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Somatic and Gametic Embryogenesis to Improve Chilli Pepper (Capsicum Annum L.)

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SOMATIC AND GAMETIC EMBRYOGENESIS TO IMPROVE CHILLI PEPPER (*CAPSICUM ANNUM* L.)

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By

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Dedicated
To
My Parents

DECLARATION

I hereby declare that the research work embodied in this thesis entitled **"SOMATIC AND GAMETIC EMBRYOGENESIS TO IMPROVE CHILLI PEPPER (*CAPSICUM ANNUM* L.)"** has been carried out by me for the degree of Doctor of Philosophy. The resaerch work was carried our under the guidance of Professor Dr. S. M. Shahinul Islam, 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 **Tushar Kanti Mondal** worked under my supervision as a Ph.D Fellow, Session: 2012-2013, Institute of Biological Sciences (IBSc), University of Rajshahi, Bangladesh. It is my great pleasure to forward his thesis entitled **"SOMATIC AND GAMETIC EMBRYOGENESIS TO IMPROVE CHILLI PEPPER (*CAPSICUM ANNUM* L.)"** which is a bonafide record of research carried out at Plant Biotechnology and Genetic Engeneering Laboratory, IBSc, University of Rajshahi. He has fulfilled all the requirements of the regulations relating to the nature and prescribed period of research submission of the thesis for awarding the degree of **Doctor of Philosophy.**

This is also to certify that the thesis represents the independent work of the candidate.

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ABSTRACT

Under this study, three experiments were conducted on somatic embryogenesis (SE), i.e., (i) *in vitro* improvement of calluses and its subsequent regeneration, (ii) direct organogenesis from different explants, and (iii) effect of PGRs and $AgNO_3$ on callus induction and regeneration of chilli cultivars in Bangladesh. Another two experiments were done on gametic embryogenesis (GE), i.e., (i) embryoids induction and plant regeneration via anther culture, and (ii) effects of cold stress pre-treatment factors on anther culture responses in chilli cultivars.

Six chilli cultivars of Bangladesh (three local e.g Boltu, Bullet and Halda and three hybrids e.g. Kulali, California wonder and Yellow wonder) were taken for *in vitro* improvement and its subsequent regeneration. Among the studied genotypes Bullet showed highest percentage (97.10%) for seed germination in *in vitro* culture whereas in the field condition the Bullet showed 67.20% germination. The variety Yellow wonder showed best performance on callus induction (98.60%) followed by Boltu (97.80%) and Bullet (96.20%) with 0.5 mg/l 2,4-D + 2.0 mg/l kin. For regeneration, Bullet produced the highest regenerated plantlets (71.40%) from cotyledonary explants which cultured on MS medium in addition with 0.50 mg/l kinetin. The highest number (76.40%) of shoot elongation was observed for Yellow wonder from cotyledonary explants followed by Kulali (71.60%) and California wonder (70.80%). Among the tested PGRs combination kinetin (0.5 mg/l) and GA_3 (1.0 mg/l) showed better results on shoot elongation (76.40%). Highest rooting was observed in Halda (98%) and Boltu (98%) which cultured on MS medium supplemented with IAA (0.5 mg/l) and kinetin (0.1 mg/l). In the present study, simple and suitable protocols on callus induction of the selected chilli genotypes were established for somatic embryogenesis.

Callus induction from cotyledon using 2,4-D (0.5 mg/l) and kinetin (2.0 mg/l) showed best performance on regeneration. It was observed that MS medium in addition with 0.5 mg/l kinetin showed best on regeneration. In case of callus induction and shoot elongation kinetin (0.5 mg/l) and GA_3 (1.0 mg/l) showed best than others. Best rooting was observed when plantlets were cultured on MS medium in addition with IAA (0.5 mg/l) and kinetin (0.1 mg/l). The results clearly demonstrated that above culture condition was the most effective and suitable for somatic embryogenesis of chilli cultivars.

To develop a simple, efficient and reliable *in vitro* regeneration various explants of chilli e.g. cotyledon, hypocotyl and cotyledonary nodal region from Boltu, Bullet, Halda and Zeha were considered of this study. Cotyledonary nodal region showed significantly higher number of shoot induction (69.60%) followed by cotyledon (67.20%). From hypocotyl explants only TDZ produced multiple shoots. In respect of all explants, Boltu produced highest number of shoots (69.60%) for cotyledonary nodal region, 67.20% for cotyledon and 45.00% for hypocotyl. Highest number (82.60%) of shoot elongation was observed in Boltu followed by Bullet (82.40%) and Zeha (82.00%). Among the tested PGRs significantly higher shoot elongation (82.60%) was recorded when plantlets were cultured in MS + BAP (8.0 mg/l) + GA_3 (1.0 mg/l). Highest (99.20%) rooting was observed on MS + NAA (0.5 mg/l) + BAP (2.0 mg/l) in Bullet. The protocol established under this study is efficient, quick and highly reproducible and can be used in advance biotechnological research for chillies improvement in future.

To examine the effect of PGRs and silver nitrate on callus induction and regeneration, two chilli cultivars (Bullet and Boltu) and as explants cotyledon and hypocotyl were used. Highest callusing was recorded from hypocotyl which cultured on MS medium in addition with BAP (4.0 mg/l) + $AgNO_3$ (3.0 mg/l) for Bullet (86.40%); followed by Boltu (73.80%) and the cotyledon explants. Among the tested cultivars highest callusing was recorded in Bullet (51.74%) than Boltu (41.26%). The hypocotyl showed better callusing (48.34%) than cotyledon (44.66%). Best shooting (75%) was found in Bullet which cultured in MS

medium in addition with BAP (5.0 mg/l) and $AgNO_3$ (3.0 mg/l). The number of roots per shoot was higher when cultured them in MS medium supplemented with 2.0 mg/l BAP + 0.1 mg/l NAA. The maximum number (9.40) of roots per shoot was observed in hypocotyl where calli derived from shoots.

To determine the androgenetic responses especially for anther culture five local chilli genotypes *viz*. Boltu, Bulett, Halda, Zeha and Baromashi and three basal media (MS, N and C-R) were considered. Here, Bullet showed the highest percentage of responded anthers (20.81%), when cultured them on C-R medium. In case of indirect embryogenesis, Halda showed best performance on callus induction (1.43%) on C-R medium. Though the frequency of regenerants of all of the studied genotypes showed very low, but Bullet showed highest regeneration (0.35%) in C-R medium.

To determine the effect of heat stress pre-treatment factors six different durations e.g. 3, 5, 7, 9, 11 and 13 d were considered for variety Bullet and as medium C-R was used. In this case cultured anthers were pre-treated at 35°C for the said duration and after the incubation period transferred the cultures at 25°C chamber. It was observed that heat stress pre-treatment for 3-13 days ranges stimulated anther culture response and best callusing was recorded for 7 days. Heat shock stress for 7 days showed highest percentage of ELS in case of direct embryogenesis, callusogenesis and for regeneration 5.28, 17.42, 1.26 and 2.99% found respectively.

To evaluate the effect of cold stress pre-treatment different durations e.g. 12, 24, 36, 48 and 60 h, as genotype Boltu, Bullet and Halda were taken and excised anthers were cultured on C-R medium. It was observed that Halda showed highest embryoids induction (22.63%) for 24 h cold shock stress pre-treatment. Both cv. Bullet and Boltu showed very less but heights regeneration (0.62%) for 24 h cold stress pre-treatment duration. The highest embryoids induction was found for 24 h (2.95%), followed by the duration of 12 h (2.63%).

Under this study some promising chilli cultivars has been undertaken to evaluate their responses on somatic and gametic embryogenesis in Bangladesh. It was the first attempts on anther culture studies of chillis cultivars in Bangladesh. For further advance research on anther culture these protocols will be very much helpful for scientist in Bangladesh and also other countries. Calli derived from the way of somatic and gametic embryogenesis and evaluation their efficiency on regeneration and the message is very much helpful for biotechnologist and plants breeders for further advance research on chilli cultivars. Consequently, further research is needed to establish the anther and microspore culture procedure settings many experiments, analysis of ploidy levels plants derived from gametic cells using more local and recently released chilli cultivars in Bangladesh.

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LIST OF ABBREVIATIONS

The following abbreviations have been used through the text:

%	:	Percent
°C	:	Celsius
°K	:	Kelvin
0.1N	:	0.1 Normal
2,4-D	:	2,4-dichloro phenoxy acetic acid
$AgNO_3$	:	Silver nitrate
ANOVA	:	Analysis of variance
B_5	:	Gamborg et al. (1968) medium
BAP	:	6-Benzyl amino purine
C	:	Dumas De Valux et al. (1981) induction medium
CH	:	Casein hydrolysate
cm	:	Centimeter
$CuSO_4$	:	Copper sulphate
cv.	:	Cultivar
d	:	Days
DHs	:	Doubled haploid plants
DMRT	:	Duncan Multiple Range Test
DMSO	:	Dimethylsulfooxide
DW	:	Distilled water

EDTA	:	Ethylenedinitrilo tetra acetic acid
et al.	:	Et alia = and others
Fig.	:	Figure
g	:	Gram
g/l	:	Gram per liter
GA_3	:	Gibberellic acid
h	:	Hour (s)
HCl	:	Hydrochloric acid
$HgCl_2$	:	Mercuric chloride
i.e	:	That is (to say)
IAA	:	Indole- 3-acetic acid
IBA	:	Indole-3- butyric acid
kg	:	Kilogram
Kin.	:	Kinetin
KOH	:	Potassium hydroxide
LSD	:	Least significant difference
mg	:	Milligram
mg/l	:	Milligram per liter
MS	:	Murashige and Skoog (1962) medium
N	:	Nitsch and Nitsch (1969) medium
NAA	:	α-naphathalene acetic acid
NaOH	:	Sodium hydroxide

PGRs	:	Plant growth regulators
P^H	:	Negative logarithm of hydrogen ion (H^-) concentration
ppm	:	Parts per million
R	:	Dumas De Valux et al. (1981) regeneration medium
SE	:	Standard error
sp.	:	Species
TDZ	:	Thidiazuron
v/v	:	Volume by volume
viz.	:	Videlicet (L.); namely
w/v	:	Weight by volume

Chapter I

General Introduction

1. General Introduction

Capsicum annuum L. (chilli pepper) belongs to the family of solananceae with around nine hundred genera and two thousands species. It an important commercial crop and cultivated more or less all over the country of Bangladesh. This family includes important vegetables like tomato, brinjal and potato. Basically two chilli crops are produced in Kharif and Rabi seasons in the country. For growing chilli cultivars best temperature range is at 20-30°C. Its growth and yields suffer when temperatures exceed 30°C or drops below 15°C. More than 400 different varieties of chillis found all over the world. The genus *Capsicum* consists of about 25 wild and 5 domestic species (*Capsicum annum* L., *Capsicum frutescns* L., *Capsicum chinense* Jacq, *Capsicum baccatum* L., *Capsicum pubescens* R&P) (IBPGR 1983). Out of them *C. annuum* is the most widespread and widely cultivated in tropical and subtropical countries in the world. The fruit of chilli or *Capsicum* plants have a variety of names depending on place and type. It is commonly called chilli pepper, red or green pepper, or sweet pepper in Britain and typically just capsicum in Australian and Indian English.

Chillies are rich in vitamins, especially in vitamin A and C. They are also packed with potassium, magnesium and iron. Bangladesh is the 3rd largest chilli producing country in the world. But there are are some limitations which attributed to its low productivity. Chilli seeds have poor limits of their germination (58%) in the field. Seeds usually germinate in dark condition. The seedlings damaged at the seed bed are another burning question. Considering the importance of chilli production, conservation of different local indigenous varieties and improvement of chilli of Bangladesh this research programme has undertaken with a major view to develop a reproducible technique for rapid multiplication and the germplasm conservation system of chillies of our country.

Preliminary studies have intensely indicated various inherent problems associated with *in vitro* culture of capsicum such as severe recalcitrant morphogenic nature, formation of rosette shoots or ill-defined shoot buds, genotypic dependence, which alone or together put at risk the whole tissue culture efforts and in turn plant improvement through biotechnological approaches. Tissue culture responses are greatly influenced by three main factors *viz*. physiology of donor plants, *in vitro* manipulation, and stress physiology was reported by Benson (2000).

Capsicum has been a hard to work with plant as compared to other solanaceous crops like tobacco, tomato, potato frequently used as model systems because of their great capability to regenerate plants. However, genotypic dependency is an important factors that influences the organogenesis in capsicum *in vitro* cultures. The existence of strong genotype specificity in the regeneration capacity of the different cultivars represents an important limiting factor that makes development of a standard regeneration protocols a necessity for each cultivar. Tissue culture has been exploited to create genetic variability by producing haploids, somaclonal and gametoclonal variants from which crop plants can be improved. It is also utilized for improving the state of health of the planted material and to increase the number of desirable germplasm available to the plant breeder.

There are many reports on successful callus induction as well as plant regeneration requires appropriate choice of explants and age of the the donor plants, culture media, growth regulators, genotype, source of carbon sources, gelling agent, other physical and chemical stress pre-treatment factors including light regime, temperature, humidity, pH, incubation periods, sub-culturing and related other factors. Ochoa-Alejo and Ireta-Moreno (1990) marked a clear influence of chilli pepper cultivar on the capacity of hypocotyl tissues form adventitious shoots. Venkataiah et al. (2003) used thidiazuron in addition to the induction medium for organ cultures and they used ten chilli pepper genotypes and

found the response depended upon the genotype specifically. They found out of the ten genotypes *Capsicum annuum* cv. CA 960, G4 and X-235 produced highest number of adventitious shoots.

Valadez-Bustos et al. (2009) used four chilli genotypes to evaluate their efficeincy on *in vitro* organ cultures and found results depend on variations on genotypes. Growth and morphogenesis of the plant tissue under *in vitro* conditions are largely affecting using appropiriate explant in vegetable and other crops. Mostly using explants are cotyledons, hypocotyls, leaves, shoot tips, zygotic embryos, embryonal leaves, stems, internodes, mature seeds and roots have been employed for calluses induction as well as plant regeneration in capsicum was reported by Agrawal and Chandra (1983), Ramírez-Malagón and Ochoa-Alejo (1996) and Berljak (1999). The pioneering work of successful regeneration of two chilli peppers using cotyledon and hypocotyl explants was reported by Gunay and Rao (1978). Cotyledon was found to be the most responsive explant and there are some reports on *in vitro* callus induction and successful regeneration using cotyledon explants mainly from *C. annuum* seedlings (Alibert 1990, Gatz and Rogozinska 1994, Binzel et al. 1996b).

For crop improvement production of haploids and doubled haploids (DHs) through anther and isolated microspore culture (gametic embryogenesis) is an important tool in the field of biotechnology. It is particularly useful for crops like chilli to generate a great deal of heterozygosity in the progenies. There are three androgenic routes potentially leading to a haploid/DH: elimination of the female genome after egg fertilization, meiocyte derived callogenesis and microspore embryogenesis. Of these, microspore embryogenesis has been by far the most exploited DH technique because of its convenience for producing pure homozygous lines in a short period of time with acceptable yields (Forster et al. 2007). Microspore embryogenesis consists of the inducible reprogramming of pollen grains or

their precursors, the microspores, diverting them from their original pathway toward embryogenesis. Till there are some reports using anther culture methods for chilli peppers improvement.

Haploids and diploids can be successfully applied to study pepper resistance to viruses (Valux et al. 1982, Pochard et al. 1983) and transmission of resistance to *Phytophthora capsici* (Abak et al. 1982). Dumas de Valux et al. (1981) studied androgenetic methods for chilli pepper's improvement by the application of heat stresses at +35°C for the first stage of anther culture. Vagera and Havranek (1985) used charcoal and carrot extract in addition to the medium and found charcoal and carrot extract stimulated androgenetic responses in Severka and Morava chilli pepper cultivars. The positive effect of charcoal on the anther culture of pepper was also reportedby Chunling and Baojun (1993).

Microspores can switch their normal gametophytic development *in vitro* towards an embryogenic pathway. The process, known as microspore embryogenesis, represents an important tool in plant breeding, since it allows the generation of isogenic lines and new varieties through double-haploid plants (Chupeau et al. 1998). Several protocols have been reported to induce microspore embryogenesis and plant regeneration in different varieties of *Capsicum annuum* L. (Dumas de Valux et al. 1981, Mityko et al. 1995, Dolcet-Sanjuan et al. 1997, Barany et al. 2001). In this study main aimed to improve the yield of haploid embryos in pepper anther culture by using different incubation conditions and by application of the two different basal media.

The main objectives of this study are mentioned below-

- *In vitro* screening of chilli genotypes in Bangladesh.
- Optimization of media and plant growth regulators (PGRs) to improve callus induction and regeneration through somatic and gametic embryogenesis.

- Application of chemicals and physicals stress pre-treatment factors to improve callus induction and regeneration in chilli.
- Screening of androgenetic chilli cultivars in Bangladesh.
- Optimization of media and other culture conditions for anther of chilli cultivars.
- Enhancement of anther culture responses by different physical and chemical stress pre-treatment factors in chilli.

Chapter II

Review of Literature

2. Review of Literature

2.1 Origin of chilli pepper

Chilli or pepper (*Capsicum* spp.) originated from South America and then spreaded to Asia and Africa and other countries **(Fig. 1)**. The three species *C. annuum*, *C. frutescens* and *C. chinense* evolved from a common ancestor located in the North of the Amazon basin (NW-Brazil, Columbia). On that time, chillies were cultivated by the farmers together with other crops to protect the primary crop from any damage from birds or insects. It has been reported that Columbus carried chilli seed to Spain in 1493. Then the cultivation of chilli and capsicum become popular and spread rapidly from Spain to Europe. Then chillies became more popular in the whole of Asia rapidly and native Asians started cultivating this crop. The south Asian climate is very much suitable for cultivation this crop, and since its introduction in the 16 century has been increasingly cultivated in south Asia.

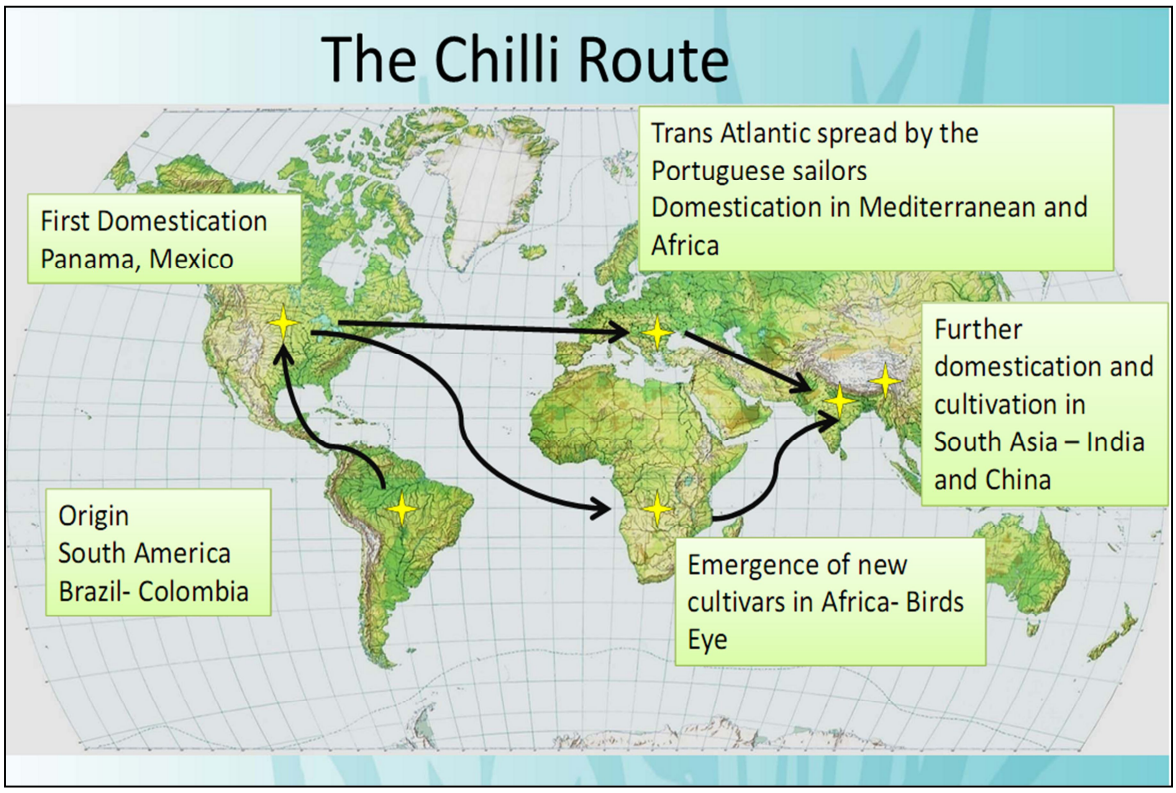

Fig. 1: Origin and domestication route of chilli (Image source: Internet).

2.2 Taxonomic position and classification of capsicum

Chilli is a fruit of the plants *C. annuum* and *C. frutecens* that come from the genus capsicum belongs to the family of solanaceae. Its scientif classification is mentioned below:

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Solanales

Family: Solanaceae (= Nightshade family)

Genus: *Capsicum*

2.2.1 Plants type

Chilli plant is an annual or perennial herbaceous or semi-woody type and its fruits varied on the basis of its shape, size, colour and degree of pungency. The plant reaches a height of one meter or more. Life cycle of chilli peepers is around 30-190 days (**Fig. 2**).

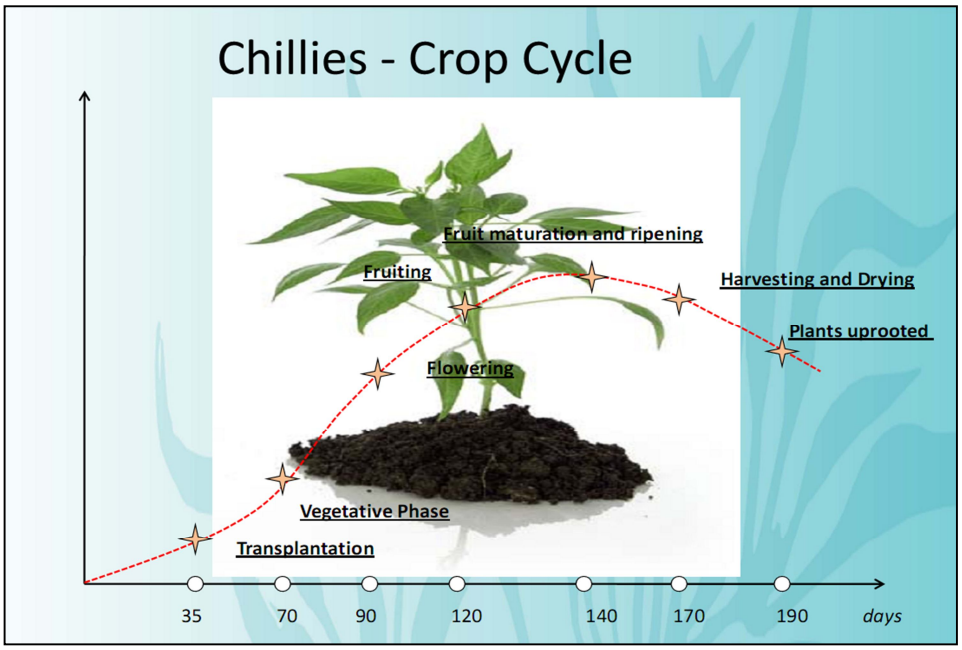

Fig. 2: Crop cycle of chilli (Image source: Internet).

2.2.2 Leaves

Leaves of chilis are ovate, tapering with sharp point, measuring up to 15 cm, dark green on the upper surface and pale green on the lower surface. The lanceolate leaves are either glabrous or pubescent ovate, tapering to a sharp point, measuring up to 15 cm, dark green on the upper surface and pale green on the lower surface.

2.2.3 Floral biology and pollination habit

The chillis flowers are develop about 3 months after planting. Colour of chilli flowers is white but in some genotypes it is violet or purple. The calyx is 5 lobed and corolla is 5-parted and white, but occasionally purple in colour.

2.2.4 Fruits

Fruits of chillis are a pod like thick peduncle and short berry types. Chilli fruits are first green, when ripening they turn yellow or red. The shape of the fruit varies from a flattened oblate to long slender and tapering.

2.2.5 Varieties

Various local and hybrid chilli varieties are available in Bangladesh and there are no recommended chilli cultivars yet. Popular chilli cultivars and their major cultivation regions of Bangladesh are Balujuri (Sherpur), Bindu (Manikganj), Irrimarich (Comilla), Baromashi, Halda, Zeha, Boltu, Bullet and Suryapati (Khulna).

