of haploids through isolated microspores can be limited by various factors and is genotype-dependent (Dunwell et al. 1985; Datta, 2005; Kunz et al. 2000).

Most efforts toward improving anther culture have focused on different stress pretreatments to induce androgenesis by redirecting the reprogrammed gametophytic

to the sporophytic development pathway (Hu and Kasha, 1999; Zamani et al. 2003; Soriano et al. 2007; Muñoz-Amatriain et al. 2009; Islam and Tuteja, 2012). Genotypic dependency still plays an important role for androgenesis and *in vitro* tissue culture work of many cereal and other crops. There are some reports on androgenetic research about the cold pretreatment of anthers either pre- or post-culture treatment (3 to 5^{o}C for 2 to 4 days), symmetric rather than asymmetric division of the microspore nuclei or division of the vegetative nucleus in some crops (Touraev et al. 1997; Slama-Ayed, 2010; Rukmini et al. 2013). Obert and Barnabás (2004) suggests that cold or heat acts as a shock treatment causing a 90° shift in division plane of microspore causing a symmetrical division that enhancing anther culture response. The induction of embryo from cultured anthers and production of haploid plants from embryoids has been reported by many researchers (Jacquard et al. 2006; Islam, 2010c; Sen et al. 2011; Khatun et al. 2012; Moqbeli et al. 2013; Khound et al. 2013).

In case of cereal crops last twenty years the number of green plants has been improved 100-1000 times (Touraev et al. 1996; Wojnarowiez et al. 2002; Asakavičiūtė and Pašakinskienė, 2006; Shariatpanahi and Touraev, 2010; Lazaridou et al. 2011; Islam et al. 2013b). But success in the anther culture depends on first genotype and then duration of cold pretreatment, genotypes and growth medium (Niroula and Bimb, 2009; Kahrizi et al. 2011; Ślusarkiewicz-Jarzina and Ponitka, 2007). It appears that optimum media and pretreatment conditions vary from genotype to genotype. Other important factor appears to be the cold pretreatment to androgenesis in the development of callus from anthers (Oleszczuk et al. 2006; Żur et al. 2009; Islam and Tuteja, 2012).

Lazaridou et al. (2005) reported that the combination of the FHG medium with 28 days cold pretreatment was the most efficient in embryoids formation, total and green plant production in Greek barley cultivars. A problem affecting the efficiency of androgenesis in barley is the production of albino plantlets in various proportions according to the cultivars and still is a great problem and it has no agronomic value (Caredda et al. 1999; 2000; Cistué et al. 2004; Lazaridou et al. 2005; Wietholter et al. 2008; Islam, 2010b). However, for enhancement of embryoids induction, its

subsequent regeneration and reducing albinism is very important about testing the influence of genotype on barley androgenesis. Therefore, the present research has been undertaken to find out a suitable androgenetic genotypes, to determine the effect of suitable duration of cold pretreatment and optimization of media on barley anther culture.

5.1.2 Plant materials

Two barley genotype: BARI barley-6 and BARI barley-3 were considered for the present experiments purpose for its good response to anther culture (Section 4.1). Plants were grown in the research field of Institute of Biological Sciences, University of Rajshahi and spikes were collected.

5.1.3 Harvesting and cold pretreatment

Harvesting procedures are described in the section of 2.3.3.3 (materials and methods, general). To optimize the cold pretreatment duration the harvested spikes were wrapped in wet tissue paper and kept them at 4°C in dark for 2 (T_1), 4 (T_2), 6 (T_3), 8 (T_4), 10 (T_5), 12 (T_6), 14 (T_7) and 16 (T_8) days. Harvested spikes without cold pretreatment were considered as control.

5.1.4 Media for embryoids induction

Five induction media *viz*., MS (Murashige and Skoog, 1962), B5 (Gamborg et al. 1968), N6 (Chu, 1975), FHG (Hunter, 1987) and AMS_3 (Islam, 2000) were used for embryoids induction are shown in previous section 2.2, **Table 7** (materials and methods, general). The pH of all media was adjusted 5.8.

5.1.5 Sterilization and inoculation of anthers

After cold pretreatment, spikes were sterilized for 45 seconds to maximum 1 minute by 70% ethanol in laminar air-flow cabinet. Anthers from the central part of the spikes were cultured in semisolid induction medium. In order to obtain semisolid medium 3 g/l phytagel (Sigma) were used before autoclaving. The petri dishes were sealed with parafilm and maintained in the culture chamber at 26 ± 2°C, in the dark for 4-8 weeks.

5.1.6 Embryoid induction and regeneration

The petri dishes used for culture were examined at weekly intervals to observe the progress in embryoid formation. Anther derived embryoids were transferred to the MSR medium (modified form of MS, Islam, 2000) for regeneration. The petri dishes were maintained in the culture chamber at 25°C with a 16/8 hour photoperiod for plant regeneration for around 2-3 weeks. To induce growth of a strong root system, well-developed plantlets (2-3 cm in length) were transferred to rooting medium (GM, modified from MS, Islam, 2000). The components of GM medium are shown in previous section 2.2, **Table 7** (materials and methods, general). When the root length of plants was around 5-7 cm and good shoot growth was observed, they were transferred to pots after a period of acclimatization.

5.1.7 Data recording and statistical analysis

Data were recorded on the basis of frequency of callus induction, total regenerated plantlet, green and albino plants. Significance of cold pretreatment, medium and genotype effects on callus formation and also medium × pretreatment, genotype × medium and genotype × pretreatment interaction were analyzed using three-way analysis of variance (ANOVA). Comparison between mean values of pretreatments was made by BONFERRONI test. Statistical analysis has been performed by SPSS programme.

5.1.8 Results

5.1.8.1 Effects of cold pretreatment and medium on embryoid induction

It was observed that in most cases embryoids observed within four to eight week of culture initiation. The effects of cold pretreatment and medium type on embryoid induction are shown in **Table 30**. For both genotypes fewer embryoids were recorded if spikes were not cold pretreated or if a long (16 days) pretreatment was used. This experiment showed that embryoid formation may be affected by the duration of cold pretreatment, media and genotype. Between the responding genotypes, BB-6 showed a higher frequency of embryoid induction (14.6%) in FHG medium with the T_5 (10 day) cold treatment, than BB-3, where the frequency was 5.89% (**Table 30**). It was observed that fewer embryogenic structures were observed in AMS_3, MS, N6 and B5 media in comparison to FHG. Among the mentioned treatments T_4, T_5 and T_6

produced the highest number of embryoids in FHG medium for both genotypes. On the basis of our findings it may be concluded that, out of five media tested, FHG was the best choice for anther culture response using two cultivars of barley. It was observed that in all cases the embryoids were yellowish in color, compact and non-friable. Their appearance was nodular, formed by globular structures, as shown in **Fig. 22**. Analysis of variance (ANOVA) showed that highly significant differences in the frequency of embryoids induction were produced depending on the duration of cold pretreatment, culture media and between the genotypes. It was also observed that the interactions of medium and treatment, and genotype and medium, were significant but the genotype and treatment interaction was not significant (**Table 31**).

Table 30: Effect of cold pretreatment and media on embryoids induction of two barley genotypes (± SE)