2.3 Cultivation of chilli

2.3.1 Soil, climate and rising of seedling

Chilli is grown in both tropical and sub-tropical areas. An ideal condition for chilli growing is a light loamy soil and for its cultivation suitabable temperature is at 20 to 25°C. The best time for sowing of seed is from 1st week of September to 15th October. It has been reported that a warm humid climate is favourable for growing chilli while dry weather enhances fruit maturity. The following doses of manures and fertilizers have been recommended for chilli cultivars in Bangladesh (**Table 1**).

Table 1: Following doses of manures and fertilizers needed for chilli cultivars

Manures and fertilizers	Doses (kg/ha)
Cowdung/Compost	15 tons /ha
Urea	200
TSP	300
MP	200
Gypsum	110 -120
Zinc oxide	5

2.4 Production of chilli

The top 10 chilli producing countries of the world is India, China, Bangladesh, Ethiopia, Myanmar, Mexico, Vietnam, Peru, Pakistan, Ghana and accounted for more than 85% of the world production in 2009. The lion's share is taken by India with 36% share in global production, followed by China (11%), Bangladesh (8%), Peru (8%) and Pakistan (6%). Bangladesh is not only a large producer but also the large consumer of chilli in the world. Bangladesh contributes about 8% to the total world Bangladesh ranks third in the world with an area of 150000 (ha.) and products 170000 (MT) per year.

Table 2 distributes the division wise cultivated area of chillies by varieties for the year 2014. Out of the three varieties, local has the highest cultivation area of chilli which is 79.09%. The second highest 16.77% of land is used for the hybrid variety of chilli. And the remaining land areas of 4.14% have been used for all other varieties of chilli. It is mentionable that 5.14%, 4.16%, 3.31% and 2.47% acres of land are used for hybrid variety in Khulna, Chittagong, Dhaka and Rangpur division respectively whereas the remaining three divisions cultivate only 1.70% land for chilli.

Table 2: Percentage distribution of chilli cultivation area by farming time and division

Division	Total area		Farming time			
			Summer		Winter	
	Hectares	%	Area	%	Area	%
Barisal	31546	7.26	-	-	31546	7.26
Chittagong	84683	19.48	31896	7.34	52787	12.14
Dhaka	112451	25.87	50400	11.59	62051	14.27
Khulna	42405	9.75	34305	7.89	8100	1.86
Rajshahi	77406	17.80	26114	6.01	51292	11.80
Rangpur	77316	17.78	40514	9.32	36802	8.46
Sylhet	8951	2.06	55	0.01	8896	2.05
All over the country in Bangladesh	434757	100	183284	42.16	251473	57.84

(Source: Global Disclosure of Econ. Business, 4(1): 2307-9592, 2015).

2.5 Importance of chilli

Capsaicin is being studied as an effective treatment for sensory nerve fiber disorders, including pain associated with arthritis, psoriasis, and diabetic neuropathy. Nutritional value of chilli peppers (per 100 g) are mentioned in **Table 3**.

Table 3: Nutritional value of chilli cultivars

Nutritional value of chilli (per 100 g)		
Parameters	Dry chilli	Green chilli
Moisture	10.00 g	85.70 g
Protein	15.00 g	2.90 g
Fat	6.20 g	0.60 g
Minerals	6.10 g	1.00 g
Fibre	30.20 g	6.80 g
Carbohydrates	31.60 g	3.00 g
Energy	246.00 kcal	29.00 kcal
Calcium	160.00 mg	30.00 mg
Phosphorus	370.00 mg	80.00 mg
Iron	2.30 mg	4.40 mg
Vitamins		
Carotene	345.00 µg	175.00 µg
Thiamine	0.93 mg	0.19 mg
Riboflavin	0.43 mg	0.39 mg
Niacin	9.50 mg	0.90 mg
Vitamin C	50.00 mg	111.00 mg
Minerals and trace elements		
Sodium	14.00 mg	-
Potassium	530.00 mg	-
Phytin Phosphorus	71.00 mg	7.00 mg
Magnesium	-	272.00 mg
Copper	-	1.40 mg
Manganese	-	1.38 mg
Molybdenum	-	0.07 mg
Zinc	-	1.78 mg
Chromium	-	0.04 mg
Oxalic acid	-	67.00 mg
Caloric values		
Chilli (dry)		297 kcal
Chilli (green)		229 kcal

2.6 Somatic embryogenesis (SE)

Somatic embryogenesis is an important tool for obtaining plants derived from calli from various explants, cell suspension culture or protoplast fusion. *In vitro* asexual embryogenesis such as parthenogenesis, adventitious embryos and somatic embryogenesis are powerful tools for mass production embryos as well as regeneration. Different methods and explants for successful completion of somatic embryogenesis are metioned below:

2.6.1 Callus induction and regeneration

Callus refers to a group of cells derived from competent source tissue that is cultured under *in vitro* conditions to form an undifferentiated mass of cells which alludes to the effectively isolating disorderly mass of cells initiated in culture. According to Skoog and Miller (1957), for the most part, induction of callus formation was found when the growth medium contained a higher concentration of auxin. A wide assortment of conditions like genotypes, explants, light/dark incubation period and development controllers may affect the quantity and quality of callus produced was reported by Patil (2001). Kintzios et al. (2000) reported that the impact of various inorganic micro-nutrients and vitamins on callus induction and development of somatic embryos from young leaves of *C. annum.*

2.6.2 Multiple shoots induction from cotyledonary explants

The most regularly utilized explants for developing shoots in chillis are cotyledonary leaf. Gunay and Rao (1978) firstly reported the successful regeneration of capsicum through *in vitro* culture and Kniitel et al. (1991) reported the successful regeneration from cotyledons of mature zygotic embryos as explants in chilli peppers. Shivegowda et al. (2002) found best result for regeneration from cotyledonary explants of chilli using MS medium supplemented with zeatin (9-18 μM) + GA_3 (2.89 μM). An efficeint protocol of direct substantial embryogenesis utilizing stem fragments and shoot tips of *C. annuum* was developed by Kintzios et al. (2000).

2.7 Gametic embryogenesis (GE)

Gametic embryogenesis (cultures of gamet cells) is a convenient alternative in plant breeding because it makes possible the development of homozygous lines, increasing efficiency and speed in conventional breeding programs. Biotechnologies provide powerful tools for plant breeding, and among these ones, tissue culture, particularly haploid and doubled haploid technology, can effectively help to select superior plants. In fact, haploids (n), which are plants with gametophytic chromosome number, and doubled haploids (2n), which are haploids that have undergone chromosome duplication, represent a particularly attractive biotechnological method to accelerate plant breeding and advance biotechnological works. Production of doubled haploids through anther (AC) or isolated microspore culture (IMC) is called androhenesis. Androgenesis is defined as the development of a haploid/doubled haploid (DH) individual whose genome originates exclusively from a male nucleus. Androgenic haploids and DHs present many important advantages for basic research and commercial applications (Forster et al. 2007). There are some reports using traditional methods for chilli peppers improvement through anther and microspore culture.

2.7.1.1 Anther culture (AC)

Anther culture is very quick as compared to microspore culture and it also influences development of microspores acting as a conditioning factor. Uncontrolled effect of anther wall (possibility of diploid plants and secretion of growth inhibiting substances) and other associated tissue are eliminated but anther wall has a stimulatory effect. The anther wall provides the nourishment in microspore development of a number of plant species. During anther culture there is always the possibility that somatic cells of the anther that are diploid will also respond to the culture condition and so produce unwanted diploid calli or plantlets.

The most important issue affecting embryogenesis is the age of donor plant. Anthers extracted from buds of the primary inflorescences have higher embryogenic potential (Powell 1990, Reinert and Bajaj 1992). There were less responding anthers when used around 12 weeks old donor plants were reported by Kristiansen and Andersen (1993). There are some reports about the most significant factors for influencing androgenic responses in chilli pepper by using a suitable genotypes (Comlekcioglu et al. 2001, Rodeva 2001, Wang and Zhang 2001, Rodeva et al. 2004, Gudeva et al. 2007, Liu et al. 2007). However, certain pepper genotypes are recalcitrant on the way to induction of androgenesis and establishment of haploid regenerants. The frequency of direct embryogenesis differs from 0.5 to 75 embryoids per 100 anthers and the response is dependent on genotypes was reported by Qin and Rotino (1993), Ltifi and Wenzel (1994), Mityko et al. (1995) and Gudeva et al. (2007). They used different procedures for haploid plant production and standarized an effective DH production strategy. The problems associated with work in this field, and the influence of critical factors for successful embryo formation and plantlet development is summarize, through comparison, results in pepper anther culture.

2.7.1.2 Isolated microspore culture (IMC)

Haploid plants have the genotypic number of chromosomes that is a single set of chromosomes in sporophyte that induced by different techniques. Where the most promising and successful technique is isolated microspore culture. The IMC is an important and useful tool in plant breeding for production of homozygous line and combined study with genetic transformation to develop fertile homozygous plants can be produced rapidly. To overcome the high reactivity of somatic tissues such as anther wall, multicellular hair-type structures, anther connective and parenchymatous vascular bundle microspore culture is very elegant system.

2.8 Rooting

Plants derived from somatic and gametic cells through *in vitro* cultures were sub-cultured on MS medium which supplemeted various concentrations of auxins e.g lAA (1.0 mg/l) and kinetin (0.1 mg/l) following the methods of Christopher et al. (1986).

2.9 Hardening of plants

Well rooted plants were transferred to pots after acclimatization that contained with garden soil (with 90% survival rate).

Chapter III
Materials and Methods (General)

3. Materials and Methods (General)

All experiments of this study were carried out at the Plant Biotechnology and Genetic Engenering Lab., Institute of Biological Sciences, University of Rajshahi, Rajshahi, Bangladesh.

3.1 Plant material

3.1.1 Cultivars

The popular local cultiver *viz*. Boltu, Bullet, Halda, Zeha and Baromasi were considered because the cultivars are more popular in coastal region for its pungency, flavour and attractive colour. The other cultivars Hybrid-1, Shimla, Kulali, Yellow wonder, California wonder were chosen also for the study, because the cultivars are more popular in our country and abroad for its flavour pungency, mild or less pungency and attractive colour (**Fig. 4 & 5**). All of the cultivers are in *Capsicum annuum* (2n = 2x = 24).

Fig. 3 (a-j): Fruits of selected chilli cultivars; (a) Boltu, (b) Bulette, (c) Halda, (d) Zeha, (e) Baromasi, (f) Hybrid-1, (g) Shimla, (h) Kulali, (i) California wonder, and (j) Yellow wonder.

Fig. 4 (a-j): Morphology of selected chilli cultivers. (a) Boltu, (b) Bullet, (c) Halda, (d) Baromasi, (e) Kulali, (f) Zeha, (g) Shimla, (h) Hybreed-1, (i) California wonder, and (j) Yellow wonder.

3.1.2 Explant sources

All explants of this study were collected from 15 days old *in vitro* grown seedling of Boltu, Bullet, Halda, Zeha, Shimla, Hybreed-1, Kulali, Baromasi, California wonder and Yellow wonder to evaluate their efficiency on callus induction and regeneration (somatic embryogenesis). For androgenetic studies (gametic embryogenesis) flower buds of Boltu, Bullet, Halda, Zeha and Baromasi were collected from the experimental field of the IBSc, RU at 8:30 am to 9:30 am. The schematic diagram of these studies is shown in **Fig. 5.**

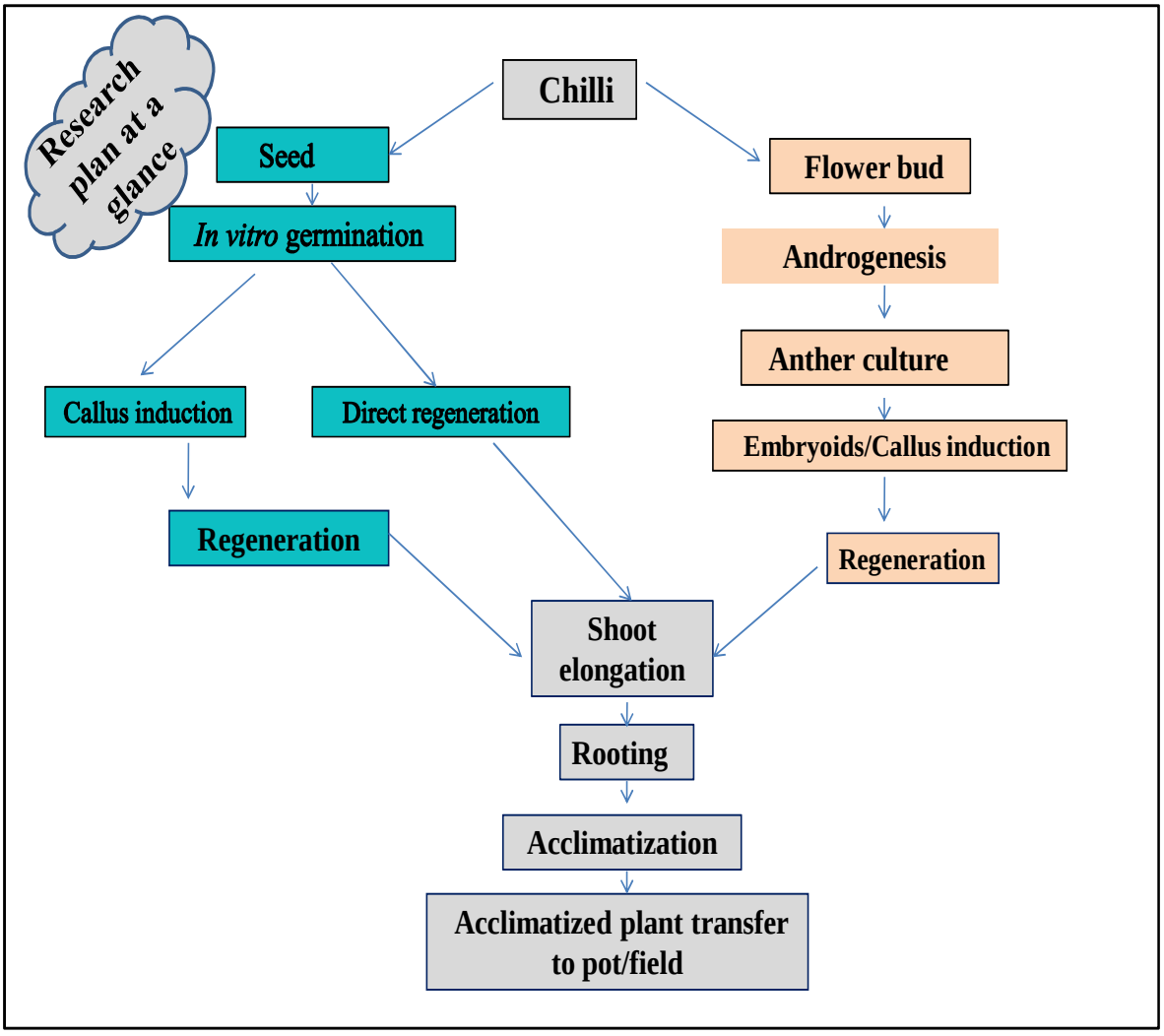

Fig. 5: Flow chart is showing details the research activities at a glance.

3.1.3 Plant growth regulators (PGRs)

As plant growths regulators major phytohormones like auxins (IAA, NAA and 2,4-D), cytokinins (BAP and Kin), gibberellins (GA_3) and TDZ (Thidiazuron) were used for this study. For experimental purpose PGRs were used either single or in combination are mentioned in **Table 4**.

Table 4: Various plant growth regulators (PGRs) and its solubility

Types	PGRs	Solubility	Molecular weight (g/mol)
Auxin	NAA	1N NaOH	186.21
	IAA	1N NaOH	175.19
	Picloram	Ethanol	241.46
	2,4-D	Ethanol	221.04
	IBA	1N NaOH	203.24
	BAP	1N NaOH	225.25
Cytokinin	Kinetin	1N NaOH	215.22
	Zeatin	1N HCl	219.20
Gibberellins	GA_3	1N KOH	346.38

3.1.4 Culture media

Two major culture media namely Murashige and Skoog (1962) and B5 (Gamborg et al. (1968) were used with certain modifications and its compositions are given below (**Table 4 & 5**).

Table 4: Composition of three culture media

Category	Constituents	Media (mg/l)		
		MS	½MS	B_5
A. Macronutrients	KNO_3	1900.00	950.00	2500.00
	NH_4NO_3	1650.00	825.00	-
	KH_2PO_4	170.00	85.00	-
	$CaCl_2.2H_2O$	440.00	220.00	150.00
	$MgSO_4.7H_2O$	370.00	185.00	122.09
	NH4SO4	-	-	134.00
	$Na(PO_4)_2H_2O$			130.42
B. Micro-nutrients	$MnSO_4.4H_2O$	22.30	11.15	-
	$MnSO_4.H_2O$	-	-	10.00
	H_3BO_3	6.20	3.10	3.00
	$ZnSO_4.7H_2O$	8.60	4.30	2.00
	KI	0.83	0.415	0.75
	$Na_2MoO_4.2H_2O$	0.25	0.125	0. 25
	$CuSO_4.5H_2O$	0.025	0.125	0.025
	$CoCl_2.6H_2O$	0.025	0.125	0.025
C. Micro-nutrients (iron)	$FeSO_4.7H_2O$	27.85	13.925	27.85
	Na_2-EDTA	37.25	18.625	37.25
D. Vitamin-I	Glycine	2.00	1.00	-
	Nicotinic acid	0.50	0.25	1.00
	Pyridoxine HCl	0.50	0.25	1.00
	Thiamine-HCl	0.10	0.05	10.00
E. Vitamin-II	Myo- inositol	100.00	50.00	100.00
F. Carbon sources	Sucrose	30,000.00	30,000.00	20,000.00
p^H	-	5.8	5.4	5.2

Table 6: Culture medium for androgenetic studied in chilli cultivars

Nutrient's catagory	Components	Media (mg/l)			
		MS	N	C	R
Macro-I	KNO_3	1900	950	2150	2150
	$MgSO_4.7H_2O$	370	185	444	444
	NH_4NO_3	1650	720	1238	1238
	KH_2PO_4	170	68	142	142
	$NaH_2PO_4.H_2O$	-	-	38	38
	$(NH4)_2\ SO_4$	-	-	34	34
	$Ca(NO_3)_2$	-	-	50	50
Macro-II	$CaCl_2.2H_2O$	440	166	313	313
	KCl	-	-	7	7
	$MnSO_4.H_2O$	-	-	22.13	20.13
	$ZnSO_4.7H_2O$	8.6	10	3.625	3.225
	H_3BO_3	6.2	10	3.150	1.550
Micro-II	KI	0.83	-	0.695	0.330
	$Na_2MoO_4.2H_2O$	0.25	0.25	0.188	0.138
Micro-III	$CuSO_4.5H_2O$	0.025	0.025	0.016	0.011
	$CoCl_2.6H_2O$	0.025	-	0.016	0.011
Vitamin- I	Glycine	02	02	0.10	0.10
	Thiamin-HCl	0.10	0.5	0.60	0.60
	Pyridoxin-HCl	0.50	0.5	5.5	5.5
	Nicotinic Acid	0.50	05	0.7	0.7
	Ca-Pantothenate	-	-	0.500	0.500
	Folic Acid	-	0.5	-	-
	Biotin	-	0.05	0.005	0.005
	Vitamin B_{12}	-	-	0.03	-
Vitamin-II	Myo-inositol	100	100	50.300	50.300
Iron	$FeSO_4.7H_2O$	27.8	27.8	13.90	13.90
	Na_2-EDTA	37.3	37.3	18.65	18.65
Carbon sources	Sucrose	30000	20000	30000	30000
	Tryptone	-	-	-	-
	Activated charcoal	1%	1%	-	-
	Carrot extract	200 ml/l	200 ml/l	-	-
Solidifying agent	Phytagel	4000	4000	4000	4000
	Agar	8000	8000	8000	8000
P^H		5.5	5.5	5.9	5.9

N = Nitsch and Nitsch (1969), C & R = Dumas De Vaulx et al. (1981) (induction and regeneration medium).

3.2 Methodology

3.2.1 Somatic embryogenesis

3.2.1.1 Preparation of explants

The fruits of chilli pepers were surface sterilized with distilled water for 4-5 times and then washed with distilled water in addition with Tween 20 + 1-2 drop of savlon. Then dip in 70% ethanol for 45-60 seconds for 1 min and washed with sterile distilled water for 3-4 times and finally shaked gently with 4% NaOCl for 5 min and washed 5-6 times. Then the seeds were placed in culture vessels with 25 ml of MS medium for germination. Around 7-10 seeds were placed in each culture vessels and then incubated in dark. The seedlings of twelve (12) days were selected for the explant sources (**Fig. 6**). Its cotyledons (1 - 2 mm), hypocotyls (2 - 3 mm) and cotyledonary nodes (1 - 2 mm) excised into pieces using sanitized surgical blades and forceps.

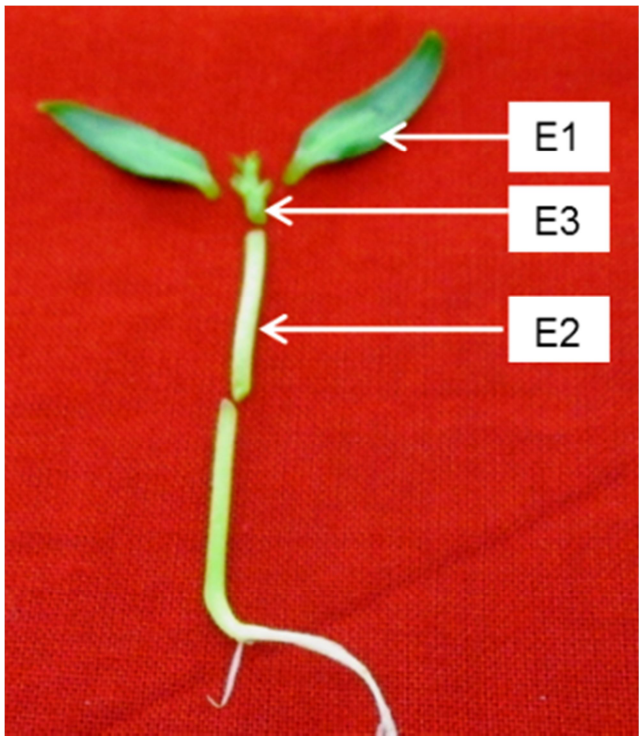

Fig. 6: Various explants (E1-cotyledon, E2- hypocotyl, and E3- cotyledonary nodal region/ shoot tip) were used for callus and multiple shoot induction.

3.2.1.2 Preparation of media

The macro and micronutrients, vitamins and myo-inositol were taken from the stock solutions according to the requirement of plant tissue culture medium. Varius plant growth regulators used of this study is mentioned in **Table 5 & 6**. In addition sucrose 30 g/l and myo-inositol 100 mg/l were added freshly in the medium before autoclaving. The p^H of all media was adjusted to 5.6 - 5.8 using 0.1N NaOH or 0.1N HCl. Then agar (8.0 g/l) or phytagel (4.0 g/l) was added and autoclaved at 121°C for 15 min.

3.2.1.3 Callus induction

Three types of explants *viz*., cotyledon, hypocotyl and shoot tips were used for this study. Various types of calluses induced e.g. friable, cottony, watery, and compact, colour of callus, and visual callus quality of this studies were observed after 3-4 weeks of culture initiation. For callus induction MS medium in addition with various growth regulators either single or in combinations were used. As auxin 2,4-D (0.1, 0.5, 1.0, 1.5, 2.0 mg/l), auxins + cytokinin (2,4-D + BAP = 1.0 + 1.0, 1.0 + 1.5, 2.0 + 2.0, 1.0 + 2.5 mg/l) and 2,4-D + kinetin (1.0 + 1.0, 1.0 + 1.5, 1.0 + 2.0, 1.5 + 2.5 mg/l) was used under this study.

3.2.1.4 Regeneration

Calli derived from various explants of this study were sub-cultured on MS medium with lower concentration of BAP (0.25, 0.5 and 1.0 mg/l), kinetin (0.25, 0.5 and 1.0 mg/l) and BAP + IAA (0.5 + 0.1 mg/l) for regeneration. Then cultures were incubated at 25±2°C for 16/8 h. Data were recorded on the basis of days to callus initiation, callus induction; fresh callus weight (g) and number of shoots and anlysed statistically.

3.2.1.5 Induction of multiple shoots and elongation

For multiple shoot induction various types of explants e.g. cotyledon, hypocotyls and cotyledonary nodal region were considered and cultured them on MS medium in addition with various concentration of BAP (2.0, 4.0, 6.0, 8.0, 10.0, 12.0,14.0 mg/l) and TDZ (0.1, 0.5, 1.0, 1.5 mg/l). For shoot elongation as basal salt MS medium was used supplemented with BAP (10.0 mg/l) and GA_3 (0.5, 1.0 or 2.0 mg/l).

3.2.1.6 Rooting and hardening

For rooting shoots derived of this study were transferred to MS medium which supplemented with various concentrations of BAP + IAA (0.1 + 0.1, 0.1 + 0.5, 0.1 + 1.0 mg/l) and BAP + NAA (0.1 + 0.1, 0.1 + 0.5, 0.1 + 1.0 mg/l). After 10 days of inoculation data were taken. Well rooted plants were transferred to pots contained with peat: soil: sand (1:1:1) mixture.

3.2.2 Gametic embryogenesis (anther culture)

3.2.2.1 Cultivation of plants and collection of flower buds

A part of seeds (selected cultivars) were shown at the experimental field of the Institute of Biological Sciences, University of Rajshahi and another part is grown in pots. The flower buds of the first inflorescences of selected cultivers of Boltu, Bullet, Halda, Zeha, Hybreed-1, Kulali, Baromasi, California wonder, Yellow wonder were collected and excised anthers in different stages of development in the morning before 9:30 AM were used as explant sources (**Fig. 7**). The immature flower buds with corolla and calyx were harvested to determine the microspor developmental stages (**Fig. 8**). Microspore developmental stages were checked by 1% acetocarmine under microscope (**Fig. 9**).

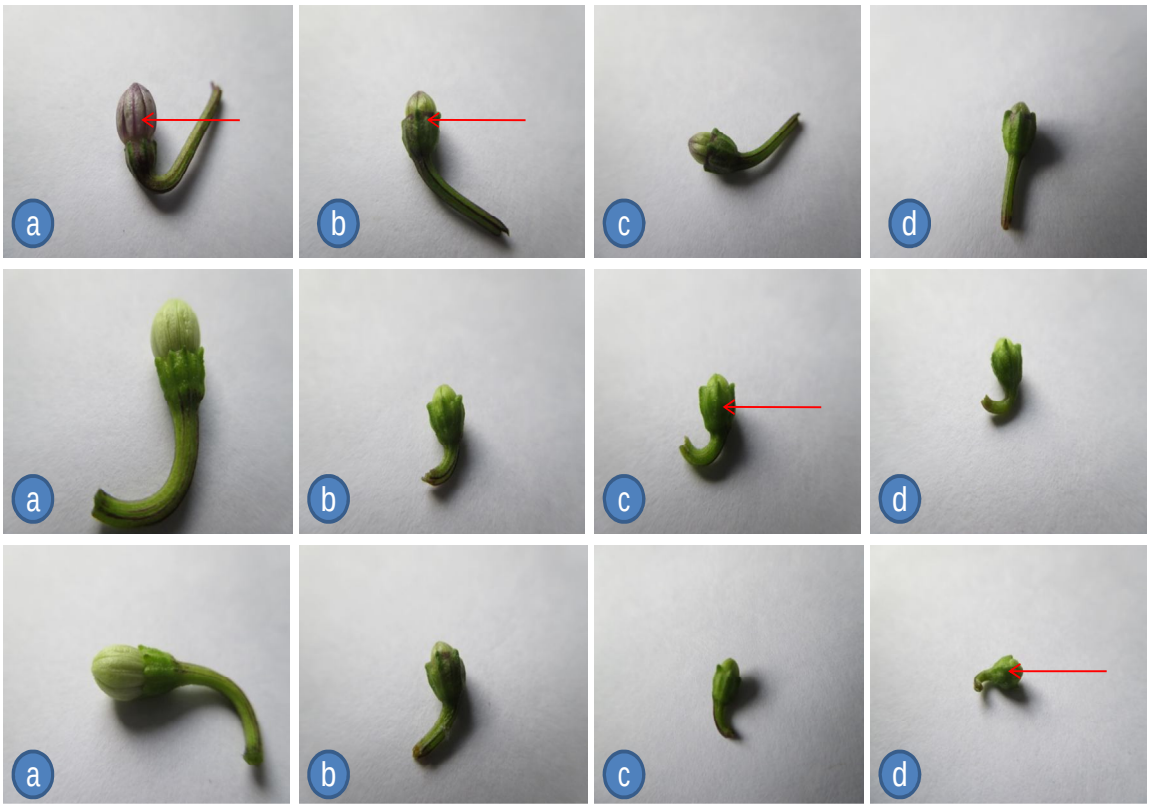

Fig. 7 (a-d): Flower buds of chilli cultivars. (a) Flower buds contain bi-neucleate microspores, (b) Flower buds contain bi-nucleate & uni-nucleate microspores, (c) Flower buds contains uni-nucleate microspores, (d) Flower buds contained early stages of microspores are not suitable for culture.

Fig. 8 (a-j): Flowers of selected chilli cultivers. (a) Boltu, (b) Bullet, (c) Halda, (d) Baromasi, (e) Kulali, (f) Zeha, (g) Shimla, (h) Hybreed-1, (i) California wonder, and (j) Yellow wonder.

Fig. 9: Selected anthers for culture (one third portion of anther is violet colour it contained uninucleate microspores).

3.2.2.2 Cold pre-treatment, surface sterilization and inoculation

Collected flower buds were wrapped with aluminum foil and incubated at 4°C chamber for 1-5 days. Harvested flower busd were surface sterilized following standard procedures under laminer air flow cabinet. Very carefully anthers were removed from the treated fowers buds in aseptics conditions and cultured them in sterile glass petri dishes.

3.2.2.3 Culture media

For this study full and half strength of MS, N, C & R media were used with certain modifications. The media was supplemented with different concentration of carbons such as sucrose (0-5%), maltose (0-5%) and glucose (0-5%) in addition with single BAP (1-10 mg/l), NAA (1-1.5 mg/l), kinetin (1.0 mg/l), 2,4-D (1.0 mg/l), peptone (2.0 mg/l) and $CuSO_4.5H_2O$ (2.2 mg/l).

3.2.2.4 Inoculation of anthers and embryoids induction

To obtain callus and/or embryods excised anthers were cultured on MS, N, C and R media which supplemented with various concentration of kinetin (0.1, 0.5, 1.0, 2.0, 2.5, 3.0 mg/l), 2,4-D (0.1, 0.5, 1.0, 2.0 mg/l) and BAP (0.1, 0.5, 1.0, 1.5 mg/l). Data were recorded on the basis of the number of anthers and induced embryoids. The experiment was repeated thrice. Inoculated anthers were incubated at 25°C ± 1°C chamber in dark for 4-6 weeks for embryoids induction. Number of proliferated embryoids was transferred and number plants were recorded.

3.2.2.5 Transfer of embryoids for shoot induction

For shoot development calli/embryoids were transferred to semi-solid ½MS medium with different concentration of carbons and as phytohormones BAP (1.0 mg/l) and NAA (1.0 mg/l) and placed them at growth chamber for shoot regeneration.

3.3.8.6 Sub-culture and root induction

The sub-cultured callus contained to proliferate and differentiate into shoots when these grew at 2-3 cm in length. They were rescued aseptically from the cultured vials and were separated from each other and cultured medium that was freshly prepared such as i) T_1 = ½MS + 0.5 mg/l NAA + 0.5 mg/l IAA, ii) T_2 = ½MS + 0.5 mg/l NAA + 1.0 mg/l IAA, and iii) T_3 = ½MS + 0.5 mg/l NAA + 1.5 mg/l IAA. The vials containing plantlet were incubated under continuous light. Observations were carried out to note the response of the growing plantlets.

3.3.8.7 Data recording and analysis

3.3.8.7.1 Seed germination

Seed germination was assessed after seven days of culture initiation following the formula mentioned below:

$$\text{Seed germination (\%)} = \frac{\text{No. of germinated seeds}}{\text{No. of inoculated seeds}} \times 100$$

3.3.8.7.2 Assessment of shoot development

Shoots derived from inoculated explants were calculated following the formula mentioned below:

$$\text{Shoot induction (\%)} = \frac{\text{No. of multiple shoots}}{\text{No. of explants}} \times 100$$

3.3.8.7.3 Assesment of shoots and its elongation

Responsed of multiple shoots were assessed after 15 days of culture initiation and calculated them on following the formula mentioned below:

$$\text{Shoot elongation (\%)} = \frac{\text{No. of explants produced multiple shoots}}{\text{No. of explants with multiple shoots}} \times 100$$

3.3.8.7.4 Assessment the days of callus initiation

After 30 days of culture initiation responded explants showed callus initiation were calculated following the formulae mentioned below:

$$\text{Callus initiation (\%)} = \frac{\text{No. of explants with callus initiation}}{\text{Total No. of explants}} \times 100$$

3.3.8.7.5 Assessment of embryogenesis

Calli derived from various explnats under this study were assessed following the formulae mentioned below:

$$\text{Embryogenesis (\%)} = \frac{\text{No. of calli contributed on embryogenesis}}{\text{Total No. of callus cultures}} \times 100$$

3.3.8.7.6 Assessment of rooting

Rooting was assessed by calculating the number of roots obtained under shoots was assessed following the formulae mentioned below:

$$\text{Rooting (\%)} = \frac{\text{No. of shoots with multiple roots}}{\text{Total No. of shoots}} \times 100$$

3.3.8.7.7 Assessment of embryos derived from anthers

Embryoids derived from cultured anthers were assessed following the formulae mentioned below:

$$\text{Embryods (\%)} = \frac{\text{No. of anthers with embryos}}{\text{Total No. of anthers}} \times 100$$

3.4.8 Statistical analysis

Each treatment had three replicates containing five explants in each culture vessel. Data collected on different parameters were subjected to analysis of variance (ANOVA) and mean values of treatments were compared by Duncan Multiple Range Test (DMRT) were calculated at the confidence level of $P < 0.05$ (Duncan 1955) using SPSS software (version 17.0) for Windows 7.

Chapter IV

Somatic Embryogenesis

4. Somatic Embryogenesis (Experimental findings)

4.1 An efficient *in vitro* micropropagation using various explants of chilli cultivars in Bangladesh

4.1.1 Introduction

The first evidence of somatic embryogenesis from cell suspension culture in carrot was reported by Steward et al. (1958). For improvement of crop through *in vitro* micropropagation a protocol is very essential, particularly for callus induction, callus multiplication and plantlet regeneration. For somatic embryogenesis MS is most widely used as basal medium for *Capsicum* cultivars. Tissue culture of chilli is very difficult compared to other dicot plants (Gammoudi et al. 2018). Induction of callus and subsequent plant regeneration are affected by the genotypes, carbohydrate metabolism-source, plant growth regulators (PGRs), culture medium and conditions, etc (Sahijram and Bahadur 2015, Kumar et al. 2019). Among the mentioned factors, genotypic differences, media, culture conditions and nutrient compositions are important factors for success of *in vitro* regeneration (Abe and Futsuhara 1986, Rueb et al. 1994, Kunanuvatchaidach et al. 1995, Khanna and Raina 1998, Ge et al. 2006). Moreover, effect of PGRs in marigold (Kumar et al. 2019), potato (Bhuiyan et al. 2019) and soybean (Islam et al. 2017), carbon sources in barley (Haque et al. 2015, Haque and Islam 2015) and banana (Hossain et al. 2009); light in tobacco (Yanjie 2004, Siddique and Islam 2015); silver nitrate in barley (Haque et al. 2015); callus age and size in barley (Haque and Islam 2015) and wheat (Islam 2010) were investigated in some studies on somatic and gametic embryogenesis.

Chilli is known as one of the main agricultural spice crops and commonly used as a spice for wonders. It is botanically identified as *Capsicum annuum* L. or *Capsicum frutescene* L. Chilli, commonly known as red pepper, belongs to the family of Solanaceae and the genus *Capsicum*. Traditional techniques typically use enormous quantities of seeds, resulting in a

substantial shortage of crops. Cross-pollination activity of chilli plants is a threat to agricultural and commercial seed development propagation characteristics. Also the limitations of seed propagation are limited viability and the poor germination rate. Methods of tissue culture offer a means to asexually multiply chilli pepper because there is no naturally occurring vegetative propagation. Hence, *in vitro* tissue culture may be an easy and cost effective way to produce a large number of disease free and true type plants in a short time to increase reliably standardized yield and quality. For somatic embryogenesis different explants like cotyledons, hypocotyls, epical meristem and direct shoot organogenesis from 15 days seedling were reported by Gunay and Rao (1978), Diaz et al. (1988) and Christopher and Rajam (1996). Moreover, those approaches were not or could not be changed as used to regenerate plants from certain cultivars of pepper. As a consequence, the regeneration from different explants, the main influence is pepper variety and the optimization of techniques for *in vitro* propagation of those forms is important. In a few instances, spontaneous shoot regeneration has been reported from different explants cultured on medium lacking of phytohormone (Ezura et al. 1993, Binzel et al. 1996a). The present work has been considered to develop somatic embryogenesis as well as screening of the most popular chilli cultivars using several explants of this study.

4.1.2 Materials and Methods

4.1.2.1 Plant materials

Mature seeds of chilli cultivars were used to grow *in vitro* seedlings of this study. Various explants like cotyledon, hypocotyl and cotyledonary nodes were taken from the *in vitro* grown plants. The ripe fruits of Boltu, Bullet, Baromasi, Halda and Zeha were collected from the local farmers of Dumuria in the District of Khulna, Bangladesh. California wonder, Hybreed-1, Kulali, Shimla and Yellow wonder were collected from India and Thailand. In this experiment six chilli cultivars *viz.* Boltu, Bullet, Halda, Kulali, California wonder and Yellow wonder were selected on the basis of germination performance.

4.1.2.2 Methods

4.1.2.2.1 Preparation of explants

Described briefly in Chapter III, Section 3.2.1.

4.1.2.2.2 Culture media and growth regulators (PGRs) for callus induction

Callus induction medium (CIM) were prepared by MS basal salt which supplemented with thirteen different concentration and combinations of PGRs either singly or in combinations (T_1 - T_{13}). Where T_0 = Control (without PGRs), T_1 = 0.1 mg/l 2,4-D, T_2 = 0.5 mg/l 2, -D, T_3 = 1.0 mg/l 2,4-D, T_4 = 1.5 mg/l 2,4-D, T_5 = 2.0 mg/l 2,4-D, T_6 = 1.0 mg/l 2,4-D + 1.0 mg/l BAP, T_7 = 1.0 mg/l 2,4-D + 1.5 mg/l BAP, T_8 = 2.0 mg/l 2,4-D + 2.0 mg/l BAP, T_9 = 1.0 mg/l 2,4-D + 2.5 mg/l BAP, T_{10} = 1.0 mg/l 2,4-D + 1.0 mg/l Kin, T_{11} = 1.0 mg/l 2,4-D + 1.5 mg/l Kin, T_{12}= 1.0 mg/l 2,4-D + 2.0 mg/l Kin, and T_{13} = 1.5 mg/l 2,4-D + 2.5 mg/l Kin. Five - six pieces of explants of six studied cultivars were placed horizontally in every vessel and quietly placed into the surface of the sterilized culture medium with thirteen different treatments mentioned herein (**Fig. 12a**). The culture vessels were incubated under fluorescent light in a room with controlled temperature at 25±1°C with 16 h photoperiod. After 10 - 12 days data on callus initiation were recorded.

4.1.2.2.3 Optimization of media for callus induction (CIMs)

To observe the effect of media on callusing, the suitable PGRs concentration and combination T_8 (2.0 mg/l 2,4-D + 2.0 mg/l BAP) was considered as constant growth regulator, and augmented independently with three basal media, *viz.* MS, N and B_5 to prepare the CIMs. Sterilized mature seeds of all the studied cultivars were inoculated on the CIMs for callus induction.

4.1.2.2.4 Culture media and growth regulators for plant regeneration

Calli derived from various explants reached in a suitable size (1.0 - 1.4 cm) were removed aseptically and placed them on fresh medium contained with suitable growth regulators for shoot induction. For regeneration, media were prepared by MS basal supplemented with eight different concentration and combinations of PGRs either singly or in combination (R_1 - R_8), where R_0 = control (free from PGRs), R_1 = 0.5 mg/l BAP, R_2 = 1.0 mg/l BAP, R_3 = 1.0 mg/l BAP + 0.5 mg/l IAA, R_4 = 1.0 mg/l BAP + 0.5 mg/l NAA, R_5 = 1.0 mg/l BAP + 0.5 mg/l Kin + 0.5 mg/l IAA, R_6 = 1.5 mg/l BAP + 1.0 mg/l Kin + 1.0 mg/l NAA, R_7 = 1.0 mg/l BAP + 1.0 mg/l Kin + 0.5 mg/l NAA, and R_8 = 1.0 mg/l BAP + 0.5 mg/l Kin + 0.5 mg/l NAA. After every 21 days, primary cultures were sub-cultured on semi-solid MS medium augmented with same growth regulators. After 45 days of sub-culture the somatic embryos started germinating and the shoot buds achieved appropriate size after two months from callus culture. The vessels were kept under white fluorescent lamp of 32 Watt with light intensity of 5000 lux at 16/8 hours light/dark cycle. The temperature of the culture room or growth chamber was maintained at 26±1°C. The calli which produced the shoots were counted and considered to determine the frequency of plant regeneration.

4.1.2.2.5 Optimization of media for plant regeneration

To observe the effect of media on plant regeneration, the suitable PGRs concentration and combination R_6 (1.5 mg/l BAP + 1.0 mg/l Kin + 1.0 mg/l NAA) was considered as constant growth regulator, and added separately with three basal media, such as *viz.* MS, N and B_5 to prepare the regeneration media and cultured the calli for plant regeneration.

4.1.2.2.6 Culture condition for root induction

To induce sufficient roots MS basal medium supplemented with 1.0 mg/l IBA was used as root induction media (RIM). After regeneration when the shoot buds achieved appropriate size (4 - 5 cm) after two months from callus culture and then detached aseptically from the

vessels on a sterilized glass plate inside in the laminar air flow cabinet and were placed into culture vessels containing RIM. The culture vessels were kept under white fluorescent lamp of 36 Watt with light intensity of 5000 lux at 16/8 hours light/dark cycle. The temperature of the culture room or growth chamber was maintained at 25±1°C. The shoots which produced roots considered for determining the frequency of root induction (RI).

4.1.2.2.7 Acclimatization and transferred of plants to field

Plantlets with healthy shoot and root systems were washed (especially the root portions) under running tap water to clear off the entire residual agar medium so as to check the chances of contamination in soil. The plantlets were then transferred to small (about 5 cm diameter) plastic cups containing pre-soaked sterilized vermiculite and kept inside the moist chamber and covered with polyethylene bag to avoid evapo-transpirational losses of water for one-two weeks. Then they were kept under the shade conditions for further period of two weeks. After hardening the plants were transferred to larger pot (about 20 cm diameter) containing the mixture of vermicompost: soil: sand (1:1:1); and kept under open light maintaining the natural environmental conditions for field culture.

4.1.2.2.8 Data recording and statistical analysis

Data were recorded as number of calli to determine the frequencies of callus induction and plant regeneration (%) and calculate using the formulae followed by Zaidi et al. (2006). Data were analysis proceures were done and the formulae mentiond in Chapter III (3.4.1, 3.3.3, 3.4.4, 3.4.6) and for statistical analysis the procedures are briefly described in Chapter III (3.4.8).

4.1.3 Results

4.1.3.1 Seed germination

Experiment on *in vitro* seed germination was conducted using ten chilli cultivars *viz.* Boltu, Bullet, Baromasi, California wonder, Halda, Hybreed-1, Kulali, Shimla, Yellow wonder and Zeha. Seeds of selected cultivars were cultured on MS semi-solid medium without any PGRs (**Fig. 10**). Among the ten studied genotypes Bulet showed highest percentage (97.10%) for seed germination whereas in the field condition the Bullet showed 67.20% germination. For all cases in field condition studied genotypes were showed low response for seed germination. The results are presented in **Table 7.**

Table 7: Seed germination index of ten chilli cultivars

Name of chilli cultivars	No. of inoculated seed	Weight/ per seed (mg)	% of germinated seeds (*in vitro*) (Mean ± S.E)	% of germinated seeds(field) (Mean ± S.E)
Baromasi	70	2.5	66.80 ± 0.37^{c}	51.40 ± 0.37^{c}
Boltu	85	4.1	95.60 ± 0.97^{g}	65.10 ± 0.97^{g}
Bullet	90	6.0	97.10 ± 0.31^{h}	67.20 ± 0.31^{h}
California wonder	90	5.5	91.60 ± 0.50^{f}	54.00 ± 0.38^{d}
Halda	70	4.8	89.20 ± 0.86^{f}	60.20 ± 0.37^{e}
Hybreed-1	80	9.2	64.20 ± 0.38^{b}	45.40 ± 0.50^{c}
Shimla	85	9.6	60.80 ± 0.37^{a}	39.20 ± 0.37^{a}
Kulali	75	2.0	90.20 ± 0.96^{f}	62.20 ± 0.96^{f}
Yellow wonder	75	4.8	96.20 ± 0.86^{gh}	66.80 ± 0.86^{gh}
Zeha	70	4.6	61.10 ± 0.92^{a}	43.10 ± 0.92^{b}

Values represent mean ± S.E (standard error). Values in a column with similar superscripts are not significantly different at $p \leq 0.05$ levels according to DMRT.

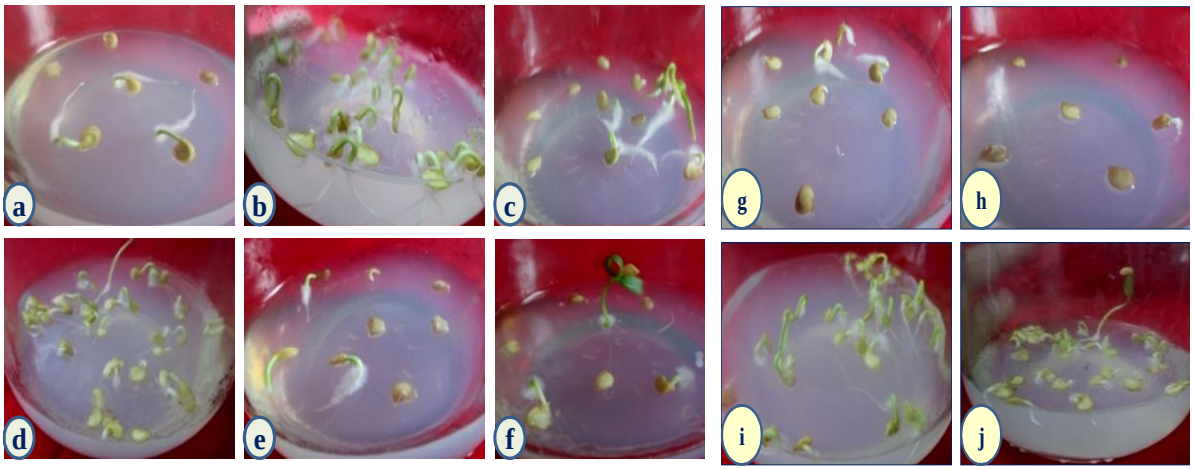

Fig. 10 (a-j): *In vitro* germination of ten chilli cultivars. (a) Boltu, (b) Bullet, (c) Baromasi, (d) California wonder, (e) Halda, (f) Hybreed-1, (g) Kulali, (h) Shimla, (i) Yellow wonder, and (j) Zeha.

4.1.3.2 Effect of PGRs on callus induction

Thirteen PGRs combinations (T_1 - T_{13}) were supplemented to MS medium were applied on three different explants (cotyledon, hypocotyl and cotyledonary node) and tested their efficiency on callus induction for all the studied six chilli cultivars (**Table 8**). Required minimum duration (days) for callus initiation were presented in **Fig. 11**. The results indicated that all the responding cultivars showed well performance to callus induction (CI). Out of six cultivars, Yellow wonder performed highest frequency of CI (98.60%) followed by Boltu (97.80%), Bullet (96.20%), Halda (95.80%), California wonder (94.80%) and Kulali (91.60) in T_8 (2.0 mg/l 2, 4-D + 2.0 mg/l BAP) from cotyledon explant (**Table 8**). Conversely, hypocotyl explant gave the lowest frequency of CI in T_1 (0.1 mg/l 2,4-D) for all studied genotypes (**Table 8**). On the other hand, minimum duration (10.33 days) was recorded for callus initiation in T_4 (1.5 mg/l 2,4-D) and maximum (12 days) was in T_{13} (0.5 mg/l 2,4-D + 2.5 mg/l Kin) (**Fig. 11)**. In this experiment, non-embryogenic friable calli mass were produced from the single uses of the PGR 2,4-D (without combination of other PGR) but compact emryogenic calli mass were formed

when 2,4-D was combinely used with kinetin ro BAP (**Fig. 12 b&c**). On an average of the PGRs (treatments), Boltu showed the highest (45.08%) callusing performance; followed by 44.92, 44.26, 44.11, 43.24 and 42.12% for Yellow wonder, California wonder, Bullet, Kulali and Halda respectively, though the explants cotyledon exhibited highest 45.55% CI rate; subsequently 43.34 and 42.97% from cotyledonary node and hypocotyl explant correspondingly (**Fig. 13**).