Variety	Treatment	Media				
		MS	B5	N6	FHG	AMS_3
BB-3	Cont.	0.21 ± 0.10	0.10 ± 0.10	0.11 ± 0.11	0.44 ± 0.11	0.21 ± 0.10
	T_1	0.76 ± 0.10	0.48 ± 0.12	0.8 ± 0.23	1.11 ± 0.29	0.98 ± 0.18
	T_2	0.80 ± 0.23	0.68 ± 0.13	0.93 ± 0.18	1.72 ± 0.12	1.13 ± 0.21
	T_3	1.23 ± 0.17	0.77 ± 0.29	1.28 ± 0.25	3.46 ± 1.31	1.54 ± 0.11
	T_4	2.54 ± 0.21	1.98 ± 0.23	2.58 ± 0.36	3.14 ± 0.12	2.75 ± 0.11
	T_5	3.79 ± 0.34	2.06 ± 0.19	3.01 ± 0.21	5.89 ± 0.33	4.24 ± 0.32
	T_6	2.03 ± 0.28	1.17 ± 0.39	1.63 ± 0.42	3.22 ± 0.18	2.73 ± 0.21
	T_7	1.04 ± 0.13	0.48 ± 0.12	1.06 ± 0.13	1.88 ± 0.22	1.31 ± 0.18
	T_8	0.43 ± 0.10	0.48 ± 0.12	0.93 ± 0.26	1.33 ± 0.19	0.98 ± 0.18
BB-6	Cont.	0.21 ± 0.10	0.12 ± 0.12	0.13 ± 0.13	0.55 ± 0.11	0.43 ± 0.10
	T_1	0.75 ± 0.10	0.43 ± 0.10	0.66 ± 0.19	1.49 ± 0.11	1.66 ± 0.19
	T_2	0.97 ± 0.12	0.53 ± 0.13	0.76 ± 0.22	2.69 ± 0.22	1.83 ± 0.11
	T_3	1.56 ± 0.22	0.98 ± 0.18	1.60 ± 0.23	3.82 ± 0.10	2.41 ± 0.19
	T_4	3.07 ± 0.17	2.26 ± 0.21	2.41 ± 0.19	8.26 ± 0.13	5.03 ± 0.33
	T_5	4.21 ± 0.40	2.43 ± 0.12	3.21 ± 0.44	14.6 ± 0.12	7.14 ± 0.20
	T_6	2.30 ± 0.22	1.33 ± 0.24	1.78 ± 0.35	8.42 ± 0.20	5.35 ± 0.21
	T_7	1.88 ± 0.21	0.97 ± 0.43	1.60 ± 0.12	2.90 ± 0.21	2.06 ± 0.19
	T_8	1.00 ± 0.12	0.43 ± 0.10	0.95 ± 0.13	1.92 ± 0.22	1.19 ± 0.19

Cont. = without cold pretreatment, T_1 = 2, T_2 = 4, T_3 = 6, T_4 = 8, T_5 = 10, T_6 = 12, T_7 = 14 and T_8 = 16 days at 4°C.

Table 31: Analysis of variances (ANOVA) showing the effect of cold pretreatment, media and genotypes

Sources	Sum of squares	df	Mean square	F. value
Genotype (G)	16.77	1	16.77	19.27**
Medium (M)	83.25	4	20.81	23.92**
Cold (C)	185.06	8	23.13	26.58**
G × M	18.91	4	4.72	5.42**
G × C	14.87	8	1.86	2.13^{NS}
M × C	60.88	32	1.90	2.18*
Error (G × M × C)	28.10	32	0.87	
Total	774.71	90		

* and ** indicating significant at 0.05 and 0.01 level respectively, NS = Non significant.

5.1.8.2 Effects of cold pretreatment on regeneration

The effect of cold pretreatments on plant regeneration, using MSR medium, was recorded and results are presented in **Table 32**. Anther-derived embryoids and their subsequent regeneration are illustrated in **Fig. 22 (A-F**). The highest proportion of green plants was recorded in BB-6 (10.72%) and in BB-3 (6.73%). The percentage of total regenerated plantlets, green and albino plants are shown in **Table 32**. The results indicate that cold pretreatment was essential for the induction of embryos and regeneration of plants in barley anther culture. We observed that regeneration may be affected by the duration of cold pretreatment and also by the use of a suitable genotype. Between the genotypes tested, BB-6 showed better performance in terms of plant regeneration (13.8%) when subjected to cold pretreatment for 10 days (T_5) than did BB-3 (10.85%). **In Table 33**, most of the treatment groups (T_3-T_7) showed significantly increased embryoid induction in comparison to the control, T_1, T_2 and T_8. However, in most of the treatments the number of regenerated plantlets, green and albinos plants for both genotypes did not differ significantly from the control except in the number of regenerated plantlets in T_3, T_4, T_5 and T_6 (**Table 33**). The

frequencies in T_3, T_4 and T_5 treatment groups were significantly different at 0.05 probability levels for embryoid induction and regeneration. The T_5 treatment group proved to be the most effective condition for embryoid induction and regeneration, with frequencies significantly different from other treatments and the control.

Table 32: Regeneration frequency from cold treated anther derived embryoids (% ± SE)

Treatment (Cold)	Variety					
	BB-6			BB-3		
	TRP	GRP	ALP	TRP	GRP	ALP
Cont.	1.84 ± 0.13	0.12 ± 0.10	1.72 ± 0.24	1.25 ± 0.12	0.10 ± 0.09	1.15 ± 0.16
T_1	2.69 ± 0.23	0.32 ± 0.53	2.37 ± 0.33	1.93 ± 0.23	0.17 ± 0.12	1.76 ± 0.25
T_2	3.15 ± 0.57	0.93 ± 0.12	2.22 ± 0.31	2.18 ± 0.57	0.48 ± 0.23	1.70 ± 0.24
T_3	4.58 ± 0.11	2.54 ± 0.10	2.04 ± 0.29	3.26 ± 0.21	1.98 ± 0.10	1.28 ± 0.18
T_4	7.81 ± 0.13	4.21 ± 0.57	3.60 ± 0.51	6.14 ± 0.10	3.15 ± 0.13	2.99 ± 0.42
T_5	13.80 ± 0.53	10.72 ± 0.13	3.08 ± 0.42	10.85 ± 0.13	6.73 ± 0.96	4.12 ± 0.58
T_6	10.41 ± 0.23	5.35 ± 0.23	5.06 ± 0.71	8.42 ± 0.13	4.96 ± 0.71	3.46 ± 0.49
T_7	3.63 ± 0.57	1.06 ± 0.13	2.57 ± 0.36	3.06 ± 0.12	1.73 ± 0.24	1.33 ± 0.19
T_8	2.90 ± 0.12	0.43 ± 0.09	2.47 ± 0.34	2.23 ± 0.13	0.98 ± 0.19	1.25 ± 0.17

TRP= total regenerated plants, GRP = green regenerated plants, ALP= albino plants, Cont. = without cold pretreatment.

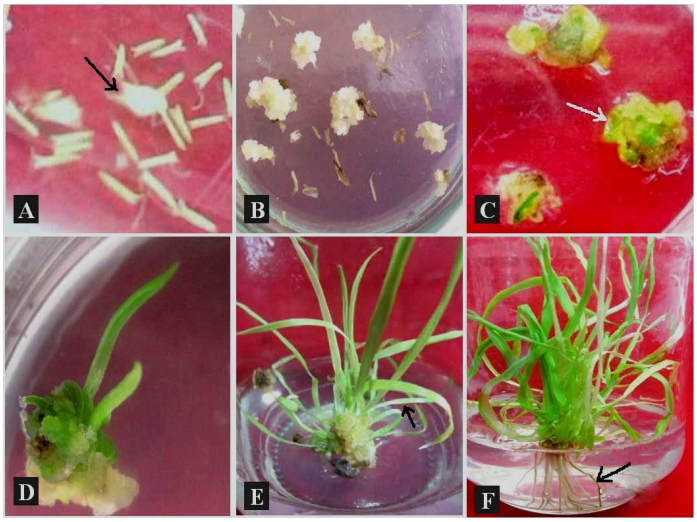

Fig. 22 (A-F): Anther culture response in barley. A-B: Embryogenic structures in semisolid induction medium, C: Embryoids with green regenerable structure, D: Regenerated green plantlets from anther-derived embryoids, E: Regenerated green and albino plants, F: Well developed root and shoots from anther derived plants.

Table 33: Effect of cold pretreatments on embryoids induction and its regeneration efficiency for barley anther culture

Treatment (Cold)	Embryoids formation		Total regenerated plantlets		Green plantlets		Albinos	
	Mean	Diff.	Mean	Diff.	Mean	Diff.	Mean	Diff.
T_1	0.91	0.66 ^{NS}	2.31	1.12 ^{NS}	0.24	0.13 ^{NS}	2.06	0.63 ^{NS}
T_2	1.20	0.95 ^{NS}	2.66	2.37 ^{NS}	0.70	0.59 ^{NS}	1.96	0.52 ^{NS}
T_3	1.86	1.61*	3.92	5.43*	2.26	2.15 ^{NS}	1.66	0.22 ^{NS}
T_4	3.40	3.15*	6.97	10.78*	3.68	3.57 ^{NS}	3.29	1.86 ^{NS}
T_5	5.05	4.80*	12.32	7.87*	8.72	8.61*	3.60	2.16 ^{NS}
T_6	2.99	2.74*	9.41	1.80 ^{NS}	5.15	5.04 *	4.26	2.82*
T_7	1.51	1.26*	3.34	1.02 ^{NS}	1.39	1.28 ^{NS}	1.95	0.51 ^{NS}
T_8	0.96	0.71 ^{NS}	2.56	1.12 ^{NS}	0.70	0.59 ^{NS}	1.86	0.42 ^{NS}
Cont.	0.25	-	1.54	-	0.11	-	1.43	-

*Indicating the mean difference is significant at the 0.05 level. Cont. = without cold pretreatment, T= Treatment, NS = Non significant and Diff. = Difference.