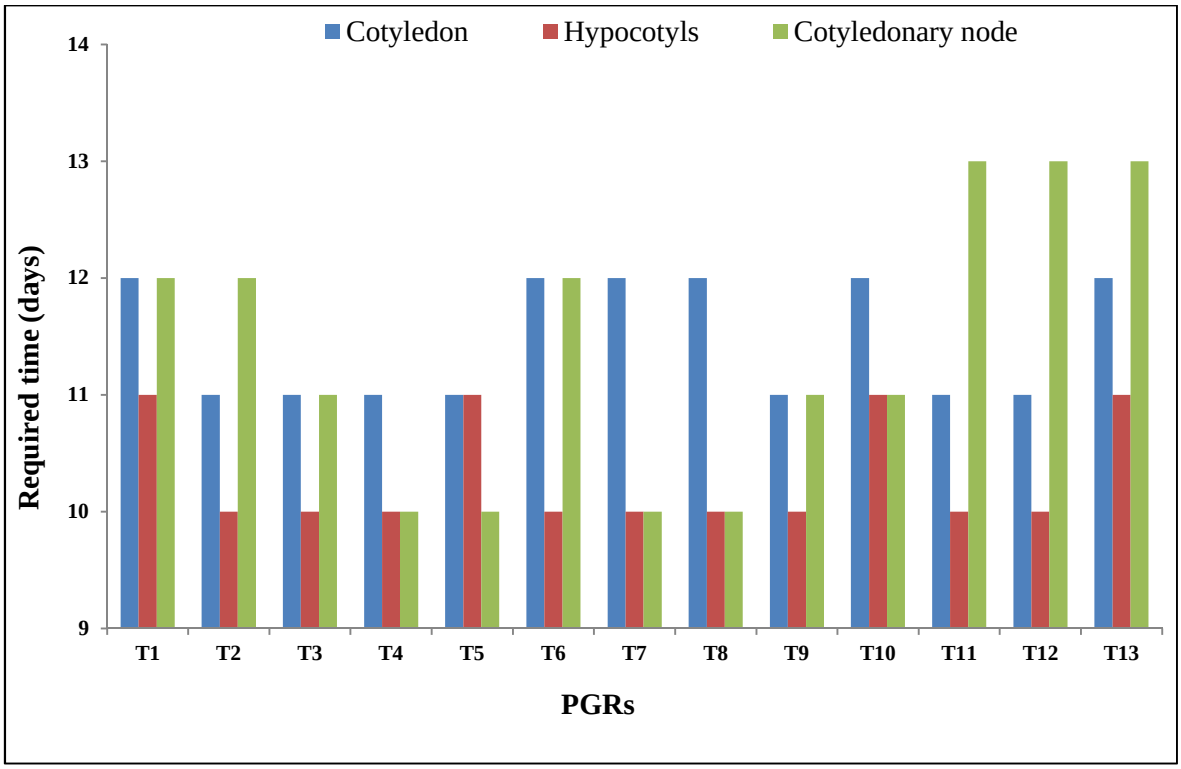

Fig. 11: Effects of plant growth regulators with various explants on callus induction at different days of inoculation.

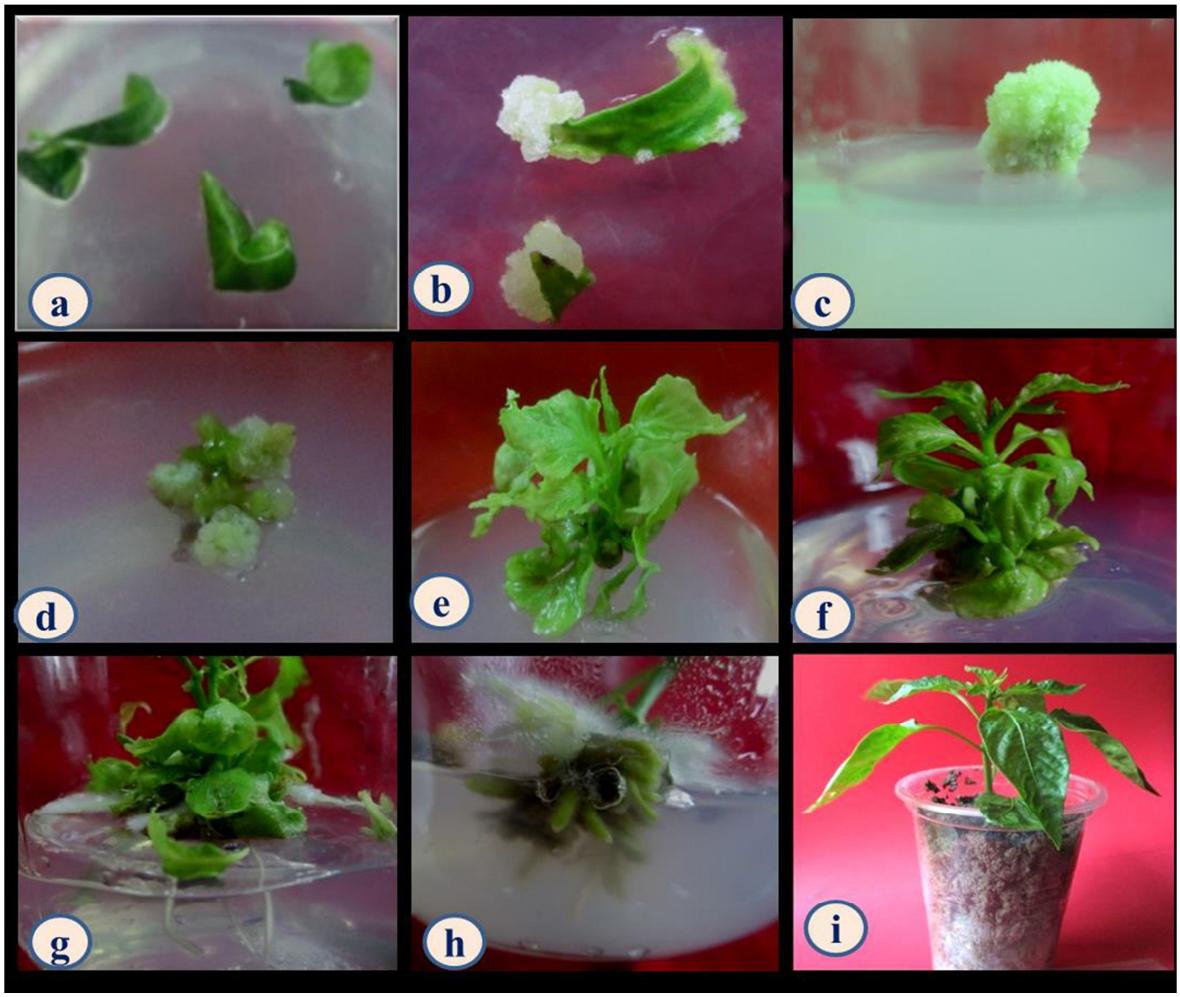

Fig. 12 (a-i): *In vitro* regeneration of chilli cultivars. (a) cotyledon culture on callus induction media, (b-c) embryogenic calli formation, (d) somatic embryo formation, (e-f) regenerate plants (shoots) from embryo, (g-h) well rooted plantlet, and (i) plantlet transferred to pot.

Table 8: Effect of PGRs on callus induction from three explants of six chilli cultivars

Explants	PGRs	Callus induction (% ± SE)					
		Boltu	Bullet	Halda	Kulali	California wonder	Yellow wonder
Cotyledon	**T_0**	$0.00\pm0.00_n$	$0.00\pm0.00_m$	$0.00\pm0.00_m$	$0.00\pm0.00_o$	$0.00\pm0.00_m$	$0.00\pm0.00_o$
	T_1	7.20±0.66m	$7.02\pm0.45_l$	$5.20\pm0.37_k$	7.10 ±0.44$_m$	7.00±.31$_l$	$7.20\pm0.65_l$
	T_2	$18.80\pm0.66_l$	$17.80\pm0.86_k$	$18.60\pm0.51_k$	$16.80\pm0.37_k$	$17.40\pm0.50_k$	$16.80\pm0.65_l$
	T_3	$20.20\pm0.66_k$	$19.20\pm0.49_j$	$22.80\pm0.58_j$	$21.00\pm0.44_j$	$20.20\pm0.37_j$	$19.20\pm0.65_l$
	T_4	$25.60\pm0.87_j$	$25.00\pm0.71_i$	$24.20\pm0.58_i$	$23.20\pm0.37_i$	$27.00\pm0.44_i$	$25.60\pm0.88_j$
	T_5	$29.60\pm0.67_i$	$28.00\pm0.71_h$	$27.80\pm0.5_h$	$27.40\pm0.50_h$	$30.40\pm0.81_h$	$29.60\pm0.65_i$
	T_6	$42.80\pm0.66_g$	$41.40\pm0.51_f$	$31.00\pm0.44_f$	$40.00\pm0.70_f$	$43.00\pm0.44_f$	$42.80\pm0.67_g$
	T_7	$65.20\pm0.37_d$	$64.20\pm0.37_d$	$62.20\pm0.36_d$	$60.80\pm0.58_d$	$66.20\pm0.37_d$	$65.20\pm0.35_d$
	T_8	**$97.80\pm0.48_a$**	**$96.20\pm0.37_a$**	**$95.80\pm0.37_a$**	**$91.60\pm0.50_a$**	**$94.80\pm0.32_a$**	**$98.60\pm0.49_a$**
	T_9	$79.20\pm0.58_b$	$78.40\pm0.51_b$	$76.20\pm0.37_b$	$77.20\pm0.37_b$	$80.20\pm0.58_b$	$79.20\pm0.56_b$
	T_{10}	$40.60\pm0.74_h$	$40.20\pm0.37_f$	$38.80\pm0.37_g$	$38.80\pm0.73_f$	$41.60\pm0.74_g$	$40.60\pm0.73_h$
	T_{11}	$48.60\pm0.87_f$	$48.20\pm0.58_g$	$39.80\pm0.58_g$	$35.40\pm0.50_g$	$37.00\pm0.31_g$	$48.60\pm0.85_f$
	T_{12}	$73.60\pm0.50_c$	$72.00\pm0.45_c$	$70.60\pm0.50_c$	$69.80\pm0.58_c$	$74.20\pm0.37_c$	$73.60\pm0.48_c$
	T_{13}	$61.40\pm0.67_e$	$59.40\pm0.60_e$	$59.20\pm0.58_e$	$56.80\pm0.37_e$	$61.80\pm0.58_e$	$61.40\pm0.66_e$
Hypocotyl	**T_0**	$0.00\pm0.00_n$	$0.00\pm0.00_m$	$0.00\pm0.00_m$	$0.00\pm0.00_o$	$0.00\pm0.00_m$	$0.00\pm0.00_o$
	T_1	$6.20\pm0.66_m$	$6.30\pm0.40_l$	$5.60\pm0.50_l$	$5.80\pm0.37_n$	$7.40\pm0.50_l$	$6.10\pm0.66_n$
	T_2	16.00±0.70l	$14.20\pm0.73_k$	$16.60\pm0.40_j$	$13.20\pm0.73_{jk}$	14.60±.24$_k$	$12.00\pm0.69_k$
	T_3	19.40±0.50k	$18.20\pm0.37_k$	$19.00\pm0.44_i$	$18.20\pm0.37_j$	17.20±.37$_j$	$18.40\pm0.48_k$
	T_4	23.00±0.70j	$22.20\pm0.58_j$	$22.20\pm0.37_h$	22.20 ±0.58$_i$	23.80±.58$_i$	$23.00\pm0.69_j$
	T_5	26.60±0.87i	$26.00\pm0.71_i$	$22.20\pm0.58_h$	$26.00\pm0.70_i$	26.00±.54$_h$	$26.60\pm0.86_i$
	T_6	40.60±0.50g	$39.60\pm0.40_g$	$40.40\pm0.81_e$	$39.60\pm0.40_f$	41.60±.50$_f$	$40.60\pm0.49_g$
	T_7	62.80±0.37d	$62.00\pm0.45_d$	$61.00\pm0.54_d$	$62.00\pm0.44_d$	63.80±.37$_d$	$62.80\pm0.36_d$
	T_8	87.00±0.44a	$86.20\pm0.37_a$	$87.20\pm0.37_a$	$86.20\pm0.37_a$	88.00±.44$_a$	$86.00\pm0.43_a$
	T_9	69.60±0.50c	$69.00\pm0.45_c$	$70.00\pm0.70_c$	$69.00\pm0.44_c$	70.40±.50$_c$	$69.60\pm0.48_c$
	T_{10}	34.80±0.37h	$34.00\pm0.32_h$	$35.40\pm0.50_f$	$34.00\pm0.31_h$	35.80±.37$_g$	$34.80\pm0.36_h$
	T_{11}	48.80±0.58f	$48.60\pm0.68_f$	$30.40\pm0.50_g$	$48.60\pm0.67_g$	35.20±.37$_g$	$48.80\pm0.57_f$
	T_{12}	$74.60\pm1.40_b$	$72.20\pm0.37_b$	$74.60\pm1.40_b$	$72.20\pm0.37_b$	$75.60\pm1.40_b$	$74.60\pm0.40_b$
	T_{13}	59.40±0.60e	$58.80\pm0.58_e$	$59.60\pm0.50_d$	$58.80\pm0.58_e$	60.40±.60$_e$	$59.40\pm0.58_e$
Cotyledonary node	**T_0**	$0.00\pm0.00_n$	$0.00\pm0.00_m$	$0.00\pm0.00_m$	$0.00\pm0.00_o$	$0.00\pm0.00_m$	$0.00\pm0.00_o$
	T_1	$9.80\pm0.66_m$	$9.40\pm1.21_k$	**$4.80\pm0.37_l$**	9.40 ±1.20$_l$	$8.00\pm0.31_k$	$9.80\pm0.64_m$
	T_2	$15.40\pm0.50_l$	$14.60\pm0.93_j$	$14.80\pm0.37_k$	14.60 ±0.92$_k$	$13.60\pm0.24_j$	$15.40\pm0.48_l$
	T_3	$19.60\pm0.87_k$	$18.60\pm0.51_i$	$16.00\pm0.70_j$	17.60 ±0.50$_j$	$19.80\pm0.37_i$	$21.60\pm0.85_k$
	T_4	$23.80\pm0.37_j$	$22.80\pm0.37_h$	$20.80\pm0.37_j$	$22.80\pm0.37_i$	$24.80\pm0.37_h$	$23.80\pm0.37_j$
	T_5	$25.60\pm0.67_i$	$24.60\pm0.51_h$	$21.00\pm0.44_i$	24.60 ±0.50$_i$	$24.60\pm0.50_h$	$25.60\pm0.66_i$
	T_6	$47.80\pm0.66_f$	$47.80\pm0.37_g$	$30.40\pm0.50_f$	$47.80\pm0.37_g$	$34.20\pm0.37_g$	$47.80\pm0.65_f$
	T_7	$62.80\pm0.37_d$	$61.60\pm0.68_d$	$59.80\pm0.58_d$	$61.60\pm0.67_d$	$63.80\pm0.37_d$	$62.80\pm0.35_d$
	T_8	$90.80\pm0.58_a$	$89.60\pm0.51_a$	$89.20\pm0.66_a$	$89.60\pm0.50_a$	$91.60\pm0.50_a$	$90.80\pm0.57_a$
	T_9	$78.20\pm0.58_b$	$76.20\pm0.58_b$	$75.00\pm0.31_b$	$76.20\pm0.58_b$	$79.00\pm0.44_b$	$78.20\pm0.58_b$
	T_{10}	$32.40\pm0.24_h$	$31.80\pm0.37_g$	$31.00\pm0.44_h$	$31.80\pm0.37_h$	$33.40\pm0.24_g$	$32.40\pm0.22_h$
	T_{11}	$40.40\pm0.50_g$	$38.80\pm0.37_f$	$38.20\pm0.58_g$	$38.80\pm0.37_f$	$41.00\pm0.70_f$	$40.40\pm0.50_g$
	T_{12}	$72.00\pm0.54_c$	$71.00\pm0.32_c$	$69.20\pm0.37_c$	$71.00\pm0.31_c$	$70.60\pm0.40_c$	$72.00\pm0.52_c$
	T_{13}	$60.00\pm0.44_e$	$59.00\pm0.45_e$	$56.20\pm0.37_e$	$59.00\pm0.44_e$	$60.00\pm0.54_e$	$60.00\pm0.43_e$

Values in a column with similar subscripts are not significantly different at $p\leq0.05$ levels according to DMRT.

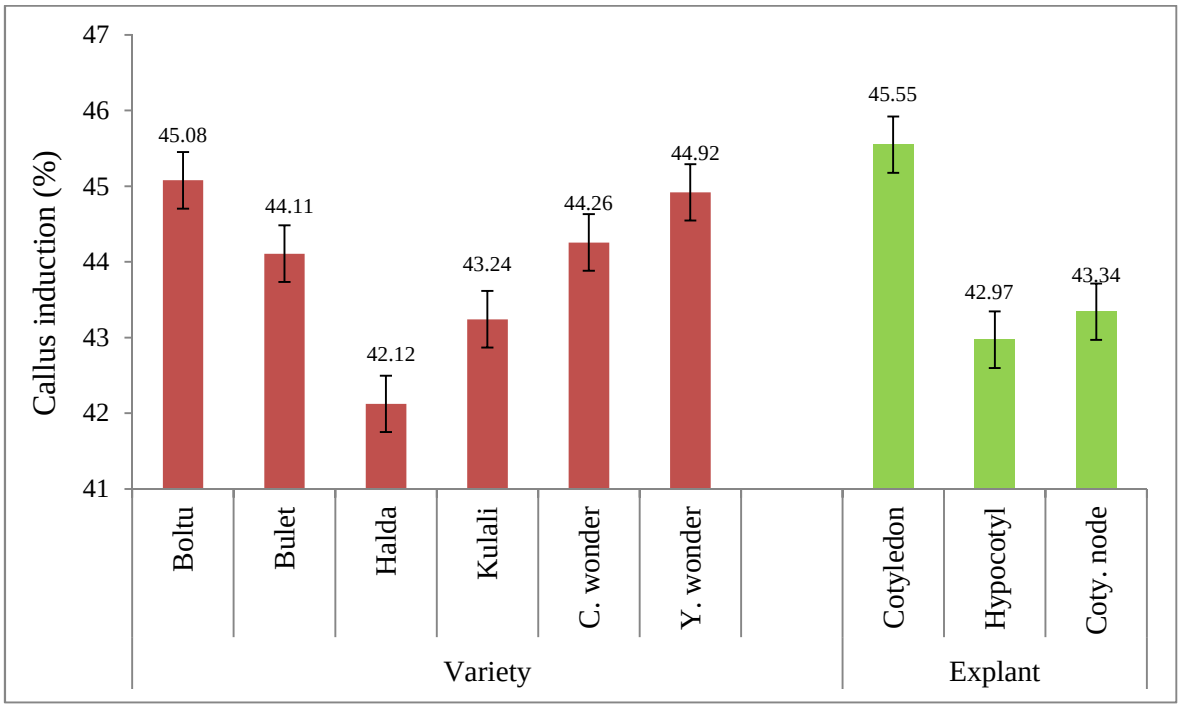

Fig. 13: An efficient callus induction of the studied chilli cultivars using various explants.

4.1.3.3 Optimization of media for callus induction

To test the effect media on callus induction, a suitable growth regulator and optimum concentration and combinations T_8 (2.0 mg/l 2,4-D + 2.0 mg/l BAP) was considered as constant and added individually with three basal media namely MS, N and B_5; and all the studied genotypes were cultured to induce calli. The results showed that all the genotypes performed the maximum callusing in MS medium. Boltu induced calli at the highest rate 45.08% in MS; and followed by 44.92%, 44.26%, 44.11%, 43.24% and 42.02% for Yellow wonder, California wonder, Bullet, Kulali and Halda respectively (**Table 9**). Average 43.94% callusing was measured from MS the highest value out of three media and the lowest was in N (34.39%). Within the medium the genotypes showed significant difference at $p \leq 0.05$ (**Table 9**).

Table 9: Effect of three basal media on callus induction of six chilli cultivars

Variety	Media Callus induction (% ± SE)		
	MS	N	B_5
Boltu	**45.08 ± 1.61$_a$**	36.33 ± 1.49$_a$	41.10 ± 2.11$_a$
Bullet	44.11 ± 1.33$_{ab}$	34.67 ± 1.83$_b$	39.67 ± 2.19$_{ab}$
Halda	42.02 ± 2.17$_c$	31.00 ± 1.39$_d$	36.54 ± 2.67$_c$
Kulali	43.24 ± 1.22$_{bc}$	32.57 ± 2.18$_c$	38.23 ± 2.49$_b$
California wonder	44.26 ± 1.87$_{ab}$	35.22 ± 1.37$_{ab}$	39.33 ± 2.39$_{ab}$
Yellow wonder	**44.92 ± 1.19$_a$**	36.57 ± 1.65$_a$	41.05 ± 1.63$_a$
Mean	43.94	34.39	39.32

Values in a column with similar subscripts are not significantly different at $p \leq 0.05$ levels according to DMRT.

4.1.3.4 Effect of PGRs on regeneration

Diffreent types of eight hormonal combinations (R_1 - R_8) were added to modified MS medium which known as regeneration media (RM) and the embryogenic calli (less friable and compact) of studied genotypes were transferred to test their effectiveness on plant regeneration (**Table 10**). After 21 days cultured on RM greenish globular shaped somatic embryos were developed from the embryogenic calli (**Fig. 12d)**. After 45 days of sub-culture the somatic embryos on RMs with R_6 (1.5 mg/l BAP + 1.0 mg/l Kin + 1.0 mg/l NAA) started germinating and complete regenerate plants were formed after 90 days of culture on same RMs (**Fig. 12 e&f)**. Through the results it was also observed that the tested cultivars showed significant difference at $p \leq 0.05$, considering their efficiency to

regenerate plants (**Table 10**). Among the cultivars, Bullet performed highest number of plant regeneration (74.40%) followed by Yellow wonder (73.00%), Boltu (70.80%), Halda (68.80%), Kulali (67.60%) and California wonder (58.80%) in R_6 (1.5 mg/l BAP + 1.0 mg/l Kin + 1.0 mg/l NAA) from cotyledon explant and the lowest value was found for the cultivar California wonder (13.20%) in R_1 (0.5 mg/l BAP) from cotyledonary node. The treatment R_6 (1.5 mg/l BAP + 1.0 mg/l Kin + 1.0 NAA) carried the highest values of effectiveness to influence the cultivars for producing plants and the lowest values of plant regeneration were recorded from R_1 (**Table 10**).

Average of eight tested PGRs concentration and combinations with three explants intended for Bullet (46.98%) uttered that the cultivar was the most efficient genotype for plant regeneration (**Fig. 14**). In case of other cultivars the plant regeneration frequencies were recorded as 46.58%, 44.53%, 44.38% and 43.59% for Yellow wonder, Boltu, Halda and Kulali respectively. The cultivar California wonder showed the lowest frequency to regenerate plants (34.48%) when the calli were transferred to RM. On the other hand, the explant cotyledon performed highest regeneration efficiency (46.52%) where other explants, cotyledonary node and hypocotyl were 43.22% and 40.51% correspondingly (**Fig 14**). Among the explants, cotyledon expressed superiority over others for plants regeneration.

Table 10: Effect of PGRs on plant regeneration from three explants of six chilli cultivars

Explants	PGRs	Genotypes Plant regeneration (% ± SE)					
		Boltu	Bullet	Halda	Kulali	California wonder	Yellow wonder
Cotyledon	R_0	0.00±00i	$0.00±00_i$	$0.00±00_h$	$0.00±00_h$	$0.00±00_h$	$0.00±00_i$
	R_1	$25.60±0.87_g$	$28.00±0.44_g$	$25.40±0.50_g$	$23.20±0.37_g$	$14.40±0.50_g$	$27.20±0.48_h$
	R_2	$45.20±0.37_e$	$47.20±0.37_e$	$44.40±0.50_e$	$42.40±0.24_e$	$33.80±0.37_e$	$47.20±0.37_e$
	R_3	$39.00±0.44_f$	$40.20±0.37_f$	$37.40±0.50_f$	$35.20±0.37_f$	$26.80±0.37_f$	$40.80±0.37_f$
	R_4	$52.00±0.44_d$	$52.80±0.37_d$	$50.40±0.50_d$	$48.00±0.44_d$	$38.40±0.40_d$	$52.20±0.37_d$
	R_5	$26.40±0.50_g$	$39.40±0.50_f$	$36.20±0.37_f$	$34.20±0.37_g$	$27.80±0.58_f$	$39.40±0.50_g$
	R_6	$70.80±0.37_a$	$74.40±0.40_a$	$68.60±0.60_a$	$67.60±0.40_a$	$58.80±0.66_a$	$73.00±0.31_a$
	R_7	$62.80±0.37_b$	$63.80±0.37_b$	$61.00±0.31_b$	$60.00±0.31_b$	$50.80±0.37_b$	$64.20±0.37_b$
	R_8	$59.20±0.37_c$	$57.80±0.66_c$	$57.80±0.37_c$	$56.20±0.37_c$	$44.40±0.50_c$	$61.00±0.44_c$
Hypocotyl	R_0	$0.00±00_i$	$0.00±00_i$	$0.00±00_h$	$0.00±00_h$	$0.00±00_h$	$0.00±00_i$
	R_1	$21.60±0.67_h$	$22.20±0.58_h$	$20.80±0.37_g$	$20.40±0.24_g$	$16.80±0.37_g$	$22.40±0.50_h$
	R_2	$39.20±0.58_e$	$40.80±0.73_e$	$38.20±0.73_e$	$38.40±0.50_e$	$28.20±0.37_e$	$39.20±0.58_e$
	R_3	$32.60±0.40_f$	$33.20±0.37_g$	$30.80±0.37_f$	$31.60±0.50_f$	$22.20±0.37_f$	$32.60±0.40_f$
	R_4	$47.00±0.44_d$	$47.40±0.40_d$	$45.40±0.50_d$	$43.00±0.31_d$	$34.20±0.37_d$	$45.00±0.31_d$
	R_5	$24.20±0.37_g$	$35.40±0.50_f$	$31.80±0.37_f$	$31.40±0.24_f$	$23.00±0.44_f$	$34.20±0.37_g$
	R_6	$63.60±0.50_a$	$64.60±0.50_a$	$61.40±0.50_a$	$62.80±0.37_a$	$52.20±0.37_a$	$63.60±0.50_a$
	R_7	$55.20±0.37_b$	$56.60±0.50_b$	$53.20±0.37_b$	$52.20±0.37_b$	$43.20±0.58_b$	$55.20±0.37_b$
	R_8	$51.20±0.37_c$	$52.20±0.58_c$	$48.40±0.50_c$	$47.20±0.37_c$	$37.20±0.37_c$	$51.20±0.37_c$
Cotyledonary node	R_0	$0.00±00_i$	$0.00±00_i$	$0.00±00_h$	$0.00±00_i$	$0.00±00_h$	$0.00±00_i$
	R_1	$23.00±0.44_h$	$24.00±0.31_h$	$22.80±0.37_g$	$22.00±0.31_h$	$13.20±0.37_g$	$23.80±0.37_h$
	R_2	$42.00±0.44_e$	$43.20±0.37_e$	$40.80±0.37_e$	$41.20±0.37_e$	$30.80±0.37_e$	$43.20±0.37_e$
	R_3	$34.80±0.37_f$	$35.60±0.50_g$	$34.20±0.37_f$	$33.80±0.37_f$	$25.20±0.37_f$	$35.60±0.50_f$
	R_4	$48.60±0.24_d$	$49.40±0.50_d$	$48.00±0.44_d$	$45.20±0.37_d$	$36.20±0.37_d$	$48.60±0.24_d$
	R_5	$24.60±0.24_g$	$36.80±0.37_f$	$34.20±0.37_f$	$32.60±0.24_g$	$27.20±0.22_f$	$37.40±0.24_g$
	R_6	$66.40±0.40_a$	$67.00±0.31_a$	$64.80±0.37_a$	$65.80±0.37_a$	$54.80±0.37_a$	$67.80±0.37_a$
	R_7	$58.20±0.37_b$	$59.40±0.50_b$	$56.80±0.37_b$	$58.20±0.37_b$	$47.00±0.31_b$	$58.20±0.37_b$
	R_8	$55.40±0.24_c$	$55.20±0.37_c$	$52.20±0.58_c$	$53.60±0.50_c$	$40.80±0.37_c$	$54.80±0.58_c$

Values in a column with dissimilar subscripts are significantly different at $p \leq 0.05$ levels according to DMRT.

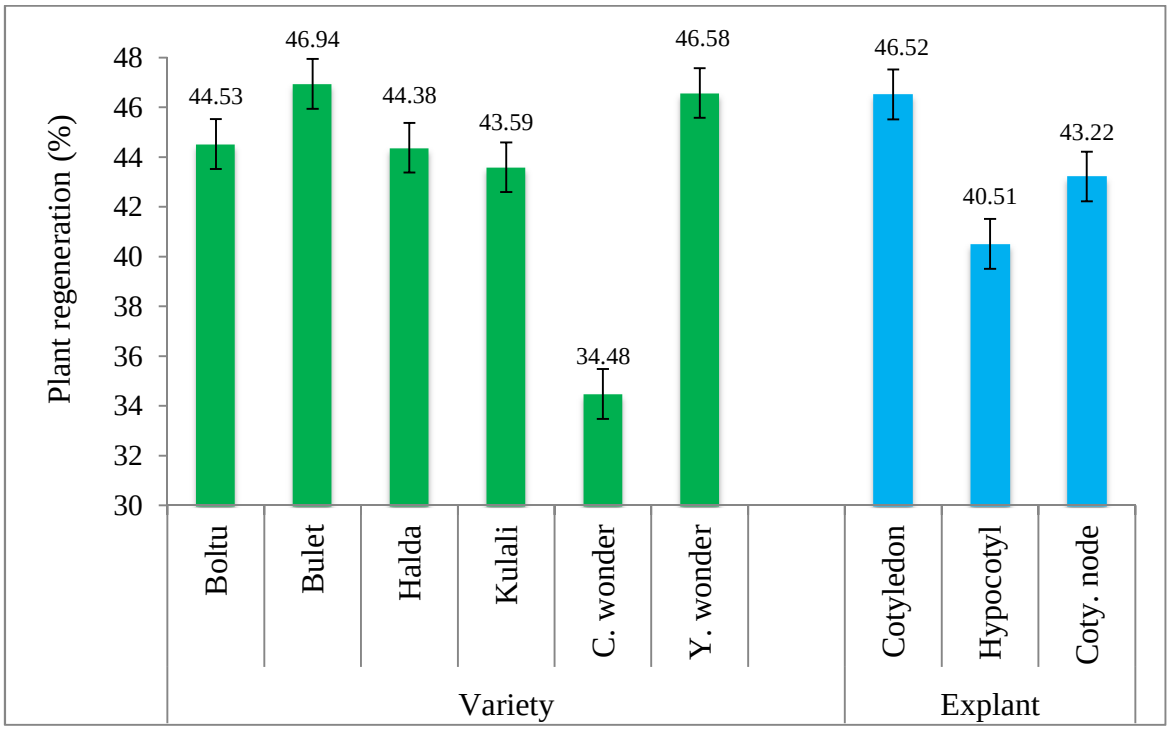

Fig. 14: Plant regeneration efficiency of studied chilli cultivars and explants on an average of tested PGRs treatments.

4.1.3.5 Optimization of regeneration medium

To test the effect of media on regeneration, a suitable PGRs concentration and combinations R_6 (1.5 mg/l BAP + 1.0 mg/l Kin + 1.0 NAA) was considered as constant and augmented individually with three basal media MS, N and B_5; and all the studied cultivars were cultured to regenerate plants. The results showed that all the genotypes accomplished the maximum regeneration in MS medium. The calli of Bulet regenerate plants at the highest rate (46.94%) followed by Yellow wonder (46.58%), Boltu (44.53%), Halda (44.38%), Kulali (43.59%) and California wonder (34.48%) in MS (**Table 11**). Within the medium the genotypes showed significant difference at $p \leq 0.05$ (**Table 11**). Average regenerating plants were measured as 43.42% from MS the highest value out of three media and the lowest 27.11% was in N medium.

Table 11: Effect of three basal media on plant regeneration of six chilli cultivars

Variety	Media Plant regeneration (% ± SE)		
	MS	N	B_5
Boltu	$44.53 \pm 1.12_{b}$	$23.34 \pm 1.44_{cd}$	$36.36 \pm 1.28_{bc}$
Bullet	**$46.94 \pm 1.25_{a}$**	$32.67 \pm 1.84_{a}$	$38.28 \pm 1.42_{a}$
Halda	$44.38 \pm 1.29_{b}$	$26.30 \pm 1.49_{b}$	$35.24 \pm 1.62_{cd}$
Kulali	$43.59 \pm 1.36_{b}$	$24.57 \pm 1.78_{c}$	$34.28 \pm 2.12_{d}$
California wonder	$34.48 \pm 1.42_{c}$	$22.22 \pm 1.47_{d}$	$31.33 \pm 2.39_{e}$
Yellow wonder	**$46.58 \pm 1.22_{a}$**	$33.57 \pm 1.64_{a}$	$37.25 \pm 1.43_{ab}$
Mean	43.42	27.11	35.46

Values in a column with similar subscripts are not significantly different at $p \leq 0.05$ levels according to DMRT.

4.1.3.6 Root induction

To identify the efficiency of root induction 4 - 5 cm length of shoot of all the genotypes were placed into the culture vessels of test tubes containing MS medium supplemented with 1.0 mg/l IBA. The highest root induction rate was recorded for Boltu (99.27%) and the lowest (82.33%) was found in Kulali (**Table 12**). The results showed that the genotypes were significantly differed on responsible to induction rate of roots. The highest length was recorded 8.40 cm for Boltu (**Fig. 12g**); followed by California wonder (7.72 cm), Yellow wonder (7.65 cm), Bullet (7.34 cm) and Halda (7.25 cm). Whereas, the lowest length of roots were observed for Kulali (6.16 cm) when the plantlets were 9 weeks old

after placed in root induction medium. On the other hand a significance differences were found on the number of roots per plant at $p \leq 0.05$. In this case, the highest average number of roots (6.32) was recorded for Boltu (**Fig. 12h**); followed by Bulet (5.88), Yellow wonder (5.65), California wonder (5.61) and Halda (4.31). The lowest average value 3.24 was calculated for Kulali (**Table 13**). Well rooted plants (**Fig. 12g**) were then transferred to pots containing the mixture of vermicompost: soil: sand (1:1:1) for hadening (**Fig. 12i**).

Table 12: Efficacy of six chilli cultivars on root induction and development per plant

Variety	Frequency of root induction (%)	Length of roots (cm)	No. of roots per plant
Boltu	**$99.27 \pm 1.23_{a}$**	**$8.40 \pm 0.24_{a}$**	**$6.32 \pm 0.17_{a}$**
Bullet	$96.67 \pm 1.33_{bc}$	$7.34 \pm 0.27_{b}$	$5.88 \pm 0.16_{ab}$
alda	$95.10 \pm 1.62_{cd}$	$7.25 \pm 0.23_{b}$	$4.31 \pm 0.12_{c}$
Kulali	**$82.33 \pm 1.63_{e}$**	$6.16 \pm 0.22_{c}$	$3.24 \pm 0.15_{f}$
California wonder	$94.67 \pm 1.32_{d}$	$7.72 \pm 0.21_{ab}$	$5.61 \pm 0.12_{ab}$
Yellow wonder	$97.23 \pm 1.58_{b}$	$7.65 \pm 0.24_{ab}$	$5.65 \pm 0.11_{ab}$

Values in a column with dissimilar subscripts are significantly different at $p \leq 0.05$ levels according to DMRT.

4.1.4 Discussion

4.1.4.1 Effect of PGRs and media for callus induction

In this present study, five different concentrations of 2,4-D (0.1, 0.5, 1.0, 1.5, and 2.0 mg/l), four types of combination of 2,4-D + BAP (1.0 mg/l 2,4-D + 1.0 mg/l BAP, 1.0 mg/l 2,4-D + 1.5 mg/l BAP, 2.0 mg/l 2,4-D + 2.0 mg/l BAP, 0.5 mg/l 2,4-D + 2.5 mg/l BAP) and another four types of combination of 2,4-D + Kin (0.5 mg/l 2,4-D + 1.0 mg/l Kin, 0.5 mg/l 2,4-D + 1.5 mg/l Kin, 0.5 mg/l 2,4-D + 2.0 mg/l Kin, 0.5 mg/l 2,4-D + 2,5 mg/l Kin) were added to MS and examined the effect on three different explants (cotyledon, hypocotyl, cotyledonary node) for callus induction of six chilli cultivars. The results showed that the frequencies of callus induction were ranged as 4.80 - 98.60% (**Table 8**). And the highest efficiency was found for Yellow wonder in T_8 = 2.0 mg/l 2,4-D + 2.0 mg/l BAP) from cotyledon explant. In this experiment, non-embryogenic friable calli mass were produced from the single uses of the PGR 2,4-D (without combination of other PGR) but compact emryogenic calli mass were formed when 2,4-D was combinely used with kinetin ro BAP. 2,4-D is one of the well known auxins for callus formation. There are several reports quoting the importance of 2,4-D in callus formation in various crops including *Capsicum* spp. (Rao and Sangapure 2014). Retort of *in vitro* grown plantlets towards plant growth hormones depends upon type of explants and genotype of the plant in concern. Various studies reveal that induction of callus from leaf, hypocotyls or cotyledonary explants of *Capsicum* spp. respond best under the influence of 2,4-D than any other forms of auxin (Umameshwari and Lalitha 2007, Suthar and Shah 2015, Hegde et al. 2017). Another observation was testified in sweet potato by Bett et al. (2015) approving that highest percentage of callusogenesis from leaf explants was found at 2.0 mg/l of 2,4-D whereas stem explants generated best results at quite a higher range of 5.0 mg/l. Bora et al. (2018) also reported that highest callus induction from the leaf explants of *Capsicum chinense* var. Naga chilli was observed in MS supplimented with 2,4-D (3.5 mg/l), $AgNO_3$

(3.0 mg/l), tryptophan (3.0 mg/l) along with BAP at a fixed concentration of 1.5 mg/l. Highest degree of callus formation was recorded by Bhuiyan et al. (2019) in three potates varieties namely, Esprit (78%), Lady Rosseta (88%) and in Meridian (80%) from nodal explants on MS added with 2.0 mg/l BAP and 1.0 mg/l NAA and they also published that nodal, internodal and leaf explants of Lady Rosseta showed better performance to callus induction. Present investigation differs with their reports, and mention that studied varieties responded with high efficiency of callus induction in 2,4-D. Hence, the present investigation claimed that 2.0 mg/l 2,4-D + 2.0 mg/l BAP combinely influenced better to induce efficient calli than 2,4-D individually or combined with Kin (**Table 8)**. Similar types of results were reported to produce embryogenic callus in *Oryza sativa* var. Kitaake a japonica rice cultivar on MS medium supplemented with 3.0 mg/l 2,4-D + 0.25 mg/l BAP (Sah et al. 2014). Results in this study revealed that cotyledon, hypocotyl and cotyledonary nodal explants of Boltu showed better enactment in induce callus induction which might be due to genotypic differences. Davletova et al. (2001) reported that 2,4-D exerted the primary control on endogenous synthesis and metabolism of IAA and cytokinin in cells which played significant role in the process of callus induction. In this experiment explants performed various percentages of callusing; cotyledon (45.55%) was more responsive than hypocotyl and cotyledonary nodal explants for callus induction (**Fig. 13**). An analogous result has been reported in *Capsicum* spp. by Grozeva et al. (2012), although the contrary result was observed by Ashrafuzzaman et al. (2009).

From this present study it was also observed that the basal medium MS performed the best callus induction than N and B_5 when a suitable hormonal concentration T_8 (2.0 mg/l 2,4-D + 2.0 mg/l BAP) was added to CIM. The genotype Boltu gave the maximum CI 45.08% in MS; while it was 41.10% and 36.33% in B_5 and N media respectively (**Table 9**). Abe and Futsuhara (1986) mentioned that potentiality for callus induction and in culture condition

depends on a number of factors, like genotype, physiological and biochemical status of the explants, composition and concentration of different ingredients of culture medium etc. while among these factors, genotypic difference is the most important one.

4.1.4.2 Effect of PGRs and media for plant regeneration

Plant regeneration efficiency for the six tested genotypes in eight different PGRs combinations (R_1 - R_8) were fluctuated 13.20% to 74.40% (**Table 10**). The treatment R_6 (1.5 mg/l BAP + 1.0 mg/l Kin. + 1.0 NAA) carried the highest values of effectiveness to influence the genotypes as well as explants for producing plants aand the lowest values of plant regeneration were recorded from R_1 (0.5 mg/l BAP). The highest value was recorded for Bulet in R_6 combination from cotyledon explant and the lowest for California wonder in R_1 from cotyledonary nodal segment (**Table 10**). Therefore, the analysis of variance indicated that the studied varieties were significantly differed at $p \leq 0.05$ on respect of variability to regenerate plant *in vitro*. On the other hand, the concentration and combinations of PGRs which verified to study the effect on plant regeneration also showed significant difference influencing the varieties in addition to explants for plant regeneration. Hence, it could be suggested that the treatment R_6 were the most effective combination of plant growth regulators to produce chilli plants through somatic embryogenesis for studied genotypes.

Auxins and cytokinin are playing key roles in plant regeneration from somatic embryos of many plant species by the regulation of plant cell cycling, division and differentiation. (Feher et al. 2003, Jiménez 2005, Pinto et al. 2011, You et al. 2011) including *Capsicum* species (Steinitz et al. 2003, López-Puc et al. 2006, Zapata-Castillo et al. 2007, Santana-Buzzy et al. 2009). In the present study, nonsynchronous development of somatic embryo was noticed as reported earlier in *C. annuum* (Zapata-Castillo et al. 2007, Kaparakis and Alderson 2008, Harini and Sita 1993 and *C. chinense* (Solís-Ramos et al. 2010). Similar

observations were reported in *C. annuum* (Steinitz et al. 2003) and *C. chinense* (Binzel et al. 1996 a&b, López-Puc et al. 2006, Santana-Buzzy et al. 2009). This result is agreed with the findings of song et al. (2010). Results in this experiment partially ratified with from Singh and Shukla (2001), Gatz (2002), Gururaj et al. (2004) with respect to the media composition and shoot regeneration. Bora et al. (2018) reported that highest number regeneration (multiple shoots) was obtained with treatment T_5 (BAP 3.5 mg/l + NAA 1.5 mg/l + $AgNO_3$ 4.0 mg/l + tryptophan 1.5 mg/l) on MS. Maximum percentage (55%) of somatic embryo germination and plantlet formation was found at 1.0 mg/l BA in *Capsicum baccatum* by Venkataiah et al. (2016). Kumar et al. (2011) achieved highest regeneration of plants on MS medium containing 5.0 mg/l BAP in combination with 0.5 mg/l IAA in three chilli pepper genotypes (*Capsicum annuum* L. cv. LCA-206, cv. G-4 and cv. PC-1). However, these results differ from the observations has been reported by Ahmad et al. (2006); Siddique and Anis (2006) who found that TDZ alone or combined with IAA ranks as the best shoot bud inductor in nodal explants of the cultivar L. cv. Pusa Jwala. This may be due to auxins or cytokinins used alone or in combination, which is supposed to be the result of the promotion of biosynthesis or inhibition of degradative metabolism, theory shared also by Singh and Shukla (2001).

It was also observed in this study that the basal medium MS led the best regeneration frequency than N and B_5 when a suitable PGRs concentration R_6 (1.5 mg/l BAP + 1.0 mg/l Kin + 1.0 NAA) was added to media. The genotype Bullet performed to regenerate plants at the highest rate (46.94%) in MS; while it was 38.28% and 32.67% in B_5 and N media respectively (**Table 11**). Consequently it is recommended that suitable genotypes and explants along with proper growth regulators with suitable basal media should be used to increase the plant regeneration frequency in chilli tissue culture.

4.1.4.3 Root induction

After regeneration of shoots, root induction was obligatory to produce plantlets as well as entire plants *in vitro.* For this purpose commonly used medium MS added with 1.0 mg/l IBA was used to observe the efficiency of root induction for all of the studied varieties. Among the genotypes the range of root induction rates was 82.33 - 99.27% (**Table 12**). Therefore, it could be sued that the studied genotypes were genetically able to produce sufficient root for taking nutrient supplements in their plant body, resulting be adapted in adverse environmental conditions. However, the genotypes indicated significance difference on the subject to the length of roots and the rage of root length was recorded as 6.16 - 8.40 cm when the plants were 9 weeks old after placing the shoots on root induction medium. The highest length of roots was found for Boltu and the genotype Kulali carried the lowest. Moreover significance differences were found among the varieties considering the feature of the number of roots per plants. In this case, the values of root number per plant were ranged as 3.24 - 6.32 where Kulali variety carried the minimum and the maximum was for Boltu.

Primarily the experiment on rooting was carried with three different forms of auxin, NAA and IAA and IBA. However, in the later stages it was reduced to IBA as the roots cultured in NAA and IAA led to very fragile roots with shorter length. Several previous instances on root initiation prefer IBA more than NAA and IAA (Sanatombi and Sharma 2007, Otroshy et al. 2011, Kumari et al. 2012 and Hedge et al. 2017). Conversely the other preferred NAA and IAA over IBA (Christopher et al. 1986, Bodhipadma and Leung 2003, Siddique and Anis 2006, Sanatombi and Sharma 2008). Similar types of results were observed while MS medium supplemented with IBA (0.5 mg/l) in the cultivars of *Capsicum annum* L. (Kumar et al. 2011, Sanatombi and Sharma 2006).

4.1.5 Conclusion

The results in this experiment provide a simple and suitable protocol on *in vitro* induction of somatic embryogenesis and its subsequent regeneration of chilli cultivars. For callus induction thirteen types of concentration and combinations of PGRs influenced the three explants applied to produce callus for all the six chilli genotypes. Among them T_8 (2.0 mg/l 2, 4-D + 2.0 mg/l BAP) was remarkably effective for most of the cases. Considering the capability of callusing the variety Yellow wonder (98.60%) showed supremacy to others in T_8. Moreover, for plant regeneration eight different hormonal concentration and combinations were tested and the genotype Bullet (74.40%) considered as the supreme member regarding its remarkable able to regenerate plant in R_6 (1.5 mg/l BAP + 1.0 mg/l Kin + 1.0 mg/l NAA). Such variations in results might have occurred due to differences in genotypes and type of explants used. On the other hand, among the three explants cotyledon was efficiently produced somatic emrbyos as well as plantlets regeneration. The regenerated shoots were rooted on MS medium fortified with 1.0 mg/l IBA. Thus, the results clearly demonstrated that above culture condition was the most effective for mass multiplication of most recalcitrant chilli genotypes and this protocol will be helpful for researcher intended for androgenetic studies and genetic transformation of chilli peppers.

4.2 Direct organogenesis using different explant of four Bangladeshi chilli cultivars

4.2.1 Introduction

Chilli is the most important spice in our country. It is a commercial crop and generally utilized as spice and it's named as miracle spice. It has limited ability to focus practicality and low germination rate and multiplication through seeds. A tissue culture technique about chillis provides a route to the asexual multiplication as the plants lack natural vegetative propagation. The genus capsicum is recalcitrant through *in vitro* regeneration potential (Liu et al. 1990). Plant regeneration through *in vitro* culture has been accomplished by various methods like protoplast, hypocotyls, cotyledons, young leaves, direct physical embryogenesis and shoot organogenesis from seedling explants of various chilli cultivars (Gunay and Rao 1978, Diaz et al. 1988, Christopher and Rajam 1996). However, these procedures failed or to be modified when they were used to regenerate plants from other chilli pepper cultivars. Thus, the strong influence is pepper variety in regeneration from various explants and this makes it necessary to optimize *in vitro* propagation protocols for the specific cultivars. Tissue culture is an amazing method to keep up the ideal ways to improve capsicum through *in vitro* micropropagation procedures (Sharma et al. 2008). Despite the fact that there are a few reports of *in vitro* recovery of various tamed types of capsicum (Christopher and Rajam 1994, Bodhipadma and Leung 2003, Peddaboina et al. 2006, Ashrafuzzaman et al. 2009, Kothari et al. 2010, Kumari et al. 2012, Gogoi et al. 2014). The current work has been considered to show contemporary advancement on *in vitro* recovery and study the impacts distinctive with hormonal combinations of the most chilli pepper cultivars in Bangladesh by utilizing cotyledon, hypocotyl and shoot tips. An endeavor has been made to build up a suitable and an efficient *in vitro* method of chilli cultivars in Bangladesh.

4.2.2 Materials and Methods

4.2.2.1 Plant material

About plant material and explants are briefly described in Chapter III (Section 3.1.1 and 3.2.1.1)

4.2.2.2 Methods

Four local chilli varieties *viz*. Boltu, Bullet and Halda were surface sterilized with distilled water for 3 - 5 minutes to reduce the level of surface organisms and then treated with distilled water mixed with Tween 20. Later the seeds were taken out and the healthy seeds were selected for surface sterilized by immersion 70% ethanol for 5 minutes with vigorous shaking followed by 5 minutes in 4% NaOCl and rinsed 5 times with sterile distilled water. Then the sterilized seeds were inoculated in culture bottles containing 25 ml MS medium for germination. MS medium was fortified with 3% (w/v) sucrose, 0.8% agar. The p^H of the culture medium was adjusted to 5.8 before autoclaving at 121ºC and at 15 lb pressure. Seeds were placed in culture vessels then incubated them in dark at for germination. After five days the seeds germination culture vessels were placed in a culture room with a 16 hour photoperiod under an illumination of 20 mmol $m^{-2}s^{-1}$ photosynthetic photon flux density provided by cool white florescent light. The seedlings of (14) days were selected for explant, its hypocotyls (1-2 mm in length), cotyledons (1.5-2 mm length) and cotyledonary nodes excised into pieces using sterilized surgical blades. Four-six segments were placed horizontally in each vessel and gently plessed into the surface of the sterilized culture medium with various combinations of growth regulators *viz.* BAP (2.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 14.0 mg/l) and TDZ (0.1, 0.5 and 1.0 mg/l).