5.1.9 Discussion

Embryoid formation and their subsequent regeneration were affected using different media and their composition. The duration of cold pretreatment and use of a suitable genotype were also very important for success of androgenetic study. A good number of embryoids were produced by suitable barley genotypes in FHG medium. For wheat anther culture AMS_3 medium produced better performance than other media, as reported by Islam (2000), but in the case of barely this study showed poor embryoids induction in AMS_3. On the contrary, it was observed that on MS, N6 and B5 media for the androgenetic response was poor in comparison to FHG (**Table 30**).

On the basis of embryoids induction in **Table 30**, it may be concluded that for barley anther culture FHG and AMS_3 are more suitable than MS, N6 and B5. Furthermore, Ritala et al. (2001) reported satisfactory frequency on green plant regeneration from barley microspores (cv. Kymppi) on modified N6 medium. The FHG medium seems to be preferable, because it guaranteed a higher induction frequency with a good regeneration capacity. Similar results were reported by other researchers in barley (Lezin et al. 1996; Castillo et al. 2000; Lazaridou et al. 2005, 2011). In this result indicated that cold pretreatment is essential and influenced frequency of embryoids induction in barley anther culture. The duration of the cold pretreatment of the harvested spikes had a significant effect on the androgenic response of the two genotypes tested.

The beneficial effect of cold pretreatment of the harvested spikes had a positive effect, as reported by several researchers in various cereal crops (Lazar et al. 1985; Kiviharju and Pehu, 1998; Bárnabas, 2003; Obert et al. 2004). In anthers from Indica rice genotypes, a cold pretreatment of 5 to 8 days produced the best performance (Alejar et al. (1995). Khatun et al. (2012) mentioned that cold pretreatment at 4ºC for 3-7 days is also very effective in rice anther culture. Kahrizi et al. **(**2011) observed that after 14 days of cold pretreatment in barley, anthers produced the highest frequency of embryoid induction. Lezin et al. (1996) reported that a 14 days cold pretreatment of barley anthers prior to culture increased embryogenesis and regeneration. In this case that cold pretreatment for 14 days to the harvested spikes of BARI barley-6 and BARI barley-3 reduced the frequency of embryoid induction and reduced green plant regeneration (**Table 32**). These observed differences may be due to the genotypes selected, or the media or other culture conditions.

5.2 Effect of Drought Stress on Anther Culture Using Two Barley (*Hordeum valgare* L.) Genotypes in Bangladesh

5.2.1 Introduction

Anther culture is one of the most demanding methods for *in vitro* culture of barley and carried out in haploid production (Lazaridou et al. 2011). Barley is generally recalcitrant to *in vitro* culture and there is imitation also on green plant regeneration. Stress pre-treatments such as cold and heat may enhance induction and regeneration efficiency in androgenesis of various cereal crops such as barley (Hou et al. 1993; Haque and Islam, 2014), rice (Khatun et al. 2012; Rukmini et al. 2013), wheat (Islam, 2010; Islam and Tuteja, 2012), maize (Genovesi, 1990), rye (Tenhola-Roininen et al. 2005) and triticale (Immonen and Robinson, 2000).

The stimulatory effect of thermal shocks for anther culture has been successfully adopted to *Datura* (Nitsch and Norreel, 1973), tomato (Debergh and Nitsch, 1973) and tobacco (Bajaj and Reinert, 1975) to enhance androgenesis. Heat pretreatment of inoculated anthers or microspores at 30–35ºC was effective in induction of androgenesis in cereals are reported by Li et al. (1988) and Reddy et al. (1985). Evidence of the beneficial effects of thermal shocks has been reported in oats (Rines, 1983) and Proso millet (Wu et al. 2012). Islam (2010) reported that drought stress (1-5 hrs) significantly better for anther culture in wheat. Shen and Veilleux (1995) reported that in potato a treatment combining a high temperature shock (35°C for 12 h) elevated 11 times higher embryo production in comparison to control. Ponitka and Slusarkiewicz-Jarzina (2007) studied the efficiency of anther culture induction in solid and liquid medium of Triticale. Guo et al. (1999) tested both the solid and liquid forms of FHG media for anther culture in Timothy. Till there is no report about the effect of drought stress pre-treatment along with different culture media on the barley anther culture. Under this study as pre-treatment factors cold and drought has been considered to evaluate their effects on embryoids induction as well as regeneration efficiency in barley.

5.2.2 Methods

As plant materials spikes of two barley genotypes namely BARI barley-3 and BARI barley-6 were considered for its good androgenetic response (Haque and Islam, 2014). Anther culture procedures are briefly described in the section of 2.3.3.1 (materials and methods, general).

For this study, anthers were carefully removed from spikelets and around 45-50 anthers were taken in a dry and sterile petri dishes (60 mm) in the laminar airflow cabinet. Drought stress of different durations such as T_1 = 30, T_2 = 60, T_3 = 90, T_4 = 120, T_5 = 150, T_6 = 180, T_7 = 210 and T_8 = 240 minutes were applied to excised anthers. For control, anthers were directly inoculated to liquid and semi-solid induction medium of FHG (Hunter, 1987) that supplemented with maltose (62 g/l) instead of sucrose, 2,4-D (2.0 mg/l), Kin (0.5 mg/), L-glutamine (730 mg/l) and as solidifying agent 3 g/l phytagel were used. All media were sterilized by autoclaving at 121ºC for 15 minutes and pH was adjusted to 5.8. Petri dishes were sealed with parafilm and incubated them in dark at 26 ± 2°C for up to 4-6 weeks. Then embryos were transferred to a semi-solid regeneration (MSR, modified form MS; Islam, 2000) medium and placed them in a growth chamber of 16/8h light/dark regimes for shoot induction.

Data were recorded on the basis of embryogenesis, regeneration on the following traits: embryo induction, expressed as the number of embryos per 100 anthers; total plants regeneration (TRP), green and albino plantlets per 100 embryos; green plant regeneration (GPR), green plants per 100 embryos; albino regenerated plants (ARP) and the number of albino plants per 100 embryos.

5.2.3 Statistical analysis

Data were performed analysis by using the SPSS (16) programme for analyzed their multiplicative inter-effects of the traits and subjected to ANOVA. LSD tests were used to compare androgenenic responses of different drought stress durations on anther culture of barley genotypes.

5.2.4 Results

It was observed that the effect of drought stress duration (minutes) using two barley genotypes (BARI barley-6 and BARI barley-3) with selected two inductions medium (semi-solid and liquid; Section 2.2) have been standardized previously and evaluated their response to embryoids induction and plant regeneration. For all treatments, the two studied genotypes were capable of inducing and developing androgentic embryos and green plants (**Fig. 23**). Drought stress for 150 minutes (T_5) showed highest percentage of embryo induction 26.50, 14.67 and green plantlets 13.58, 6.16 respectively in BARI barley-6 and BARI barley-3 (**Table 34**). In this case BARI barley-3 gave embryoids in liquid medium also. Among two genotypes BARI barley-6 showed better response on embryoids induction and green plants than BARI barley-3. Embryoids induction was found after 3-4 weeks of culture initiation on liquid medium, and after 5-6 weeks on semi-solid medium. It was observed that the differences among genotypes for both induction and regeneration ability. Generally, embryo productions as well as green plant were higher for both genotypes that embryoids developed in liquid medium. The mean frequency of embryos ranged was 6.98 to 10.07 per 100 anthers on liquid medium and from 5.34 to 7.55 per 100 anthers on semi-solid medium. The mean frequency of green plants ranged (2.36 to 3.79) per 100 embryos are shown in **Fig. 24**. Drought stress might be considered as parallel treatment of thermal shock. Least significant difference (LSD) values were calculated for undertaking the parameters and the results are presented in **Table 35**. All the evaluating traits of T_3, T_4, T_5 and T_6 were found significantly different from control. However, most of the traits in T_1, T_2 and T_8 did not show any significant differences than control. Analysis of variance (ANOVA) showed significant differences among the drought pretreatment, culture media and between the genotypes (**Table 36**).