4.2.2.3 Maintaining cultures and temperature

The culture vessels containing explants were placed under fluorescent light in a room with controlled temperature (25 ± 2°C) using 16 h photoperiod. After 20-23 days of inoculation the explants induced multiple shoots, the shoots attained convenient size after four weeks and then they were removed aseptically from the vial on a sterilized glass plate inside the laminar air flow cabinet and were placed again on freshly prepared sterilized medium containing appropriate hormonal supplements for shoot elongation. For shoot elongation BAP + GA_3 (8.0 + 0.5, 8.0 + 1.0, 8.0 + 2.0, 8.0 + 2.5, 8.0 + 3.0 mg/l) and were used. Elongated shoots were transferred to MS medium supplemented with BAP + IAA (2.0 + 0.1, 2.0 + 0.5, 2.0 + 1.0, 2.0 + 1.5, 2.0 + 2.0 mg/l) and BAP + NAA (2.0 + 0.1, 2.0 + 0.5, 2.0 + 1.0 mg/l) for root induction. Well rooted plants were transferred to pots and covered them with transparent polythene membrane to ensured high humidity and watered every third day with ½MS solution for two weeks in order to acclimatize plants to field condition. After two weeks acclimatized plants were transferred to pots.

4.2.2.4 Multiple shoots induction

Briefly described in Chapter III (Section 3.3.3).

4.2.2.5 Experimental design and data analysis

Briefly described in Chapter III (Section 3.4.8).

4.2.3 Results

4.2.3.1 Multiple shoots induction

Cotyledonary nodal region showed significantly highest frequency of shoot induction (69.60%) followed by cotyledon (67.20%) and least response was observed in hypocotyls (62.00%) (**Table 13-15, Fig. 15a-g**). Irrespective of media BAP at 10 mg/l induced shoots in significantly highest 69.60% and 65.00% by TDZ at 0.5 mg/l (**Table 13**). The least response (6.0%) was observed at 4 mg/l BAP (**Table 14**). In case of hypocotyls explants were induced shoots when TDZ was added at 0.1, 0.5, 1.0 mg/l. TDZ at 0.5 mg/l induced

shoots in significantly higher (45.00%) than other concentrations and lowest 10.00% response was observed at 0.1 mg/l TDZ (**Table 15**). The interaction effect of explant and growth regulator combination given significantly higher number in cotyledonary nodal region explants around 69.60 percent of the explants showed shoots by Boltu (**Fig. 15d**). TDZ at 0.5 mg/l induced shoots on nearly 65.00% of cotyledonary nodal region explants at the cultivar of Boltu, significantly highest shoot formation rate (69.60%) showed was by cotyledonary nodal region explants at 10.0 mg/l BAP (**Table 13**). BAP has induced shoots on cotyledon and cotyledonary nodal region explants but not on hypocotyls at any concentrations. The response of shoot induction was observed at TDZ from hypocotyls; and 0.5 mg/l TDZ induced shoots in significantly higher (45.0%) in Boltu (**Table 15**).

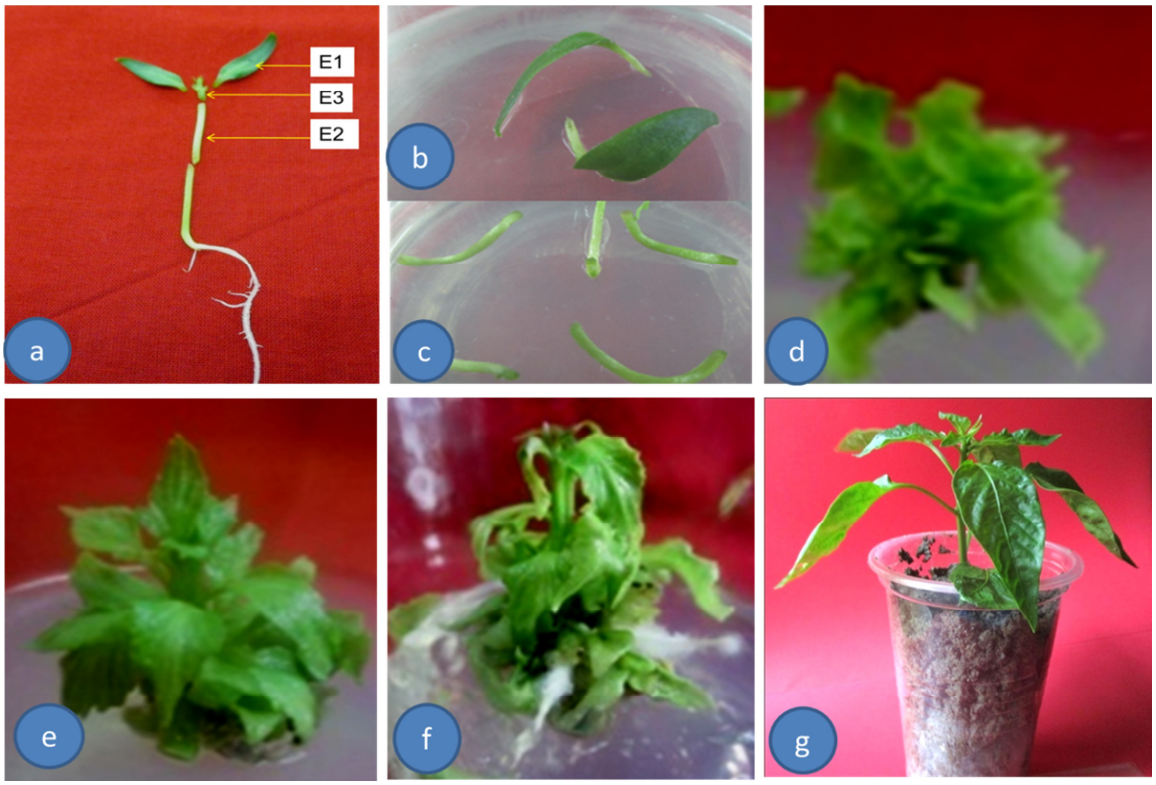

Fig. 15 (a-g): Direct regeneration of chilli cultivars. (a) Different explants (E1 = Cotyledon, E2 = hypocotyl, E3 = Cotyledonary node), (b and c) explants inoculated on culture media, (d) multiple shoot formation, (e) shoot elongation, (f) well rooted plant, and (g) plant transferred to pot.

Table 13: Effect of different PGRs on multiple shoots formation from cotyledonary nodal explants of four chilli cultivars

PGRs (mg/l)		Cultivars			
		Boltu	Bullet	Halda	Zeha
		(M±SE)			
Control	-	$0.00\pm0.00_{i}$	$0.00\pm0.00_{i}$	$0.00\pm0.00_{i}$	$0.00\pm0.00_{i}$
BAP	2.0	$0.00\pm0.00_{i}$	$0.00\pm0.00_{i}$	$0.00\pm0.00_{i}$	$0.00\pm0.00_{i}$
	4.0	$9.00\pm0.20_{h}$	$7.00\pm0.30_{h}$	$8.00\pm0.30_{h}$	$8.60\pm0.80_{h}$
	6.0	$38.06\pm0.50_{f}$	$36.60\pm0.62_{f}$	$36.00\pm0.37_{f}$	$35.00\pm0.63_{f}$
	8.0	$62.23\pm0.38_{c}$	$61.20\pm0.37_{b}$	$61.40\pm0.23_{b}$	$58.20\pm0.28_{bc}$
	10.0	$69.60\pm0.67_{a}$	$63.60\pm0.50_{a}$	$64.40\pm0.35_{a}$	$62.50\pm0.55_{a}$
	12.0	$60.80\pm0.70_{c}$	$58.74\pm0.61_{bc}$	$58.60\pm0.67_{bc}$	$60.60\pm0.74_{ab}$
	14.0	$48.60\pm0.70_{d}$	$44.80\pm0.68_{d}$	$45.20\pm0.37_{d}$	$46.80\pm0.34_{d}$
TDZ	0.1	$25.00\pm0.30_{g}$	$21.00\pm0.50_{g}$	$23.60\pm0.35_{g}$	$21.50\pm0.37_{g}$
	0.5	$65.00\pm0.50_{b}$	$61.30\pm0.20_{b}$	$62.30\pm0.37_{b}$	$60.00\pm0.34_{ab}$
	1.0	$42.00\pm0.20_{e}$	$40.20\pm0.30_{e}$	$41.00\pm0.35_{e}$	$41.20\pm0.50_{e}$

Table 14: Effect of different PGRs on multiple shoots formation from cotyledon of four chilli cultivars

PGRs (mg/l)		Cultivars			
		Boltu	Bullet	Halda	Zeha
		(M±SE)			
Control	-	$0.00\pm0.00_{i}$	$0.00\pm0.00_{i}$	$0.00\pm0.00_{i}$	$0.00\pm0.00_{i}$
BAP	2.0	$0.00\pm0.00_{i}$	$0.00\pm0.00_{i}$	$0.00\pm0.00_{i}$	$0.00\pm0.00_{i}$
	4.0	$7.00\pm0.20_{h}$	$9.00\pm0.30_{h}$	$6.00\pm0.30_{h}$	$8.60\pm0.80_{h}$
	6.0	$35.30\pm0.50_{f}$	$36.20\pm0.50_{f}$	$34.00\pm0.37_{f}$	$35.00\pm0.20_{f}$
	8.0	$61.23\pm0.30_{c}$	$60.20\pm0.37_{b}$	$61.20\pm0.20_{b}$	$58.40\pm0.20_{bc}$
	10.0	$67.20\pm0.60_{a}$	$62.30\pm0.50_{a}$	$63.30\pm0.35_{a}$	$61.00\pm0.50_{a}$
	12.0	$60.30\pm0.60_{c}$	$57.74\pm0.60_{bc}$	$59.60\pm0.60_{bc}$	$60.20\pm0.74_{ab}$
	14.0	$46.60\pm0.70_{d}$	$41.80\pm0.60_{d}$	$44.20\pm0.30_{d}$	$44.80\pm0.30_{d}$
TDZ	0.1	$23.20\pm0.50_{g}$	$22.00\pm0.50_{g}$	$21.60\pm0.35_{g}$	$20.40\pm0.30_{g}$
	0.5	$64.80\pm0.30_{b}$	$60.00\pm0.20_{b}$	$61.40\pm0.37_{b}$	$60.50\pm0.34_{ab}$
	1.0	$41.50\pm0.20_{e}$	$40.30\pm0.20_{e}$	$40.30\pm0.35_{e}$	$41.00\pm0.50_{e}$

Table 15: Effect of different PGRs on multiple shoot formation from hypocotyl explants of four chilli cultivars

PGRs (mg/l)		Cultivars			
		Boltu	Bullet	Halda	Zeha
		(M±SE)			
Control	-	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$
BAP	2.0	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$
	4.0	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$
	6.0	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$
	8.0	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$
	10.0	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$
	12.0	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$
	14.0	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$	$0.00\pm0.00_{d}$
TDZ	0.1	$12.00\pm0.30_{c}$	$10.50\pm0.50_{c}$	$10.00\pm0.20_{c}$	$11.50\pm0.30_{c}$
	0.5	$45.00\pm0.40_{a}$	$38.00\pm0.20_{a}$	$40.30\pm0.30_{a}$	$39.00\pm0.34_{a}$
	1.0	$22.00\pm0.40_{b}$	$17.00\pm0.20_{b}$	$20.30\pm0.30_{b}$	$18.00\pm0.34_{b}$

4.2.3.2 Elongation of shoots

Analysis of variance indicated significant differences for elongation of shoots using various explants, growth regulators and its combinations and interaction. Here highest number (82.6%) of shoot elongation was observed in Boltu followed by Bullet (82.4%) and Zeha (82.0%). The least number of elongations of shoots (72.6%) showed in Halda. Among the growth regulators and its combinations shoot elongation found significantly higher (82.6%) in MS + 8.0 mg/l BAP + 1 mg/l GA_3 after 45 days of inoculation (**Fig. 15e**); and followed by 8.0 mg/l BAP + 1.5 mg/l GA_3 (73.4%), 8.0 mg/l BAP + 2.0 mg/l GA_3 (68.8%), 8.0 mg/l BAP + 2.0 mg/l GA_3 (68.8%), 8.0 mg/l BAP + 2.5 mg/l GA_3 (62.4%). The minimum response was found for shoot elongation (22%) when BAP 8.0 mg/l used singly in addition with MS medium. MS basal medium with 8.0 mg/l BAP + 1.0 mg/l GA_3 supported shoot elongation in highest number of explants 82.6%, 82.4%, 82.0% and 72.6% in Boltu, Bullet, Zeha and Halda. On the other hand shoot elongation was found lowest in Zeha with MS + 8.0 mg/l BAP (**Fig.16**).

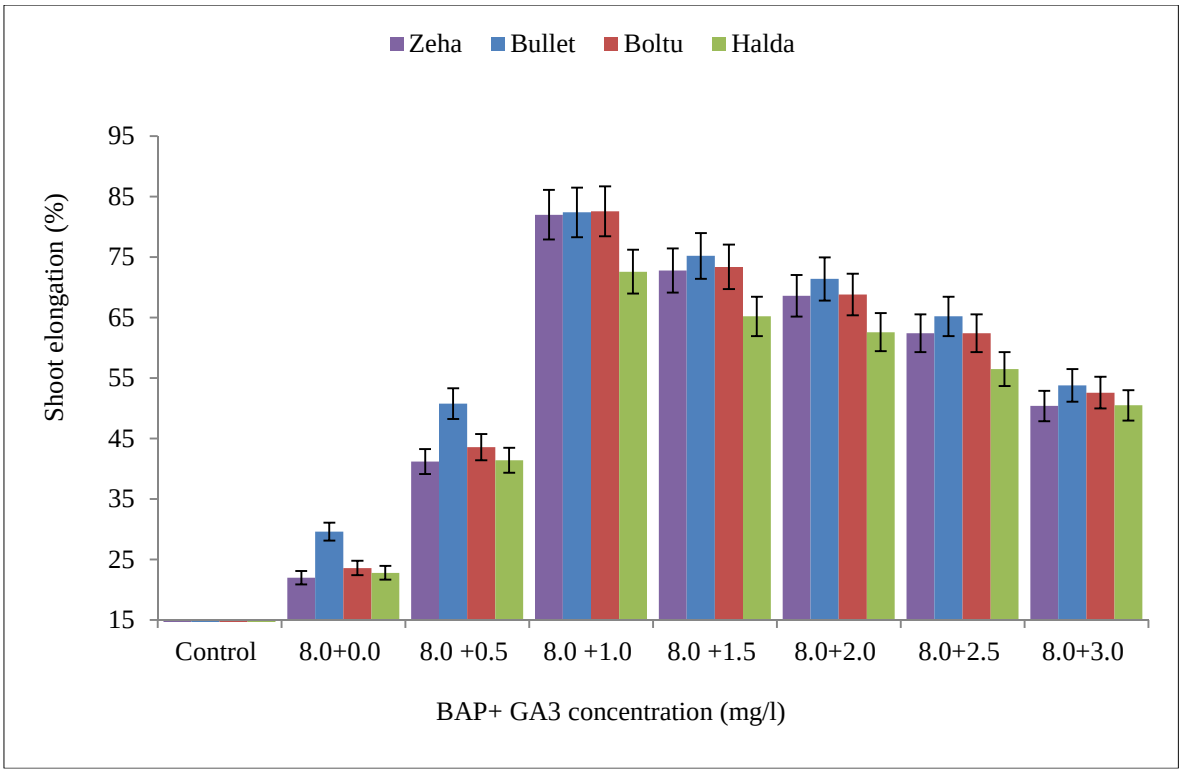

Fig.16: Shoot elongation of selected varieties of chilli on various concentrations of PGRs.

4.2.3.3 Rooting and hardening of regenerated plant

Roots were induced in all media combinations within 10 days of culture and it was looking shorter and thick on MS medium that contained with NAA; and longer and thin roots were found when the medium was contained with IAA (**Table 16**). The frequency of root induction is presented in **Fig.17 & 18**. Highest (99.2%) rooting was observed on MS medium containing 0.5 mg/l NAA and 2.0 mg/l BAP in Bullet (**Fig. 15f**); and followed by Boltu (97.4%), Halda (95.2%) and Zeha (94.2%). In case of another PGRs medium (MS + IAA 1.0 mg/l and BAP 2.0 mg/l) showed higher 95.2%, 90.8%, 89.4% and 84.4% rooting respectively on Bullet, Boltu, Halda and Zeha. The culture media MS with IAA 2.0 mg/l and BAP 2.0 mg/l showed the lowest (59.8%) rooring in Zeha. The plantlets with well developed roots were transferred to pots (**Fig. 15g**).

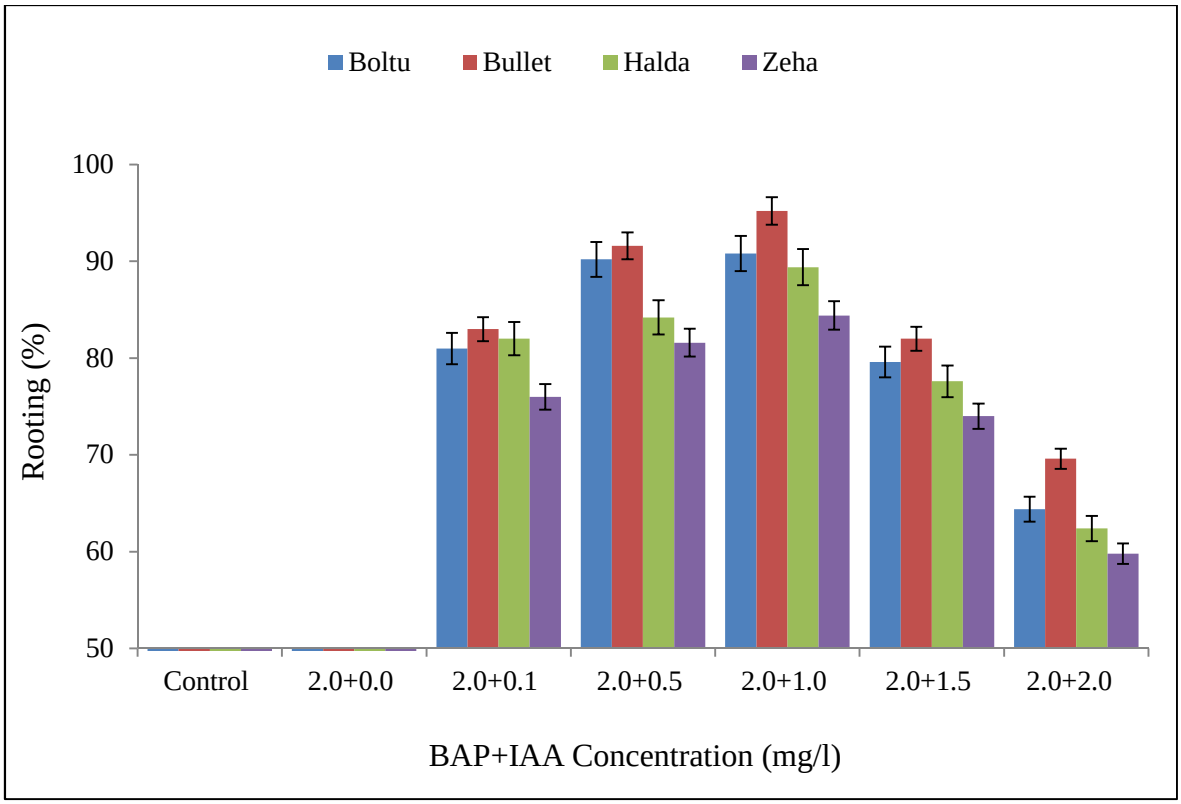

Fig. 17: Rooting of selected varieties of chilli on various concentrations of PGRs.

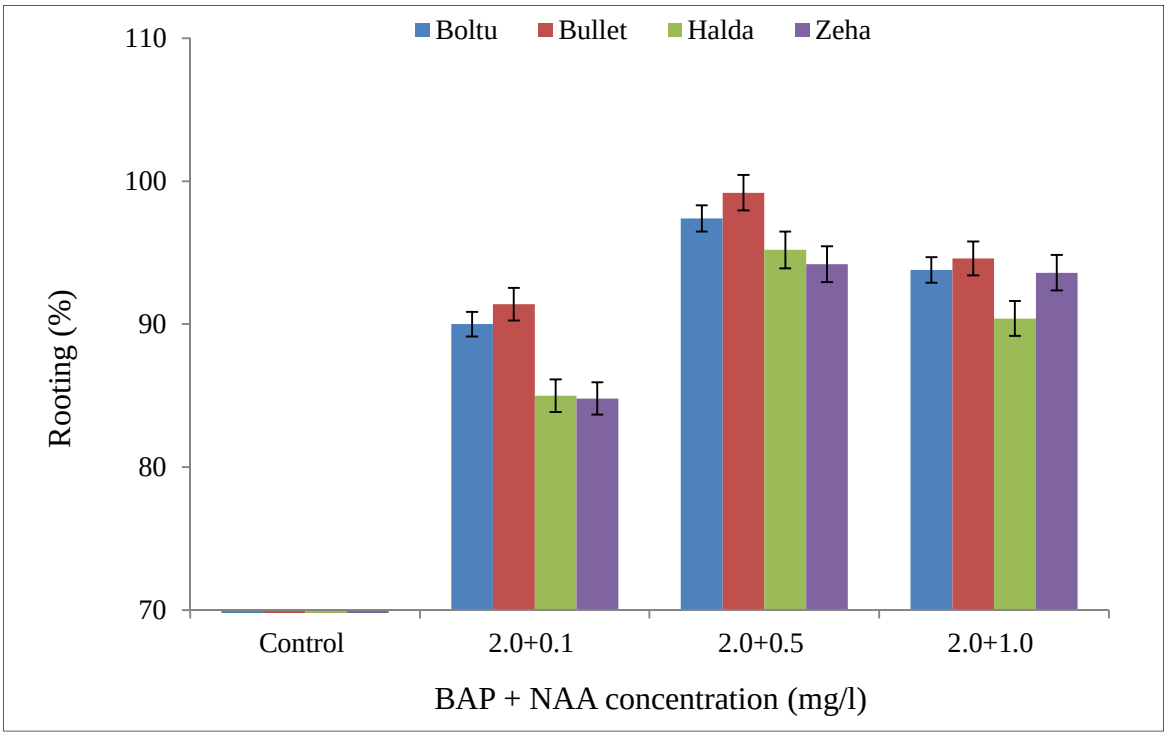

Fig. 18: Rooting of selected varieties of chilli on various concentrations of PGRs

Table 16: Response of shoots on MS medium with various concentrations of PGRs

Concentrations of PGRs (mg/l)	Responses
NAA + BAP	
0.1 + 2.0	Short and thick roots
0.5 + 2.0	Do
1.0 + 2.0	Do
IAA + BAP	
0.1 + 2.0	Long and thin roots
0.5 + 2.0	Do
1.0 + 2.0	Do

4.2.4 Discussion

Direct regeneration on shoot multiplication system, most often it occurs through shoot proliferation from pre exting mesristem. Cotylodonary leaf is the commonly used explant for shoot multiplication was reported by Gunay and Rao (1978). Chaudhari (2004) described that direct somatic embryogenesis is better than callus mediate regeneration because of genetic and cytological variations often associates. In the present staudy it was tried to regenerets plants from various explants of chilli peppers. As explant the axillary shoot-tips were proliferated the maximum number of shoot buds on a medium containing 5-10 mg/l BAP single uses. Cotyledonary node showed significantly highest number of shoot induction (69.60%) followed by simple cotyledon (67.20%) and no response was observed in hypocotyls in MS fortified with 10.0 mg/l BAP (**Table 13-15**). The least response was observed at 4.0 mg/l BAP (6.0%). TDZ at 0.5 mg/l induced higher number of shoots significantly with shoot buds (45.0%). The least response (10.0%) was observed when 0.1 mg/l TDZ used in addition with MS medium. The effect of explants and growth regulators were given significantly higher number in cotyledonary nodal region around 69.60%. The explants showed shoots at the cultiver Boltu and irrespective of growth regulators TDZ at 0.5 mg/l induced shoots on nearly 65% of cotyledonary nodal region explants at the cultiver Boltu. A significant highest shoot multiplication frequency 69.60% was observed from cotyledonary nodal explant at 10.0 mg/l BAP (**Table 13**). Hypocotyl induced significantly higher shoots (45.0%) in MS + TDZ (0.5 mg/l) for Boltu.

4.2.5 Conclusion

A simple and an efficient protocol for induction of adventious shoots of four local chilli varieties were standardized under this study. For direct regeneration from shoot tips, MS medium was supplimented with 10.0 mg/l BAP showed best results. Direct regeneration from hypocotyls the MS medium supplimented with 0.5 mg/l TDZ may be useful for large scale multiplication for chillies and other agricultural crops. The protocol established under this study is efficient, quick and highly reproducible and can be used in advance biotechnological research for chilis improvement. Regeneration system through direct somatic embryogenesis is suitable for further studying on physiological and morphological status of embryos development, mass mass propagation, genetic transformation and advance biotechnological works in future.

4.3 Effect of PGRs and silver nitrate on callus induction and regeneration of two local chilli cultivars

4.3.1 Introduction

Somatic embryogenesis in recent years has shown enormous potential in providing efficient clonal propagation system. Plant regeneration through somatic embryogenesis has many advantages over other routes of *in vitro* plant production and appears to be the most promising area of research for large scale production, rapid plant propagation and conservation. Plant regeneration through somatic embryogenesis is one of the main pre-requisites for potential use of clonal propagation in woody plants (Park et al. 1998). Chilli is a recalcitant plant and the phenomenon of somatic embryogenesis through *in vitro* callus development is rare. Therefore, the success of somatic embryogenesis via callus culture is interesting in plant regeneration of chilli. But capsicum species are highly recalcitrant to tissue culture techniques (Sharma et al. 2008). There are some reports have been mentioned that use of silver nitrate ($AgNO_3$) in media, plays an important role to improve callus induction, shoot and root formation in various plants; such as Naga chilli (Sharma et al. 2008, Bora et al. 2014), wheat (Wua et al. 2006, Bouiamrine et al. 2012), sorghum (Pola et al. 2009) and pearl millet (Oldach et al. 2001). Some authors also reported that regeneration can be improved by $AgNO_3$ in both dicots and monocots (Wua et al. 2006, Kumar et al. 2009). So far as we know that there is not enough work has been done on Bangladeshi chilli cultivars using biotechnological approach. Therefore, this study has been conducted to provide a suitable and efficient protocol for feeicient callus induction and regeneration using $AgNO_3$ and PGRs through cotyledon and hypocotyl explants.

4.3.2 Materials and Methods

4.3.2.1 Plant material and explants

Red sundried fruits of popular two local chilli cultivars *viz.* Bullet, Boltu were collected from Dumuria, Khulna used as seed source. Seedlings were grown in *in vitro* at the Plant Biotechnology and Genetic Engineering Laboratory, IBSc, University of Rajshahi, Bangladesh. As explants cotyledon and hypocotyl were excised form the *in vitro* grown seedlings and used for the present investigation.

4.3.2.2 Methods

4.3.2.2.1 Sterilization and preparation of explants

The seeds were germinates within five days of inoculation. The young cotyledon and hypocotyl of 11 days old seedlings were cut into small pieces (approximately 0.25 - 0.50 cm) and then inoculated (**Fig. 19 a&b**). The sterilization procedure, PGRS, media preparation, pH, autoclave and inculation of cultures are briefly mentioned in Chapter IV (Section 4.2.2).

4.3.2.2.2 Media preparation and culture condition

Each culture bottle was contained 30 ml MS semi-solid medium and fortified with 3% (w/v) sucrose, 0.8% agar. Various concentrations of BAP (1-4 mg/l) and kinetin (1-4 mg/l) either single or in combination with BAP (3-4 mg/l) + $AgNO_3$ (2-4 mg/l) and Kin (3-4 mg/l) + $AgNO_3$ (2-4 mg/l) were tested for callus induction. For regeneration twelve treatment with three combination of BAP- i) (5.0 mg/l) + $AgNO_3$ (1-4 mg/l), ii) BAP (5.0 mg/l) + IAA (0.1 - 1.5 mg/l) and iii) BAP (5.0 mg/l) + NAA (0.1 - 1.5 mg/l) were evaluated and six treatment with two combination of BAP- i) (2.0 mg/l + IAA (0.1-1.0 mg/l) and ii) BAP (2.0 mg/l + NAA (0.1 - 1.0 mg/l) were tested for root formation. The p^H of medium was adjusted to p^H 5.8 and medium autoclaved at 121ºC with 15 lb pressure for 15 minutes. Culture bottles were maintained at 25 ± 1ºC with 16/8 h photoperiod using

cool white florescent light in growth chamber. Calluses were originated after 10-13 days of inoculation. The convenient size of calli obtained after 21-23 days of inoculation and then removed from the vessel and placed on freshly prepared sterilized medium containing appropriate plant growth regulators for shoot induction. The calluses were sub-cultured every 21 days and shoot regeneration started after second sub-culture. Shoots were elongated on specific media and PGRs mentioned previously. The proper size of shoots were then cultured on fresh medium which containing suitable hormonal concentration for rooting.

4.3.2.2.3 Data recording and analysis

Briefly described in Chapter III (Section 3.4.8).

4.3.3 Results

4.3.3.1 Effect of BAP, Kin, and $AgNO_3$ on callus induction

Cotyledon and hypocotyl explants of two cutivars (Bullet and Boltu) were tested with twenty different treatments which prepared on MS medium containing with four differents concentrations of BAP- i) (1- 4.0 mg /l) and ii) Kin (1- 4.0 mg /l) and six combinations with iii) BAP (3-4.0 mg/l) + $AgNO_3$ (2-4.0 mg/l) and iv) Kin (3-4.0 mg) + $AgNO_3$ (2-4.0 mg/l) for callus inductions are shown in **Table 17**. The highest frequency of callusing was recorded from hypocotyl explant in 4.0 mg/l BAP + 3.0 mg/l $AgNO_3$ for Bullet (86.40%); followed by Boltu (73.80%) and the cotyledon explants was in Bullet (81.20%) and Boltu (67.30%) in the same combination (**Fig. 19 c&d)**. The lowest value was recorded in 1.0 mg Kin for Boltu from cotyledon (10.60%) and hypocotyle (13.60%). No callus was observed in PGRs free medium (**Table 17)**. Among the cultivars highest callusing was recorded in Bullet (51.74%) than Boltu (41.26%). On the other hand it was also observed that hypocotyl showed better callusing (48.34%) rate than cotyledon (44.66%) (**Fig. 20**). Observations form the interaction among the cultivars and the explants Bullet and

hypocotyl was mostly effective for better callusing than Boltu and cotyledon (**Fig. 21)**. The stimulatory effect of $AgNO_3$ on embryogenic callus induction started to decline when the concentration of $AgNO_3$ was higher than 3 mg/l in this experiment. $AgNO_3$ at 4.0 mg/l appeared to be suppressive for embryogenic callus induction (**Fig. 19d)**.

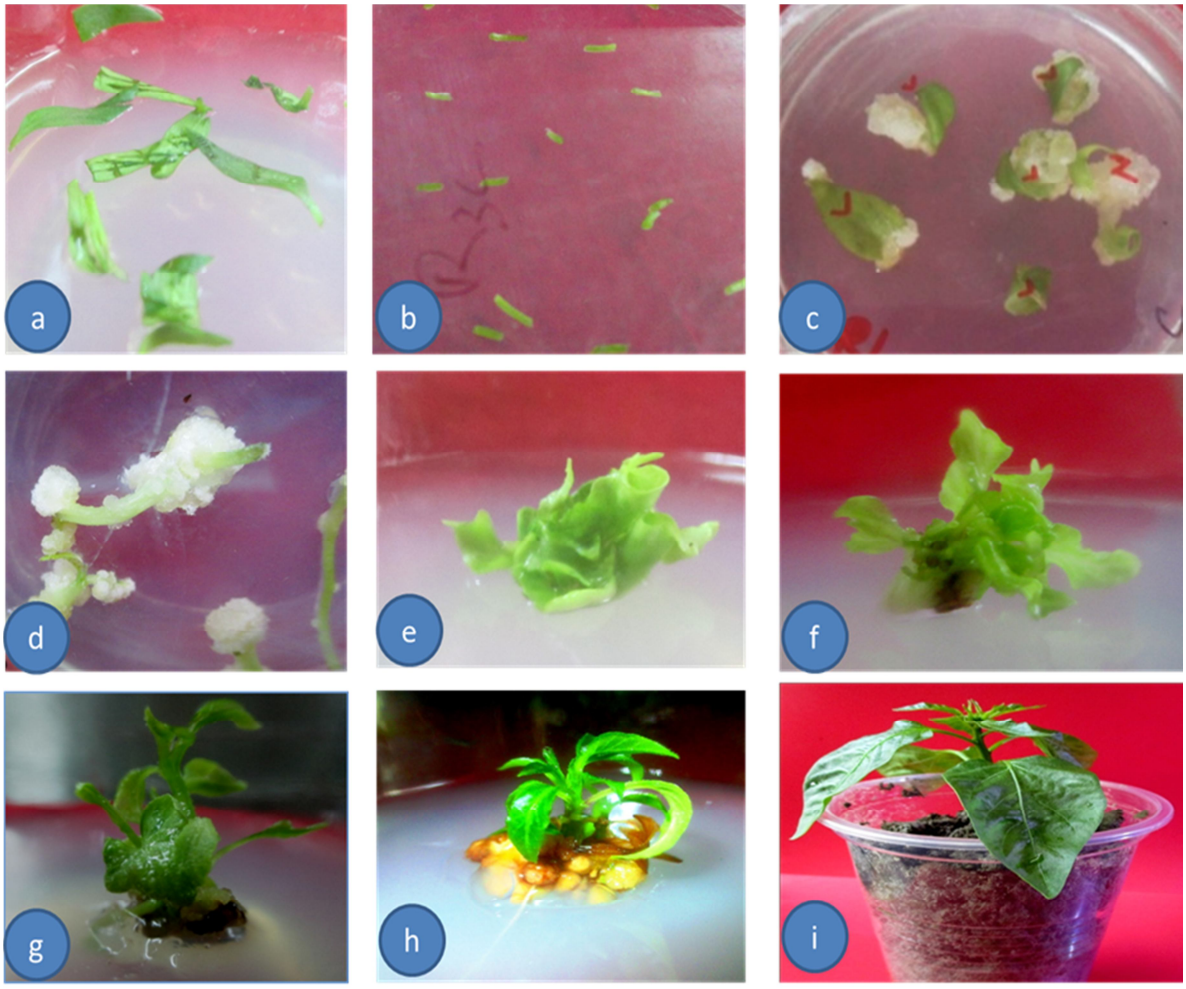

Fig. 19 (a-i): Regeneration steps of chilli cultivars. (a-b) inoculated explants, (c) callus formation from cotyledon, (d) callus formation from hypocotyl, (e-f) regeneration, (g) elongation of shoot, (h) well rooted plantlet and, (i) plant transferred to pot.

Table 17: Effect of different concentration and combination of BAP, Kinetin and $AgNO_3$ on callus induction

PGRs (mg/l)	Callus induction (% ± SE)			
	Bullet		Boltu	
	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
Control	-	-	-	-
BAP				
1.0	26.50±0.68	32.40±0.45	14.30± 58	16.70±0.62
2.0	32.10±0.92	38.30±0.70	21.40±0.82	25.60±0.70
3.0	41.60±0.54	45.10±0.62	32.40±0.72	36.80±0.66
4.0	47.60±0.32	54.40±0.44	37.10±0.45	39.40±0.65
Kinetin				
1.0	19.30± 0.43	22.60± 0.49	10.60± 0.54	13.60± 0.45
2.0	24.80±0.40	28.80±0.58	15.40±0.49	19.90±0.78
3.0	40.20±0.64	43.60±0.78	32.70±0.49	35.50±0.84
4.0	34.20±0.81	37.10±0.68	30.70±0.72	31.30±0.79
BAP + $AgNO_3$				
3.0 + 2.0	56.00±0.92	62.40±0.73	42.10±0.68	46.20±0.84
3.0 + 3.0	62.40±0.52	68.10±0.66	46.70±0.58	54.40±0.62
3.0 + 4.0	60.60±0.42	61.40±0.46	45.30±0.62	51.10±0.74
4.0 + 2.0	72.80±0.82	75.10±0.94	54.30±0.62	61.50±0.64
4.0 + 3.0	81.20±0.68	86.40±0.76	67.30±0.77	73.80±0.89
4.0 + 4.0	75.60±0.42	81.10±0.64	68.70±0.56	72.90±0.69
Kin + $AgNO_3$				
3.0 + 2.0	37.30±0.92	38.30±0.76	26.20±0.84	31.30±0.78
3.0 + 3.0	42.60±0.67	42.90±0.88	37.70±0.56	39.80±0.78
3.0 + 4.0	47.20±0.66	56.30±0.58	47.20±0.52	48.30±0.67
4.0 + 2.0	58.80±0.72	59.70±0.85	49.80±0.68	48.70±0.79
4.0 + 3.0	68.20±0.78	69.80±0.82	54.30±0.58	56.30±0.72
4.0 + 4.0	63.60±0.62	6130±0.72	53.60±0.49	52.30±0.92

Values represent mean ±SE (Standard Error). Each treatment was repeated thrice and each treatment counted of 5 replicate culture vessels.

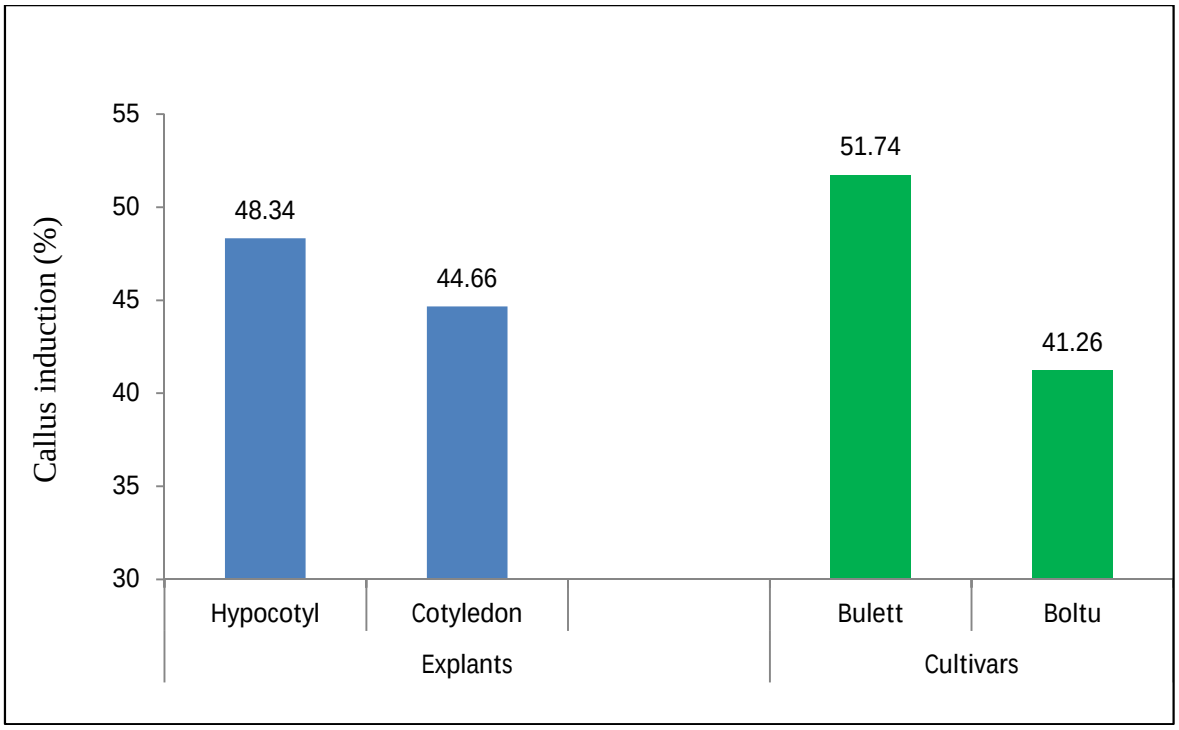

Fig. 20: Comparison of explants and cultivars on callusing.

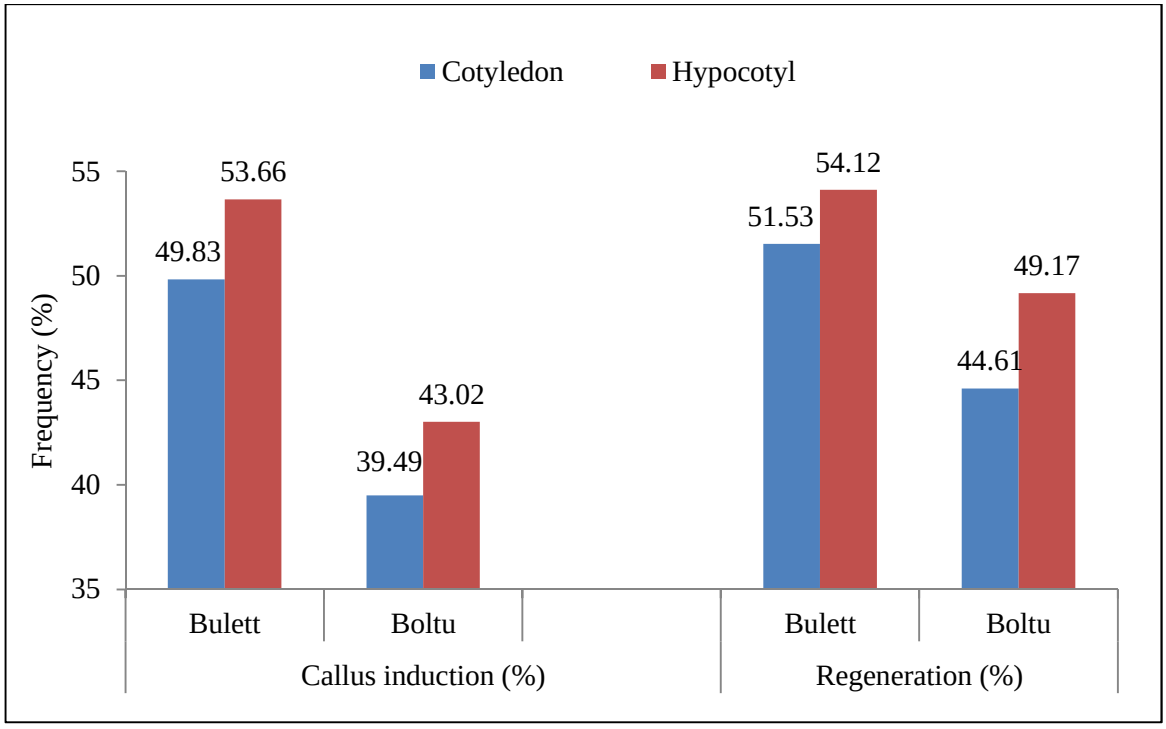

Fig. 21: Comparison of explants and cultivars on callusing and regeneration efficiency.

4.3.3.2 Regeneration and shoot elongation

Calli derived from hypocotyl and cotyledon explants of Bullet and Boltu were cultured on the regeneration medium and best shoot regeneration (75%) was recoded from the hypocotyle that cultured in MS medium supplemented with BAP (5.0 mg/l) $AgNO_3$ + (3.0 mg/l) for Bullet and Boltu (64.60%). The explant cotyledon showed 67.20% and 64.60% regeneration efficiency in Bullet and Boltu respectively (**Table 18**, **Fig. 19 e**&**f**). Lowest regeneration (26.70%) was noted when cotyledons of Boltu were cultured in MS + BAP (5.0 mg/l) + IAA (1.5 mg/l) (**Table 18**). The explants hypocotyle showed highest shoot regeneration rate in both cultivars (Bullet and Boltu). The regeneration frequency of hypocotyle from Bullet exhibited more superiority in comparison with cotyledon and Boltu (**Fig. 21**). It was also observed that BAP (5.0 mg/l) + $AgNO_3$ (3.0 mg/l) was the best combination for induction and elongation of shoots in both cultivars (Bullet and Boltu). $AgNO_3$ an ethylene inhibitor in plant tissue culture system, is found to be an essential compound in induction and elongation of shoots in chilli cultivars (**Fig. 19g)**.

4.3.3.3 Rooting and hardening

The elongated shoots derived from hypocotyl and cotyledonary callus were transferred to rooting medium that supplemented with different combinations of fixed level of BAP (2.0 mg/l) with different levels NAA (0.1-1.0 mg/l) or IAA (0.1-1.0 mg/l). Roots were developed from the shoots cultured on all the rooting media. $AgNO_3$ into the medium reduced the rooting in Bullet and Boltu. Number of roots per shoot has been presented in **Table 19**. Number of roots per shoot varies from 5.0-9.8 in Bullet and 3.4-6.9 in Boltu. The number of roots per shoot was higher in the medium supplemented with 2.0 mg/l BAP + 0.1 mg/l NAA (**Table 19**). The maximum number (9.40) of roots per shoot was observed in hypocotyl callus derived shoot and in case of cotyledonary callus derived shoot it was 8.20 (**Fig. 19h)**. Another hormonal combination 2.0 mg /l BAP + 0.5 mg /l

IAA also had potential effect on root formation, where 7.6 and 8.10 roots initiation recorded in cotyledon and hypocotyl callus derived shoot in Bullet. However, no root was induced in PGRs free MS medium. Hormonal combination 2 mg/l BAP + 0.1 mg /l NAA was the most effective for root formation. Well rooted plants were transferred to pots after acclimatization (**Fig. 19i)**.

Table 18: Effect of PGRs and $AgNO_3$ on shoot regeneration from hypocotyl and cotyledon explants

PGRs (mg/l)	Regeneration (% ± SE)			
	Bullet		Boltu	
	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
BAP + $AgNO_3$				
5.0 + 1.0	48.10±0.48b	49.30±0.42c	41.20±0.48c	44.50±0.42c
5.0 + 2.0	56.80±0.32d	52.10±0.54d	46.40±0.43e	47.80±0.59d
5.0 + 3.0	67.20±0.68g	75.00±0.32i	52.40±0.68g	64.60±0.62h
5.0 + 4.0	59.60±0.42e	63.10±0.64h	49.70±0.42f	56.40±0.64g
BAP + IAA				
5.0 + 0.1	61.00±0.92f	60.40±0.70g	53.20±0.66	57.40±0.63g
5.0 + 0.5	55.40±0.32d	58.60±0.62f	46.70±0.52e	52.60±0.68f
5.0 + 1.0	35.60±0.32a	41.20±0.62b	33.80±0.54b	37.20±0.43b
5.0 + 1.5	34.80±0.49a	36.30±0.63a	26.70±0.65a	31.60±0.72a
BAP + NAA				
5.0 + 0.1	48.20±0.44b	50.40±0.42c	44.80±0.54d	47.50±0.64d
5.0 + 0.5	52.40±0.81c	55.60±0.68e	51.60±0.58	53.50±0.74f
5.0 + 1.0	52.00±0.68c	56.10±0. 81e	46.20±0.67e	49.60±0.76e
5.0 + 1.5	47.20±0.56b	51.30±0.64cd	42.60±0.78c	47.30±0.64d

Values represent mean ± SE (Standard Error). Each treatment was repeated thrice and each treatment counted of 5 replicate culture vessels. Means in a column with the different letter (superscript) are significantly different according to DMRT at ($P < 0.05$).

Table 19: Combined effects of PGRs on the number of root per shoot

PGRs (mg/l)	No. of roots/regenerated shoot			
	Bullet		Boltu	
	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
Control	-	-	-	-
BAP + IAA				
2.0 + 0.1	5.40±0.32ab	6.60±0.62b	3.40±0.68a	3.90±0.74a
2.0 + 0.5	7.60±0.42c	8.10±0.64c	6.20±0.69c	6.90±0.58c
2.0 + 1.0	6.00±0.92b	6.40±0.70b	4.90±0.49b	5.60±0.78b
BAP + NAA				
2.0 + 0.1	8.20±0.68c	9.40±0.32d	6.40±0.73c	6.80±0.72c
2.0 + 0.5	6.20±0.44b	6.60±0.81b	5.30±0.49bc	6.50±0.48c
2.0 + 1.0	5.00±0.81a	5.10±0.68a	4.80±0.82b	5.20±0.76b

4.3.4 Discussion

Callus induction from suitable explants is one of the most important steps for successful plant regeneration in any crops. Under this study two types of explants e.g cotyledon and hypocotyl were chosen and cultured them on MS medium supplemented with $AgNO_3$ as chemical stress pre-treatment factors along with PGRs as BAP and Kin. The interactions with different hormone concentrations and explants for both explants showed significant calluses induction. Among the hormone combinations, callus formation from hypocotyls

explants showed potentiality when cultured them in MS + BAP 4 mg/l + $AgNO_3$ 3 mg/l for both Bulett and Boltu. Bulett (51.74%) showed better callusing than Boltu (41.26%). However, in the present investigation, 10.60% to 86.40% callus induction was recorded. The wide range of variability might be occurred due to genotypic effect along with the different levels of BAP, Kin and $AgNO_3$. Simillar types of results were obatained in *Capsicum chinense* by Bora et al. (2018). 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 chilli pepper (Gammoudi et al. 2018), Tomato (Shah et al. 2014), barley (Manoharan and Dahleen 2002), maize (Jakubeková et al. 2011), wheat (Farshadfar 2014), 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). Various plant tissue cultures systems produced ethylene because of the stress during explant excision (Yang and Hoffman 1984) and the presence of auxin (Yu and Yang 1979). $AgNO_3$ is ethylene antagonist that inhibits ethylene action by competing with ethylene for its binding sites. It does not reduce the ethylene production. A number of studies reported that ethylene production either continued at the same level as controls or even increased with the addition of $AgNO_3$ (Gavinlertvatana et al. 1980, Songstad et al. 1988, Vain et al. 1989, Roustan et al. 1990, Chi et al. 1991). The stimulatory effect of $AgNO_3$ on friable embryogenic callus induction has been well documented in maize (Vain et al. 1989, Songstad et al. 1991), and results in the present experiments agreed well with these findings. This enhancement is similar to what was found in rice where the addition of 50 μM $AgNO_3$ (about 9 mg/l) improved callus growth by 60% (Adkins et al. 1993). This result differed, however, from what was found in maize by Vain et al. (1989) who reported that $AgNO_3$ did not modify the growth rate of the maize type II callus.