Table 34: Effect of drought stress on anthers for the production of embryoids in semi-solid and liquid medium in BARI barley-3 and BARI barley-6

Geno-type	Treat-ment	Semi-solid medium				Liquid medium			
		Embryo induction (%)	Total regeneration (%)	Green plants (%)	Albino plants (%)	Embryo induction (%)	Total regeneration (%)	Green plants (%)	Albino plants (%)
	Cont.	2.05	1.66	0.71	0.95	2.73	2.50	1.04	1.46
	T_1	2.91	2.29	0.87	1.42	3.84	3.47	1.49	1.98
	T_2	4.08	3.50	1.65	1.85	5.09	4.22	1.90	2.32
	T_3	6.46	4.76	2.44	2.32	8.37	5.71	2.45	3.26
BB-6	T_4	10.15	6.25	3.26	2.99	13.28	8.76	4.41	4.35
	T_5	19.56	14.80	11.42	3.38	26.50	19.79	13.58	6.21
	T_6	12.15	8.40	4.47	3.93	16.10	10.67	6.04	4.63
	T_7	7.18	3.62	1.56	2.06	9.27	4.49	2.09	2.40
	T_8	3.43	1.96	0.85	1.11	5.38	3.75	1.18	2.57
	Mean	**7.55**	**5.25**	**3.02**	**2.22**	**10.07**	**7.04**	**3.79**	**3.24**
	Cont.	1.94	1.47	0.43	1.04	2.02	1.53	0.64	0.89
	T_1	2.0	1.66	0.71	0.95	3.17	2.66	1.05	1.61
	T_2	3.06	2.38	1.18	1.20	4.66	3.12	1.47	1.65
	T_3	5.94	3.44	1.98	1.46	7.96	4.72	2.32	2.40
BB-3	T_4	7.83	4.7	2.95	1.75	9.84	5.91	3.20	2.71
	T_5	12.66	8.77	5.32	3.45	14.67	10.61	6.16	4.45
	T_6	8.17	5.26	2.89	2.37	11.29	7.61	3.95	3.66
	T_7	3.85	2.70	1.14	1.56	5.72	3.63	1.72	1.91
	T_8	2.90	1.23	0.38	0.85	3.47	2.06	0.81	1.25
	Mean	**5.34**	**3.51**	**1.88**	**1.62**	**6.98**	**4.65**	**2.36**	**2.28**

Cont. (Control) = Anthers directly cultured without any drought stress. T_1 = 30, T_2 = 60, T_3 = 90, T_4 = 120, T_5 = 150, T_6 = 180, T_7 = 210, and T_8 = 240 minutes drought pre treatment to anthers.

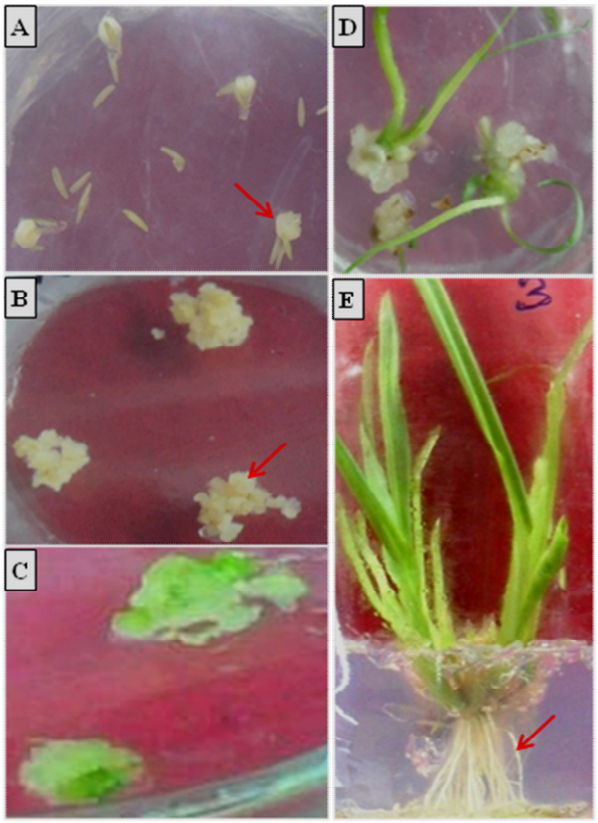

Fig. 23: A & B) Embryogenic structures from anther culture of barley, C) Embryoids with green regenerable structure, D) Regenerated green plantlets from anther-derived embryoids, E) Well rooted plants obtained from anther-derived embryoids.

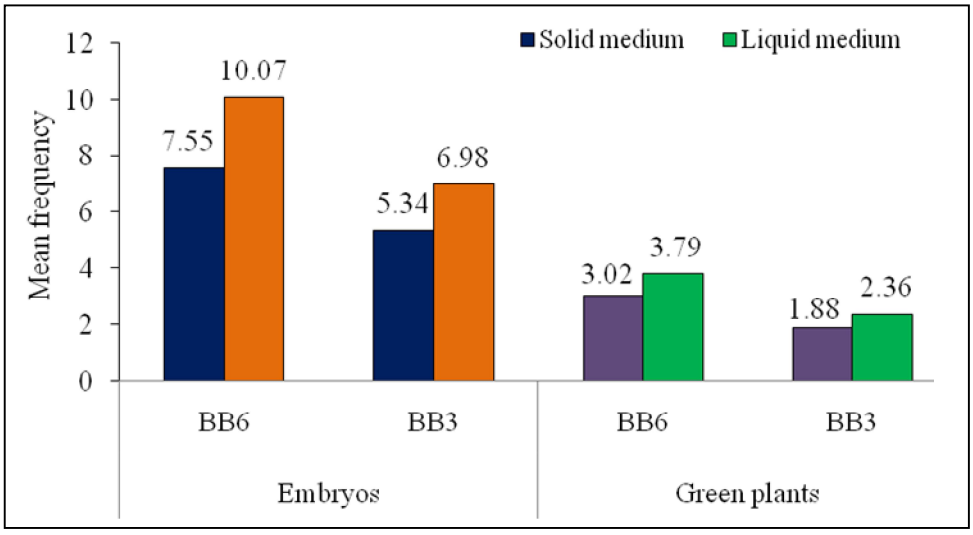

Fig. 24: Mean frequency of embryos and green plants on semi-solid and liquid induction media from two barley genotypes.

Table 35: Comparison between treatments and control using LSD test for anther response and its productivity on drought stress

Treat -ments (T)	Embryo induction (%)		Total regeneration (%)		Green plants (%)		Albino plants (%)	
	Mean	Diff.	Mean	Diff.	Mean	Diff.	Mean	Diff.
T_1	2.98	0.79 ^{NS}	2.52	0.73 ^{NS}	1.03	0.32 ^{NS}	1.49	0.40 ^{NS}
T_2	4.22	2.03 ^{NS}	3.30	1.51 ^{NS}	1.55	0.84 ^{NS}	1.75	0.67*
T_3	7.18	4.99*	4.65	2.86*	2.29	1.59 ^{NS}	2.36	1.27*
T_4	10.27	8.09*	6.40	4.61*	3.45	2.75*	2.95	1.86*
T_5	18.34	16.16*	13.49	11.70*	9.12	8.41*	4.37	3.28*
T_6	11.92	9.74*	7.98	6.19*	4.33	3.63*	3.64	2.56*
T_7	6.50	4.32*	3.61	1.82 ^{NS}	1.62	0.92 ^{NS}	1.98	0.89*
T_8	3.79	1.61 ^{NS}	2.25	0.46 ^{NS}	0.80	0.10 ^{NS}	1.44	0.36 ^{NS}
Cont.	2.18		1.79		0.70		1.08	

*Indicating the mean difference is significant at the 0.05 level. Cont. = without cold pretreatment, T= Treatment, NS = Non-significant and Diff. = Difference.

Table 36: Analysis of variance (ANOVA) showed the effect of drought stress, media and genotypic responses in barley anther culture

Subject	Sources	Sum of Squares	df	Mean Square	F
Embryo Induction	Medium (M)	38.11	1	38.11	10.77*
	Genotype (G)	62.35	1	62.35	17.63*
	Treatments (T)	876.84	8	109.60	30.99*
	M × G	1.84	1	1.84	
	M × T	13.33	8	1.66	
	G × T	67.81	8	8.47	
	M × G × T	5.40	8	0.67	
Total regeneration	Medium (M)	19.301	1	19.30	9.68*
	Genotype (G)	38.316	1	38.31	19.22*
	Treatments (T)	447.327	8	55.91	28.05*
	M × G	.960	1	0.96	
	M × T	6.849	8	0.85	
	G × T	39.647	8	4.95	
	M × G * T	2.363	8	0.29	
Green plants	Medium (M)	3.541	1	3.541	2.31*
	Genotype (G)	14.835	1	14.835	9.71*
	Treatments (T)	229.675	8	28.709	18.79*
	M × G	0.18	1	0.18	
	M × T	1.78	8	0.22	
	G × T	35.63	8	4.45	
	M × G × T	0.56	8	0.07	
Albino plants	Medium (M)	6.30	1	6.30	33.32*
	Genotype (G)	5.46	1	5.46	28.88*
	Treatments (T)	39.12	8	4.89	25.83*
	M × G	0.29	1	0.29	
	M × T	2.17	8	0.27	
	G × T	1.19	8	0.15	
	M × G × T	1.06	8	0.13	

* indicating significant at 0.05 level. T = Treatment, G = Genotype, and M = Medium.

5.2.5 Discussion

Under this study drought stress has been considered as parallel pre-treatment of thermal shock for barley anther culture. Least significant difference (LSD) was calculated for undertaking the parameters and the results are presented in **Table 35**. In the presented investigations all the evaluating traits of T_4 (120 min), T_5 (120 min) and T_6 (120 min) were found significantly different than control. Drought stress for 150 min (T5) showed highest percentage of embryoids induction (26.50, 14.67) and green plantlets (13.58, 6.16) in BB-6 and BB-3 respectively in liquid induction medium (**Table 34**). Similar type of result was reported by Islam (2010) in wheat who observed that drought stress for 3 hrs showed highest percentage of embryo production and green plantlets.

The optimal duration of a heat pretreatment varied in maize, 10 days resulted in a two-fold increase in embryo like structures (ELS) compared with continuous culture at 25ºC (Afele et al. 1992). Four days (33ºC) combined with starvation was beneficial for isolated microspores of wheat (Touraev et al. 1996). In rice, as short as 5 minutes at 35ºC followed by 10ºC for 7 days was most suitable for callus induction and green plant regeneration (Reddy et al. 1985). Genotype-dependency was reported in some cases; in wheat anther cultures Simmonds (1989) found that heat pretreatment favourable, whereas Hassawi and Liang (1990) found no significant effect. Keller and Armstrong (1979) observed in *Brassica,* embryo yield increased by subjecting excised inflorescence to short-term high temperature shock (45°C for one hr followed by 40°C for three hrs) prior to anthers planting. Similarly, Ockendon and Sutherland (1987) found that in Brussels sprouts (*B. oleracea* var. *gemmifera)* yields up to 357 embryos per 100 anthers using a thermal shock treatment of 16 hrs at 35°C at the start of the culture period.

The results of this experiment clearly demonstrated that anthers on liquid culture gave a significantly higher yield of androgenic embryos and green plants in comparison to anthers in semi-solid culture medium. The present investigation were in agreement

with Ponitka and Slusarkiewicz-Jarzina (2007) who reported that androgenic embryos and green plants efficiency was higher on liquid medium compared with semi-solid medium in Triticale. For anther culture response in other cereal species and grass various responses were found in liquid media. Henry and De Buyser (1981) determined the efficiency of embryoid production and green plant regeneration of wheat on liquid medium. Guo et al. (1999) found both the semi-solid and liquid forms of media for anther culture of Timothy. Similarly, in rye (*Secale cereale*) Ma et al. (2004) reported that anther culture in liquid medium improved the efficiency of androgenic embryos and green plant regeneration. In contrast, embryoids induced on liquid induction media had lower regeneration ability than embryoids from agar-solidified media in rice and wheat anther cultures (Zhou et al. 1991).

Chapter VI

General Discussion

6. General Discussion

Barley ranks fourth among the cereals in the worldwide but still it is a minor cereal and it can play an important role in enhancing the food security and in drainage of foreign currency in Bangladesh (Yesmin et al. 2014). The climate and epidemic condition are suitable for barley cultivation in Bangladesh except some costal districts. It is an important crop to use such fallow, char and marginal lands in Bangladesh. Morphological characteristics of barley are simple. Recently barley has been used as a genetic model species because of its true diploidy along with the similarity of its genome to that of other small-grain cereals (Nagy et al. 2011). A further advantage is that the barley genome is very similar to the D genome of wheat (Éva et al. 2008).

Through tissue culture systems of barley have great importance for its improvement (Holme et al. 2008). An efficient and reproducible plant regeneration protocols have been developed mostly based on embryogenesis (somatic embryogenesis and androgenesis) in barley. Transgenic plants could be generated by gene transfer using somatic and gametic embryos that derived from mature and immature seeds and also by anther/microspores (Islam and Tuteja, 2012). To the best of my knowledge, there is no report on *in vitro* plant regeneration using barley genotypes in Bangladesh, and none of the cultivars used in this study have been considered previously for analysis of *in vitro* plant regeneration capacity. Under this study, a highly efficient protocol has been developed using various physical and chemical pretreatment factors that showed higher plant regeneration sufficiently on barley.

For this study, seven local and eight European barley genotypes were taken to evaluate their growth and yield, *in vitro* screening of suitable genotypes for somatic and gametic embryogenesis. It was optimized media and plant growth regulators using mainly BARI barley cultivars. Salt and heat pretreatment factors were applied to improve somatic embryogenesis; as chemical copper sulphate and cobalt chloride were also applied for the same purpose. Under this study various carbon sources were

evaluated with MS and other media; standardized silver nitrate and amino acids concentration on callus induction and regeneration also. Callus age and size effect were evaluated to improve regeneration potentials. Different physical pre-treatment factors such as cold and drought stress were applied directly to targeted explants to improve anther culture responses for doubled haploid production as well as further advance biotechnological work on barley in Bangladesh. Considering all experiments under this study and its findings are mentioned below:

The first experiment was conducted using six local barley genotypes and eight European genotypes to assess performance of the genotypes, variability, genetic advances and the influence of sowing time on yield and yield contributing characters. Seeds were sown three times points e.g S_1 = 1st November, S_2 = 21st November and S_3 = 11th December. Data were recorded on the basis of their booting, maturity, plant height, spike length, spikelets, grain weight and yield. Among the studied genotype BB-5 showed the earlier booting (59.0 days) and long duration (82.0 days) was recorded in Hor-17016. It was observed that BB-5 required minimum number of days to mature (101 d) while maximum duration (129 d) was recorded in Hor-9580 (S_1). Among the genotypes Hor-9465 was the best for plant height (117.0 cm), total tillers (49.63), fertile tillers (37.43), spike length (14.33 cm), spikelets per spike (15.67), grains per spike (89.0) and grain yield (7.75 g) per plant. For grain weight BB-1 was better (34.10 g) than others. The time of sowing point S_2 produced higher grain than S_1 and S_3. In this case found that late sowing reduced grain yield. These results corroborated the findings of Alam et al. (2007) in barley and Dawson and Wardlaw (1989) in wheat. Alam et al. (2007) reported that late sowing reduces the spike length and grain yield. Dawson and Wardlaw, (1989) demonstrated that high temperatures speed up spike development from floral initiation to anthesis, and also have been found to reduce the number of spikelets formed per spike in wheat. On the other hand, Green et al. (1985) found that early sowing induced faster production of tillers and consequently gave higher maximum tillers and provided the highest grain yield led by optimum temperature during the growth period.

Under this study mature seeds of six barley genotypes *viz.* BB-1, BB-2, BB-3, BB-4, BB-5 and BB-6 were tested for callus induction. An efficient callus induction and regeneration protocol has been standardized as well as screening out of some suitable genotypes using mature embryos of studied genotypes (section 4.1). Out of six four genotypes four namely BB-6 (38.17%), BB-3 (30.11%), BB-1 (24.04%) and BB-2 (7.65%) showed better callus from mature embryos. For *in vitro* plant regeneration barley showed highly genotypic dependency reported by Castillo et al. (1998) and Han et al. (2011). Therefore, screening for highly responsive *in vitro* genotypes is very important for advance biotechnological work in barley. However, only a few barley genotypes have been identified that possesses good regeneration capacity (Lemaux et al. 1999; Aguado-Santacruz et al. 2011). In this case MS medium + 2,4-D, BAP, L-proline and casein hydrolysate with twelve different combinations (CIM_1 – CIM_{12}) were used for primary callus induction. Among the combinations CIM_8 (4.0 mg/l 2,4-D, 200 mg/l L-proline and 300 mg/l casein) showed better performance on callus induction (38.17%) in BB-6 while lowest (6.94%) was observed in CIM_{12} for BB-6 that contained 5.5 mg/l 2, 4-D, 0.2 mg/l BAP, 300 mg/l proline and 450 mg/l casein. The results obtained in this study are quite similar with the report of Ganeshan et al. (2003), who successfully induced callus from mature embryo of barley. They used similar components in the medium (2,4-D, L-proline and casein hydrolysate) but the concentration was different with the present findings. Nine different concentration of plant growth regulators (PGRs) were also added in MS media for plant regeneration purpose. It was observed that the combination of MS + 1.5 mg/l BAP + 30 g/l sucrose was proved to be the best combination for plant regeneration in BB-6 (9.26%). This result agreed well with previous works where the MS medium and BAP were successfully used in barley (Aguado-Santacruz et al. 2011). A similar type of result was found by Lee et al. (2012). They reported that sucrose has been commonly used at the concentration of 20 and 30 g/l as a carbon source in tissue culture medium. But they have not found any combined effect of different concentrations of BAP and sucrose for regeneration purpose.

In the Expt. 3 (section. 4.2), it was observed that heat pre-treatment factor along with NaCl to enhance somatic embryogenesis and their subsequent regeneration using mature embryos of BB-3, BB-6 and BHL-18. Various concentrations of NaCl (0, 1.5, 2.5, 3.5, 4.5, 5.5 and 6.5 g/l) were supplemented in MS medium to evaluate the callus viability as well as regeneration efficiency and found that the viability of calli as well as regeneration was decreased remarkably in comparison with control. Among the genotypes, BB-6 showed highest viability of callus (14.72%) and regeneration (7.69%) with high concentrations of NaCl (6.5 g/l). Different concentrations (0, 2.5, 4.5 and 6.5 g/l) of NaCl subjected to relative growth rate (RGR) and tolerance index (TI) of callus. In the top most level of NaCl stress (6.5 g/l) recorded RGR values were 0.91, 0.49 and 0.48; TI were 0.42, 0.25 and 0.28 for BB-6, BHL-18 and BB-3, respectively. Among three genotypes BB-6 exhibited the highest potentiality to survive in NaCl induced abiotic stress with maximum RGR (0.91) and TI (0.42). The results are in agreement with the previous finding that Giza 123 genotype can tolerate 5 g/l salt stress (Metwali et al. 2013). Siddique et al. (2014) reported that callus viability decreased in rice on comparison to the controls, when they were cultured in 200 mM NaCl level. To observe the effect of heat stress calli were treated at 25°C, 30, 35 and 40°C temperatures. The result revealed that calli of BHL-18 performed highest desiccation (59.70%) when it was pre-treated by heat at 40℃. The BB-6 showed best regeneration (41.66%) when calli pretreated at 35℃. Heat treatment for 35℃ led to greater regeneration percentage compared to control temperature. This result finding agree with those of Benderradji et al. (2012) who found significant effects of thermal stresses on callus induction and shoot regeneration in wheat.

To evaluate the effect of copper sulphate and cobalt chloride in addition to the medium, mature embryos of BB-3 and BB-6 were considered for this study. Various concentrations of copper sulphate and cobalt chloride (2.5, 5.0, 7.5 and 10.0 g/l) either single or in combination were tested. According to the results obtained, in addition of both chemicals at 2.5 to 7.5 mg/l (T_9 to T_{11}) were suitable on callus induction. In case of regeneration, T_{10} (5.0 mg/l copper sulphate and cobalt chloride) showed maximum plant regeneration (53.25%) in BB-6 that was around 3 fold higher than control. Some

recent reports suggested that the positive influence of copper during *in vitro* culture of various explants in barley (Bartlett et al. 2008; Yadav et al. 2011). Dahleen (1995) studied that the effect of different concentrations of $CuSO_4$ on callus culture and found that medium containing 50 µM copper regenerated significantly more plants. The rate of callus proliferation improved with synergistic effects when copper and cobalt are together in the medium reported by Al-Mayahi (2014). Amarasinghe (2009) carried out research on *in vitro* performance of nine indica rice varieties and concluded that the rate of calli production was significantly higher in all the tested varieties on MS basal medium supplemented with 5-10 mg/l cobalt chloride.

To improve callus induction and regeneration different carbon sources and plant growth regulators (PGRs) were added in the medium with another attempts. In this case only BARI barley-6 was considered for its better performance on *in vitro* culture (Haque and Islam, 2015). Seeds were pretreated with different concentrations of 2,4-D (2.5, 3.5 and 6.0 mg/l) and durations (1-6 days) prior to culture. The highest frequency of callus induction (71.38%) obtained with 3.5 mg/l 2,4-D pre-treated at 4 days. Sharma et al. (2005) used pretreatment in water plus 3.0 or 6.0 mg/l 2,4-D for barley mature embryos and resulted slightly higher primary callus induction (approximately 11% higher than control), but they did not observed the pretreatment duration. Various amount of sucrose, maltose and D-sorbitol either single or in combination with three media (MS, B5 and N6) were used. It was observed that highest frequency of primary callus (89.16%) obtained with 60 g/l D-sorbitol (T_6) but 15 g/l maltose with 15 g/l D-sorbitol (T_9) gave low callus induction (11.33%) in B5. Hassan et al. (2009) observed similar type of result in wheat. They observed that addition of sorbitol in culture media can increase the efficiency of MS medium and 20 g/l concentration of sorbitol has significant effect on callus induction frequency. They also demonstrated that sorbitol was not only improves the quantity but enhanced the quality of callus induction. The maximum embryogenic callus (70.0%) with 2,4-D (2.0 mg/l) + BAP (0.5 mg/l) and plant regeneration (47.40%) with 0.5 mg/l NAA + 1.0 mg/l BAP were recorded in MS medium. Similarly, Nawaz et al. (2013) indicated that addition of 0.5 mg/l NAA + 2.5 mg/l BAP produced maximum number of shoots

in sugarcane. Morshed et al. (2014) reported that 1.0 mg/l BAP and 0.5 mg/l IAA were suitable for regeneration in maize. For rooting GM, full & ½MS media were used that supplemented with 1.0 mg/l of NAA, IAA and IBA. The maximum number of roots obtained per plant (11.33) in GM medium supplemented with 1.0 mg/l IAA. From these study it may concluded that for efficient callus induction as carbon sources 60 g/l D-sorbitol was better than others and GM medium that contained 1.0 mg/l IAA was suitable for rooting. Similar results found in barley anther culture (Haque and Islam, 2014). On the contrary, the best growth of hairy root achieved on MS medium containing 3% or 5% sucrose in *Arnica montana* (Petrova et al. 2015).

A suitable, reproducible and efficient protocol has been established for callus induction and regeneration using $AgNO_3$ and amino acids through immature embryos of three barley genotypes *viz.* BARI barley-1, 3 and 6. Under this study, five doses of $AgNO_3$ singly and in combination with amino acids (L-proline, L-glutamine) were tested for callus induction and plant regeneration. The maximum callus induction was recorded (49.20 and 32.66%) for BARI barley-6 and BARI barley-3**,** respectively when 2.0 mg/l $AgNO_3$ and 200 mg/l L-glutamine were added to the callus induction medium. Moreover, plant regeneration remarkably increased on MS + 1.0 mg/l BAP + 1.5 mg/l $AgNO_3$ + 150 mg/l L-glutamine as 37.20% in BARI barley-6. For rooting single using of $AgNO_3$ positively affected whereas, negative influence was observed in combinations of $AgNO_3$ and amino acids (L-proline, L-glutamine). Using $AgNO_3$ and amino acids, around <4, <27 and <5 fold callus induction, regeneration and rooting were increased. Finally it may be concluded that as a stimulating agent silver nitrate and amino acids influenced callus induction and its subsequent plant regeneration. Similarly, almost doubled regeneration was recorded using $AgNO_3$ in barley (Jha et al. 2007). On the contrary, no positive effect of $AgNO_3$ on plant regeneration was found using immature embryos in barley (Hussein et al. 2004). Kumar et al. (2009) and Reddy et al. (2001) also observed that the positive effect of $AgNO_3$ for root formation in barley genotypes.

Other attempts have been done using various callus sizes, age and their fresh weight to improve regeneration efficiency using mature embryos that derived from immature embryos of BARI barley-6. Embryos size were classified into four groups e.g. (A) 0.6-1.0 mm, (B) 1.1-1.5 mm, (C) 1.6-2.0 mm and (D) 2.1-2.5 mm. Highest callusing and green plants were obtained when 1.6-2.0 mm size of embryos were used. Out of four aged groups 4-6 weeks old calli and when its weight range was 151-200 mg showed better performance on green plant regeneration with studied barley genotypes. From these findings it may be concluded that for better regeneration callus size, age and its fresh weights are important factors for increasing green plantlets as well as root developments in barley. The present results on the influence of embryo size on callus induction and regeneration agreed well with the findings of Senarath (2007). He mentioned that embryos of 1.6-2.0 mm showed the highest ability to produce callus capable of regenerating green plants in barley. Similarly, Islam (2010) reported that an early transfer of embryos into the regeneration medium, within three - five weeks, was more efficient for regeneration of green plantlets in comparison to prolonged culture (6-8 weeks) in wheat anther culture.

To identify a suitable androgenetic variety, determination of cold pretreatment duration and optimization of media experiments were conducted using barley anthers as explants sources. Two barley genotypes and five semi-solid media *viz*. MS, B5, N6, FHG and AMS_3 were considered for embryo induction and plant regeneration. Harvested spikes were pretreated with cold for 2, 4, 6, 8, 10, 12, 14 or 16 days at 4°C in the dark. Cold pretreatment for 8-12 days produced the highest frequency of embryoids on FHG medium. The genotype BB-6 performed better in terms of embryo formation and green plant regeneration in MSR medium. The highest level of embryos induction (14.6%) was observed when spikes were pretreatment at 4-7ºC cold for 10 days and a total 13.8% regenerated plantlets and 10.72% green plants were obtained. It was observed that the interactions of medium and cold pretreatment, genotype and medium were significant in determining the rate of embryoids induction. The FHG medium seems to be preferable, because it guaranteed a higher induction frequency with a good regeneration capacity. Similar results were reported

by other researchers in barley (Castillo et al. 2000; Lazaridou et al. 2005, 2011). Khatun et al. (2012) also mentioned that cold pretreatment at 4ºC for 3-7 days is also very effective in rice anther culture. Kahrizi et al. **(**2011) observed that after 14 days of cold pretreatment in barley, anthers produced the highest frequency of embryoid induction.

Another attempt as pre-treatment factor various drought stress durations such as T_1 = 30, T_2 = 60, T_3 = 90, T_4 = 120, T_5 = 150, T_6 = 180, T_7 = 210 and T_8 = 240 minutes were applied to excised anthers of BARI barley-3 and BARI barley-6. It was observed that drought stress for 150 min (T_5) showed highest percentage of embryoids induction (26.50) and green plants (13.58) in BARI barley-6.The present study demonstrated that drought stress can be influenced anther culture responses and could be used as one of the potential factors having influence on embryo yield and regeneration of barley anther culture. It was observed that the number of androgenic embryos and regenerated green plants in barley can be increased by in liquid induction medium than semi-solid medium. Similar type of result was reported by Islam (2010) in wheat who observed that drought stress for 3 hrs showed highest percentage of embryo production and green plantlets. From the mentioned experimental findings it might be summarized that the cold or drought pretreatment and their interaction clearly influence the anther culture ability of barley and this protocol will be helpful for future any advance level of biotechnological research in Bangladesh.

Chapter VII

Summary

7. Summary

For this study, seven local and eight European barley genotypes were taken to evaluate their growth and yield, *in vitro* screening on somatic and gametic embryogenesis. It was optimized that media and plant growth regulators using mainly for BARI barley cultivars. Salt and heat pretreatment factors were applied to improve somatic embryogenesis; as chemical copper sulphate and cobalt chloride were also applied for the same purpose. Under this study various carbon sources and its effect were evaluated with MS and other media. It has standardized about the effect of various concentrations of silver nitrate and amino acids on callus induction and regeneration. Callus age and size effect were evaluated to improve regeneration potentials. Different physical pre-treatment factors such as cold and drought stress were applied directly to targeted explants to improve anther culture responses for doubled haploid production as well as further advance biotechnological work on barley in Bangladesh. Considering all experiments and their findings are mentioned below:

The first experiment (Chapter 3) was conducted using six local barley genotypes and eight European genotypes to assess performance of the genotypes, variability, genetic advances and the influence of sowing time on yield and yield contributing characters. Seeds were sown three times points e.g S_1 = 1st November, S_2 = 21st November and S_3 = 11th December. Data were recorded on the basis of their booting, maturity, plant height, spike length, spikelets, grain weight and yield. Among the studied genotype BB-5 showed the earlier booting (59.0 days) and long duration (82.0 days) was recorded in Hor-17016. It was observed that BB-5 required minimum number of days to mature (101 d) while maximum duration (129 d) was recorded in Hor-9580 (S_1). Among the genotypes Hor-9465 was the best for plant height (117.0 cm), total tillers (49.63), fertile tillers (37.43), spike length (14.33 cm), spikelets per spike (15.67), grains per spike (89.0) and grain yield (7.75 g) per plant. For grain weight BB-1 was better (34.10 g) than others.

Under this study mature seeds of six barley genotypes *viz.* BB-1, BB-2, BB-3, BB-4, BB-5 and BB-6 were tested for callus induction. An efficient callus induction and regeneration protocol has been standardized as well as screening out of some suitable genotypes using mature embryos of studied genotypes (section 4.1). Out of six four genotypes [BB-6 (38.17%), BB-3 (30.11%), BB-1 (24.04%) and BB-2 (7.65%)] showed betetr callus from mature embryos. In this case MS medium + 2,4-D, BAP, L-proline and casein hydrolysate with twelve different combinations (CIM_1 – CIM_8) were used for primary callus induction. Among the combinations CIM_8 (4.0 mg/l 2,4-D, 200 mg/l L-proline and 300 mg/l casein) showed better performance on callus induction (38.17%) in BB-6 while lowest (6.94%) was observed in CIM_{12} for BB-6 that contained 5.5 mg/l 2, 4-D, 0.2 mg/l BAP, 300 mg/l proline and 450 mg/l casein. Nine different concentration of plant growth regulators (PGRs) were also added in MS media for plant regeneration purpose. It was observed that the combination of MS + 1.5 mg/l BAP + 30 g/l sucrose was proved to be the best combination for plant regeneration in BB-6 (9.26%).

In the Expt. 3 (section. 4.2), it was observed that heat pre-treatment factor along with NaCl to enhance somatic embryogenesis and their subsequent regeneration using mature embryos of BB-3, BB-6 and BHL-18. Various concentrations of NaCl (0, 1.5, 2.5, 3.5, 4.5, 5.5 and 6.5 g/l) were supplemented in MS medium to evaluate the callus viability as well as regeneration efficiency and found that the viability of calli as well as regeneration was decreased remarkably in comparison with control. Among the genotypes, BB-6 showed highest viability of callus (14.72%) and regeneration (7.69%) with high concentrations of NaCl (6.5 g/l). Different concentrations (0, 2.5, 4.5 and 6.5 g/l) of NaCl subjected to relative growth rate (RGR) and tolerance index (TI) of callus. In the top most level of NaCl stress (6.5 g/l) recorded RGR values were 0.91, 0.49 and 0.48; TI were 0.42, 0.25 and 0.28 for BB-6, BHL-18 and BB-3, respectively. Among three genotypes BB-6 exhibited the highest potentiality to survive in NaCl induced abiotic stress with maximum RGR (0.91) and TI (0.42). To observe the effect of heat stress calli were treated at 25°C, 30, 35 and 40°C temperatures. The result revealed that calli of BHL-18 performed highest desiccation

(59.70%) when it was pre-treated by heat at 40℃. The BB-6 showed best regeneration (41.66%) when calli pretreated at 35℃.

To evaluate the effect of copper sulphate and cobalt chloride in addition to the medium, mature embryos of BB-3 and BB-6 were considered for the Expt. 4 (section. 4.3). Various concentrations of copper sulphate and cobalt chloride (2.5, 5.0, 7.5 and 10.0 g/l) either single or in combination were tested. According to the results obtained, in addition of both chemicals at 2.5 to 7.5 mg/l (T_9 to T_{11}) were suitable on callus induction. In the case of regeneration, T_{10} (5.0 mg/l copper sulphate and cobalt chloride) showed maximum plant regeneration (53.25%) in BB-6 that was around 3 fold higher than control.

To improve callus induction and regeneration different carbon sources and plant growth regulators (PGRs) were added in the medium (Exp. 5; section. 4.4). In this case only BARI barley-6 variety was considered for its better performance on *in vitro* culture under this study. Seeds were pretreated with different concentrations of 2,4-D (2.5, 3.5 and 6.0 mg/l) and durations (1-6 days) prior to culture. The highest frequency of callus induction (71.38%) obtained with 3.5 mg/l 2,4-D for 4 days. Various amount of sucrose, maltose and D-sorbitol were used either single or in combination with three media (MS, B5 and N6). It was observed that highest frequency of primary callus (89.16%) with 60 g/l D-sorbitol (T_6) but 15 g/l maltose with 15 g/l D-sorbitol (T_9) gave low callus induction (11.33%) in B5. As PGRs various concentrations of 2,4-D, BAP, NAA and Kinetin were used either single or in combination. The maximum embryogenic callus (70.0%) with 2,4-D (2.0 mg/l) + BAP (0.5 mg/l) and plant regeneration (47.40%) with 0.5 mg/l NAA + 1.0 mg/l BAP were recorded in MS medium. For rooting GM, full & ½MS media were used that supplemented with 1.0 mg/l of NAA, IAA and IBA. The maximum number of roots obtained per plant (11.33) in GM medium supplemented with 1.0 mg/l IAA. From these study it may concluded that for efficient callus induction as carbon sources 60 g/l D-sorbitol was better than others and GM medium that contained 1.0 mg/l IAA was suitable for rooting.

A suitable, reproducible and efficient protocol has been established for callus induction and regeneration using $AgNO_3$ and amino acids through immature embryos of three barley genotypes *viz.* BARI barley-1, 3 and 6 (Exp. 6; section. 4.5). Under this study, five doses of $AgNO_3$ singly and in combination with amino acids (L-proline, L-glutamine) were tested for callus induction and plant regeneration. The maximum callus induction was recorded (49.20 and 32.66%) for BARI barley-6 and 3**,** respectively when 2.0 mg/l $AgNO_3$ and 200 mg/l L-glutamine were added to the callus induction medium. Moreover, plant regeneration remarkably increased on MS + 1.0 mg/l BAP + 1.5 mg/l $AgNO_3$ + 150 mg/l L-glutamine as 37.20% in BARI barley-6. For rooting single using of $AgNO_3$ positively affected whereas, negative influence was observed in combinations of $AgNO_3$ and amino acids (L-proline, L-glutamine). Using $AgNO_3$ and amino acids, around <4, <27 and <5 fold callus induction, regeneration and rooting were increased. Finally it may be concluded that as a stimulating agent silver nitrate and amino acids influenced callus induction and its subsequent plant regeneration.

Other attempts (Exp. 7; section. 4.6) have been done using various callus sizes, age and their fresh weight to improve regeneration efficiency using mature embryos that derived from immature embryos of BARI barley-6. Embryos size were classified into four groups e.g. (A) 0.6-1.0 mm, (B) 1.1-1.5 mm, (C) 1.6-2.0 mm and (D) 2.1-2.5 mm. Highest callusing and green plants were obtained when 1.6-2.0 mm size of embryos were used. Out of four aged groups 4-6 weeks old calli and when its weight range was 151-200 mg showed better performance on green plant regeneration with studied barley genotypes. From these findings it may be concluded that for better regeneration callus size, age and its fresh weights are important factors for increasing green plantlets as well as root developments in barley.

In Exp. 8 (section. 5.1) of study has been undertaken to identify a suitable androgenetic variety, to determine the effect of cold pretreatment duration and optimization of media on barley anther culture. Two barley genotypes and five semi-solid media *viz.* MS, B5, N6, FHG and AMS_3 were considered for embryo induction as well as plant regeneration. Harvested spikes were pretreated with cold for 2, 4, 6, 8,

10, 12, 14 or 16 days at 4°C in the dark. Cold pretreatment for 8-12 days produced the highest frequency of embryoids on FHG medium. BB-6 performed better in terms of embryo formation and green plant regeneration in MSR medium. The highest level of embryoids induction (14.6%) was observed at 10 days of cold pretreatment, producing a total of 13.8% regenerated plantlets and 10.72% green plants. It was observed that the interactions of medium and cold pretreatment, genotype and medium were significant in determining the rate of embryoids induction.

Another attempt as pre-treatment factor of drought stress applied to excised anthers of BARI barley-3 and BARI barley-6 (Expt. 9; section 5.2). Various drought stress durations such as T_1 = 30, T_2 = 60, T_3 = 90, T_4 = 120, T_5 = 150, T_6 = 180, T_7 = 210 and T_8 = 240 minutes were applied to excised anthers. It was observed that drought stress for 150 min (T_5) showed highest percentage of embryoids induction (26.50) and green plants (13.58) in BARI barley-6.The present study demonstrated that drought stress can be influenced anther culture responses and could be used as one of the potential factors having influence on embryo yield and regeneration of barley anther culture. It was observed that the number of androgenic embryos and regenerated green plants in barley can be increased by in liquid induction medium than semi-solid medium. From the mentioned experimental findings it might be summarized that the cold or drought pretreatment and their interaction clearly influence the anther culture ability of barley and this protocol will be helpful for future any advance level of biotechnological research in Bangladesh.

Chapter VIII

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8. References

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Curriculum Vitae
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B. Educational background

Examination	Year	Board / University
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HSC	1991	Rajshahi Board
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C. List of Publications:

i) Published articles- 04

1. **Mozidul Haque** and Islam SMS (2014) Enhancement of anther culture response by cold pretreatment and optimization of media about two barley (*Hordeum vulgare* L.) genotypes derived from Bangladesh. Asia-Pacific J. Mol. Biol. Biotech. 22(1): 127-136.
2. **Mozidul Haque** and Islam SMS (2015) Callus age and size of barley (*Hordeum vulgare* L.) improves regeneration efficiency. Notulae Scientia Biologicae 7(2): 188-191.
3. **Mozidul Haque**, Siddique AB and Islam SMS (2015) Effect of silver nitrate and amino acids on high frequency plants regeneration in barley (*Hordeum vulgare* L.). Plant Tissue Culture and Biotechnology 25(1): 37-50.
4. Islam SMS and **Mozidul Haque** (2015) Effect of Carbon Sources and Plant Growth Regulators on Efficient Callus Induction and Regeneration in Barley (*Hordeum vulgare* L.). Asia-Pacific J. Mol. Biol. Biotech. 23(3).

ii) Abstracts (Conference/Workshop/Symposium)- 03

1. **Mozidul Haque** and S. M. Shahinul Islam (2014) An efficient callus induction and regeneration by copper sulphate and cobalt chloride using mature embryos in Barley (*Hordeum vulgare* L.). Sixth Int. Bot. Conf., Dece. 6-7, Abst. 33, p 73.
2. **Mozidul Haque** and S. M. Shahinul Islam **(2014)** Enhancement of anther culture response through cold pretreatment and optimization of media for two barley (*Hordeum vulgare* L.) genotypes in Bangladesh. 7th Int. Plant Tiss. Cult. & Biotech. Conf., 1-3 March, Organized by BAPTC&B, University of Dhaka, Bangladesh. Abst. 65, p 66.
3. **Mozidul Haque** and S. M. Shahinul Islam (2015) Effect of silver nitrate and amino acids on high frequency plants regeneration by immature embryos in barley (*Hordeum vulgare* L.). Annual PTC & Biotech Conf. 2014, Org. by BAPTC&B, March 28, Abst. 24, p 37.