The highest *in vitro* shoot regeneration frequency (75% and 64.60%) and from hypocotyls and cotyledon derived calli, respectively were recorded in Bullet and Boltu cultivar. The present results clearly indicate that BAP had harmonious effect with $AgNO_3$ in the enhancement of *in vitro* shoot regeneration. Results of this experiment was simillar with Cruz de Carvalho et al. (2000) who reported 63.8% shoot development in *Phaseolus vulgaris* using BAP only but it was increased up to 100% by applying BAP in combination with $AgNO_3$. Conversely, the inhibitory effect of $AgNO_3$ on *in vitro* shoot regeneration was observed due to its higher levels (Fuentes et al. 2000). $AgNO_3$ (10 mg/l) was utilized in *Coffea canephora* and it was noticed that the number of embryos per explants were increased by 60%. This result comply with that of Ebida and Hu (1993) who reported that MS medium supplemented with additional component $AgNO_3$ has the influential effect on shoot regeneration from different explants. The promising influence of $AgNO_3$ in *in vitro* shoot regeneration has been proclaimed in some previous studies. From these studies it has been concluded that $AgNO_3$ has a stimulating effect in various crops: cauliflower (*Brassica oleracea*), cassava (*Manihot esculenta*), sunflower (*Helianthus annuus*), cabbage (*Brassica oleracea*) and Azuki bean (*Phaseolus angularis*) (Zobayed et al. 1999, Mohamed et al. 2006).

For induction of strong and stout root system, BAP and NAA was the best combination for promoting root formation. And no rooting was recorded in hormone free media (control); moreover $AgNO_3$ into the medium inhibited the root formation in Bullet and Boltu cultivars. The maximum roots (9.40) were formed in MS supplemented with 2 mg/l BAP + 0.1 mg/l NAA from hypocotyl explants in Bullet. Simillar findings were observed by Anantasaran and Kanchanapoom (2008) in *Zinnia* cultivars. Some earlier reports on root initiation prefer NAA and IAA over IBA (Siddique and Anis 2006, Sanatombi and Sharma 2008) and IBA over NAA and IAA (Otroshy et al. 2011, Kumari et al. 2012, Hedge et al. 2017). On the contrary, Sunandakumari et al. (2004) reported that 2.67 mg/l of $AgNO_3$

gave the improved frequency of rooting in *Rotula aquatica*. Conversly, the positive effect of $AgNO_3$ for root formation in barley genotypes was observed by Kumar et al. (2009). $AgNO_3$ inhibited ethylene action in plant growth and development was reprted by Beyer (1976) and Sharma et al. (2008). Since polyamines have been reported to promote embryogenesis (Feirer et al. 1984), the positive effect of ethylene inhibitors, such as $AgNO_3$, on regeneration was thought to be due to enhanced polyamine synthesis rather than reduced ethylene production (Kumar et al. 2009). There have been reports confirming the significance of $AgNO_3$ on *in vitro* processes in *C. Annuum* (Ashrafuzzaman et al. 2009) and *C. frutescens* (Sharma et al. 2008). Under this study a suitable and an efficient protocol has been standardized for callus induction and regeneration using $AgNO_3$ and PGRs through cotyledon and hypocotyl explants of chilli peppers. Results obtained of this study clearly demostrated that BAP is very effective phytohormones along with $AgNO_3$ in MS which enhanced *in vitro* shoot regeneration and its subsequent development.

Chapter V
Gametic Embryogenesis

5. Gametic Embryogenesis

5.1 An efficient methods on embryoids induction and plant regeneration of *Capsicum annum* L. via anther culture

5.1.1 Introduction

The pepper plant, which is the most important spices in Bangladesh and Indian subcontinent, it is also a most important vegetables in the world, is known as 'recalcitrant' in terms of its genetic structure. For this reason, haploidy techniques which are applied to pepper have lower levels of embryoids formation and regeneration. Anther culture is a simple and an efficient method for obtaining haploid plants in pepper. However, the frequency of obtained androgenetic plants depends on various factors such as media, plant growth regulators (PGRs) and genotype which considerably influence the effectiveness of haploidy; therefore the low rate of haploid plantlets limits the utility of anther culture in pepper breeding. The first report on induction of haploidy through *in vitro* microspore embryogenesis of pepper was reported by Wang et al. (1973). Several reports in microspore embryogenesis have been reported in different crop plants (Dumas de Valux et al. 1981, Mityko et al. 1995, Dolcet-Sanjuan et al. 1997, Barany et al. 2001, Rodeva et al. 2004 & 2006, Irikova et al. 2011, Cheng et al. 2013, Başay and Ellialtıoğlu 2013); and some reports are mentioned that the culture medium is an important factors for androgenetic studies (Sibi et al. 1979, Dumas de Valux et al. 1981, Supena et al. 2006, Cheng et al. 2013). Success on embryogenesis in chilli pepper has a great limitation especially on plant regeneration and development of normal plantlets from shoots (Nowaczyk et al. 2006, Kothari et al. 2010).

Genotype plays an important role in androgenetic reaction in chilli pepper as it is most often limiting factor stated by Comlekcioglu et al. (2001), Wang and Zhang (2001), Rodeva et al. (2001) and Rodeva et al. (2004). Mak and Maheswary (1994) found that the optimal androgenetic response under various cultivation conditions is genotype dependent. The critical point for application of temperature shock treatments is in late uni-nucleate and early binucleate stages of microspore development, a point. Mityko and Fari (1997) indicated that a decreasing androgenetic response was obtained in wax-type, dark green blocky-type, tomato shaped and Cayenne/chilli-type peppers. They also reported that although bell peppers generated a high and reproducible response, spice peppers proved to be less or non-responsive. The most important factor having an effect on the success of the *in vitro* anther culture is the genotype used. This present study was, therefore, conducted to determine the androgenetic response especially for anther culture of some pepper cultivar including some Bangladeshi genotypes.

5.1.2 Materials and Methods

5.1.2.1 Plant materials

Seeds of five local chilli genotypes *viz*. Boltu, Bullet, Halda, Zeha and Baromashi were used as explant sources for this study. Seeds were germinated and grown in winter (from October to February, 2013 to 2016) in the pot and in the experimental field of the Institute of Biological Sciences, University of Rajshahi, Bangladesh.

5.1.2.2 Harvesting stage and pre-treatment of buds

Initiation of flowering was observed around 7 weeks after sowing and young flower buds of all genotypes were harvested for about 7-8 weeks at their early flower production period for anther culture (**Fig. 24a**). Buds with equal sepal and petal in size that were containing

microspores at mid to late uninucleate developmental stages were harvested at early morning and flower buds of each genotype were refrigerated at 4°C for 24 h to induce cold stress to the anthers for better androgenic responses.

5.1.2.3 Acetocarmine staining

Prior to anther culture, precise stage of microspore (mid to late uninucleate stage) was determined by squashing anthers from a flower bud **(Fig. 24 b-e)**. Microspore development was observed at the different days of culture initiation by 1% acetocarmin staining **(Fig. 24 f&g)**. For preparing acetocarmine squash, at first 11.25 ml acetic acid added to 13.75 ml distilled water which makes it 45% glacial acetic acid. It was boiled till all the pungent smell comes out then 0.5 g of carmium was added to and then it was brought to room temperature. 1% acetocarmine stain was prepared by adding 0.25 g carmine to 25 ml of 45% acetic acid. One drop of stain was added to a slide.

5.1.2.4 Surface sterilization

The fresh and cold pre-treated flower buds were initially washed with sterile distilled water and treated with 70% ethanol for 15 seconds. Then with 2.5% sodium hypochlorite and a drop of Tween-20 for 8 minutes and rinsed in sterile distilled water 3 times or until traces of sodium hypochlorite were removed.

5.1.2.5 Anther culture process

Sterilized flower buds were carefully dissected and anthers were separated and placed them horizontally on the medium such that the anther was in contact with the medium. Care was taken to avoid anther damage. The anthers were detached and placed in (55 × 15 mm) sterile petri dishes containing 8-10 ml culture medium. Three basal media were considered for embryos induction in this study *viz.* MS, N and C. All media were supplemented with

2.0 mg/l 2,4-D, 1.0 mg/l kinetin, 8.0 g/l agar and 30 g/l sucrose. Each petri dish contained 12 anthers and inoculated them from two sterile flower buds. Inoculated anthers were incubated at 35°C, at in dark chamber for five days followed by incubation for four days at 25°C in dark chamber. Then the anthers were sub-cultured to same medium supplemented with 0.1 mg/l kinetin, 60 g/l sucrose and 8.0 g/l agar except the anthers of C culture medium. In case of C culture medium, the anthers were sub-cultured to R medium (Dumas de Valux et al. 1981) supplemented with 0.1 mg/l kinetin, 60 g/l sucrose and 8.0 g/l agar. The p^H of the media was adjusted using 1N NaOH and 1N HCl to 5.8 for all culture media and incubated at 25°C with 16 hrs photoperiod. In a separate part of the experiment, we examined the effect of different duration of heat stress on the efficiency of anther culture of the most responsive genotype of Bulett. So that anthers of two buds cultured in (55 × 15 mm) petri dishes containing C medium (according to the previous experiment) and incubated them at 35°C for 0, 3, 5, 7, 9, 11 and 13 days at dark. Then they transferred to R medium and incubated at 25°C with 16 h photoperiod. After five weeks, embryoids were transferred to hormone-free MS medium with 60 g/l sucrose and solidified with 8.0 g/l plant agar.

5.1.2.6 Data recording and statistical analysis

The present study performed in three replicates and each replication consisted of four petri dishes (each petri dish containing 12 anthers taken from two buds). The significance of the data generated at various stages of experiment was analyzed statistically using CRD (Singh and Chaudhary 1979). Replicated observations of each treatment were recorded. Standard error of mean (SE) was calculated as per the formula stated. Methods on statistical analysis were briefly mentioned in Chapter III (Section 3.4.8)

5.1.3 Results

The anthers from five genotypes (Boltu, Bullet, Halda, Zeha and Baromashi) were cultured on three induction media (MS, C-R and N). In comparison of anther response from the five studied varieties, Bullet showed the highest percentage of reacted anthers (20.81%), when cultured on C-R medium, followed by the same variety (18.70%) cultured on MS medium (**Table 20**). The variety Baromashi showed no response to anthers culturing on N medium (**Table 20**). The genotype had a significant effect on direct embryogenesis and callusing. The anthers from genotype Bullet showed the highest rate of embryoids induction (3.28%) and callusing (16.54%), when cultured them on C-R medium compared to other genotypes (**Table 20**). In case of indirect embryogenesis, Halda showed as the most responsive genotype (1.43%) among tested ones when cultured on C-R medium (**Table 20**). Though the frequency of regenerants of all studied varieties were very low, Bullet showed highest regeneration (0.35%) culturing on C-R medium among all varieties. Zeha and Baromashi showed no regeneration culturing on any type of medium (**Table 20**).

Table 20: Effect of genotype and medium on anther response, embryogenesis, callusogenesis, indirect organogenesis and regeneration of *Capsicum annum* L

Genotype	Medium	Mean ± SE				
		Anther response	Embryogenesis	Callusing	Indirect organogenesis	Regeneration
Boltu	MS	14.65±0.16d	2.47±0.09b	11.37±0.26c	0.54±0.02c	0.12±0.01c
	C-R	17.52±0.30c	2.04±0.01c	14.20±0.24b	1.04±0.03b	0.26±0.01b
	N	6.25±0.14h	1.50±0.04d	4.58±0.27g	0.56±0.04c	0d
Bullet	MS	18.70±0.29b	1.64±0.08d	16.83±0.14a	0.53±0.03c	0.25±0.03b
	C-R	20.81±0.62a	3.28±0.24a	16.54±0.11a	0.57±0.03c	0.35±0.01a
	N	4.51±0.19i	1.02±0.01e	3.51±0.27h	0d	0d
Halda	MS	12.67±0.17f	1.48±0.02d	10.46±0.28d	0.48±0.06c	0d
	C-R	13.40±0.22e	2.51±0.09b	9.53±0.19e	1.43±0.18a	0.11±0.05c
	N	3.53±0.23j	1.01±0.01e	2.59±0.28i	0d	0d
Zeha	MS	4.47±0.26i	1.02±0.01e	3.54±0.17h	0d	0d
	C-R	8.39±0.21g	1.61±0.02d	6.45±0.27f	0.53±0.02c	0d
	N	1.47±0.20l	0g	1.32±0.29j	0d	0d
Baromashi	MS	1.10±0.06l	0g	1.17±0.08j	0d	0d
	C-R	2.23±0.12k	0.57±0.01f	1.73±0.13j	0d	0d
	N	0m	0g	0k	0d	0d

The mean values in a column followed by same letter(s) are not significantly different at $p \leq 0.05$ according to DMRT.

According to the results obtained from genotype effect, the highest percentage reacted anthers were found from the variety Bullet (14.67%), followed by the variety Boltu (12.81%) (**Fig. 22**). In these two genotypes it was observed also the highest frequency of callusing (12.29 % and 10.05%, respectively). The lowest percentage of reacted anthers (1.11%) and the lowest frequency of callus formation (0.97%) in comparison with the other genotypes were registered in the variety Baromashi (**Fig. 22**). The direct embryogenesis with the highest frequency was recorded in variety Boltu (2.0%), followed by the varieties Bullet (1.98%), Halda (1.67%), Zeha (0.88%) and the lowest one in variety Baromashi (0.19%). The regeneration with the highest frequency was recorded in variety Bullet (0.2%), followed by the varieties Boltu (0.13%) and Halda (0.04%). Zeha and Baromashi showed no response of green plant regeneration.

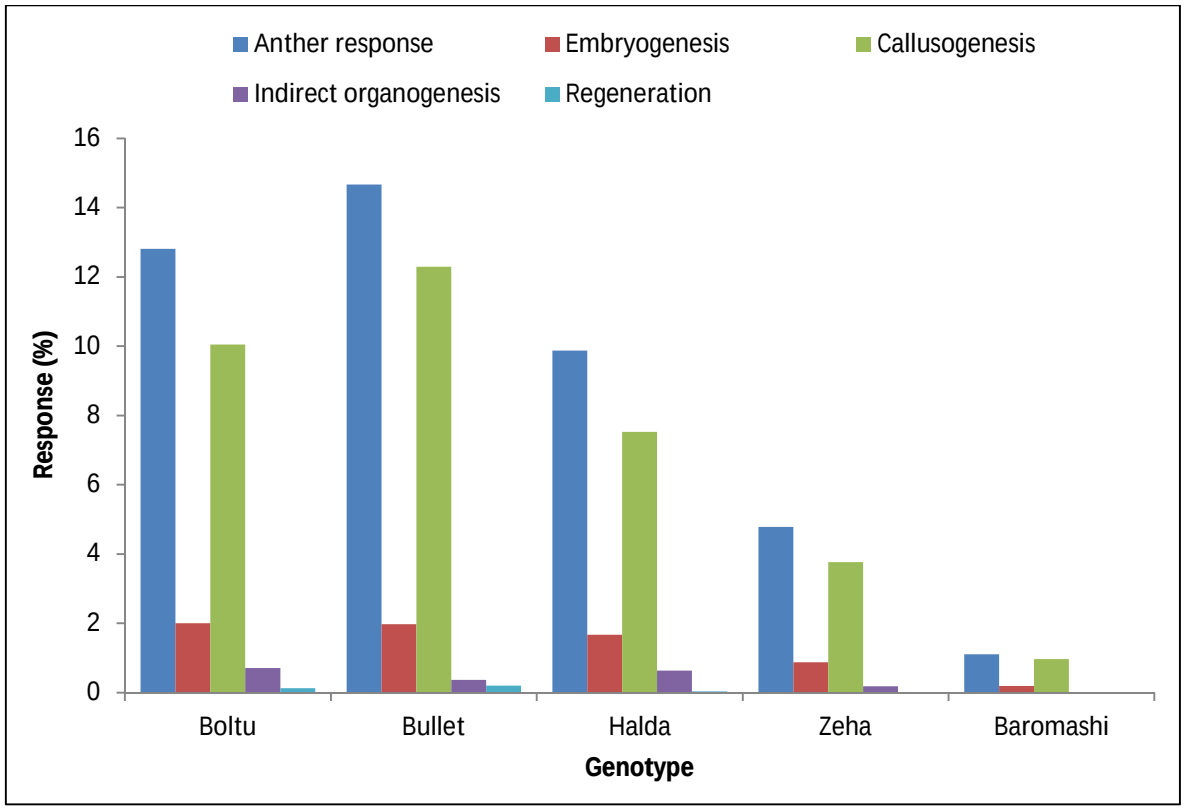

Fig. 22: Effect of genotype on anther response, embryogenesis, indirect organogenesis and regeneration of *Capsicum annum* L. on an average of three culture media.

According to the results obtained from culture media, the highest percentage reacted anthers were found from the medium C-R (12.47%), followed by the medium as MS (10.32%) (**Fig. 23**). In these two culture media we observed also the highest frequency of callusing (9.69 % and 8.67%, respectively). The lowest percentage of reacted anthers (3.15%) and the lowest frequency of callus formation (2.40%) in comparison with the other media were registered in the medium N (**Fig. 23**). The direct embryogenesis with the highest frequency was recorded in the medium C-R (2.0%), followed by the medium MS (1.32%) and the lowest one in medium N (0.71%). The regeneration with the highest frequency was recorded in the medium C-R (0.14%), followed by the medium MS (0.07%). There was no regeneration found on N culture medium.

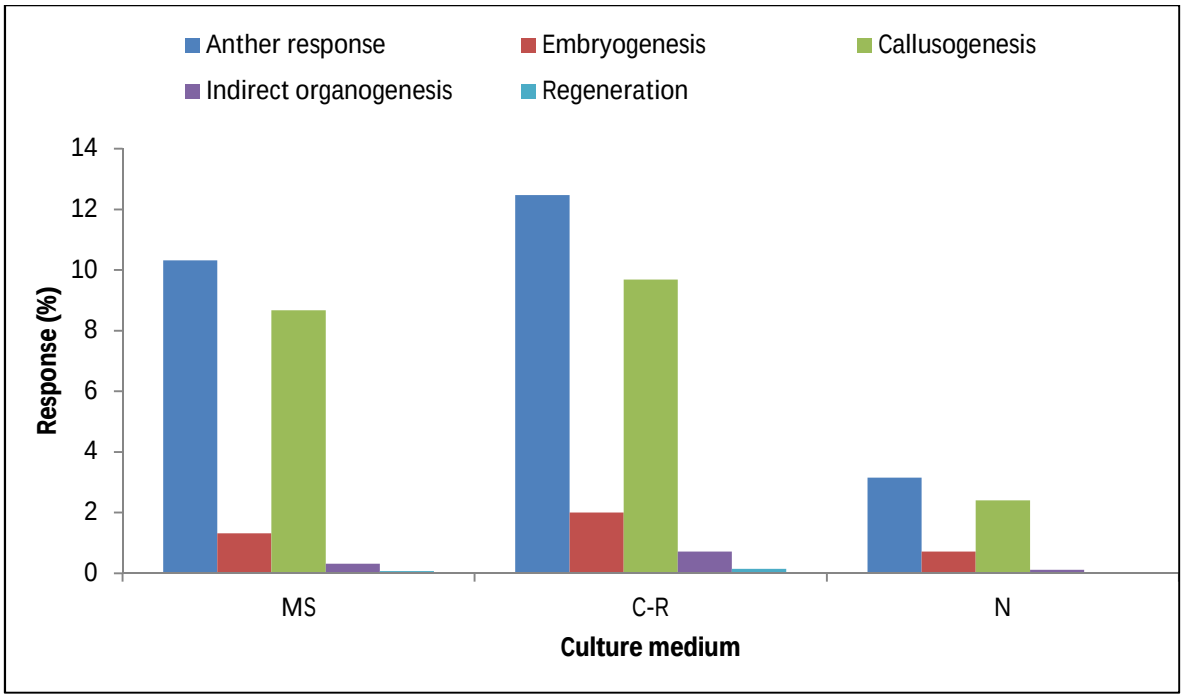

Fig. 23: Effect of culture medium on anther response and embryogenesis, indirect organogenesis and regeneration of *Capsicum annum* L. on an average of five genotypes.

The responses to heat shock duration on anther cultures were different (**Table 21**). Incubation of anther cultures at 25°C (without thermal shock) produced no embryoids production. Six different durations of heat shock stress (3, 5, 7, 9, 11 and 13 d) were applied to anther of variety Bullet culturing on C-R medium for embryogenesis and regeneration. Heat shock treatment of anther cultivations from 3 to 13 days stimulated androgenesis response and showed the best results at 7 days (**Table 22, Fig. 24**). In all the treatments, tested genotype showed varied number of direct and indirect embryogenesis, callusogenesis and regenerants production. Heat shock stress for 7 days showed highest percentage of direct embryogenesis, callusogenesis, indirect organogenesis and regeneration as 5.28, 17.42, 1.26 and 2.99% respectively (**Table 21, Fig. 24**). Increasing the duration of heat shock by more than 7 days had a detrimental effect and suppressed microspore embryogenesis as 13 days decreased callusing dramatically.

Table 21: Effect of heat stress to anther on embryogenesis, callusogenesis, indirect organogenesis and regeneration of *Capsicum annum* L.

Heat duration (days)	Embryogenesis	Callusogenesis	Indirect organogenesis	Regeneration
0	0d	14.41±0.26c	0c	0d
3	0.38±0.04d	18.30±0.49a	0c	0d
5	3.39±0.26b	16.47±0.32b	0.63±0.04b	0.39±0.03c
7	5.28±0.11a	17.42±0.30ab	1.26±0.25a	2.99±0.20a
9	2.59±0.23c	14.11±0.12c	0.34±0.06b	1.11±0.07b
11	0.47±0.09d	12.54±0.34d	0c	0d
13	0d	8.47±0.26e	0c	0d

The mean values in a column followed by same letter(s) are not significantly different at $p \leq 0.05$ according to DMRT.

Table 22: Analysis of variances of five capsicum genotypes and three nutrition media for embryogenesis, callusogenesis, indirect organogenesis and regeneration from anthers

Effect	Data sources	Source of variation	DF	Mean sum of square	F. value
Genotype × medium	Anther response (Table 20)	Genotype (G)	4	285.42	1511.47***
		Media (M)	2	357.04	1890.70***
		G × M	8	28.06	148.61***
		Error	30	0.19	
	Embryogenesis (Table 20)	Genotype (G)	4	5.61	331.61***
		Medium (M)	2	6.31	373.19***
		G × M	8	0.65	38.13***
		Error	30	0.02	
	Callusogenesis (Table 20)	Genotype (G)	4	189.88	1354.36***
		Medium (M)	2	233.86	1668.09***
		G × M	8	22.71	162.01***
		Error	30	0.14	
	Indirect organogenesis (Table 20)	Genotype (G)	4	0.82	98.30***
		Medium (M)	2	1.41	169.80***
		G × M	8	0.25	29.98***
		Error	30	0.09	
	Regeneration (Table 20)	Genotype (G)	4	0.07	93.94***
		Mediim (M)	2	0.08	101.76***
		G × M	8	0.02	27.45***
		Error	30	0	
Heat stress	Embryogenesis (Table 21)	Heat duration	6	12.74	203.81***
		Error	14	0.06	
		Total	20		
	Callusogenesis (Table 21)	Heat duration	6	33.58	113.25***
		Error	14	0.30	
		Total	20		
	Indirect organogenesis (Table 21)	Heat duration	6	0.70	24.01***
		Error	14	0.03	
		Total	20		
	Regeneration (Table 21)	Heat duration	6	3.72	181.48***
		Error	14	0.02	
		Total	20		

*** = significant at $p \leq 0.001$.

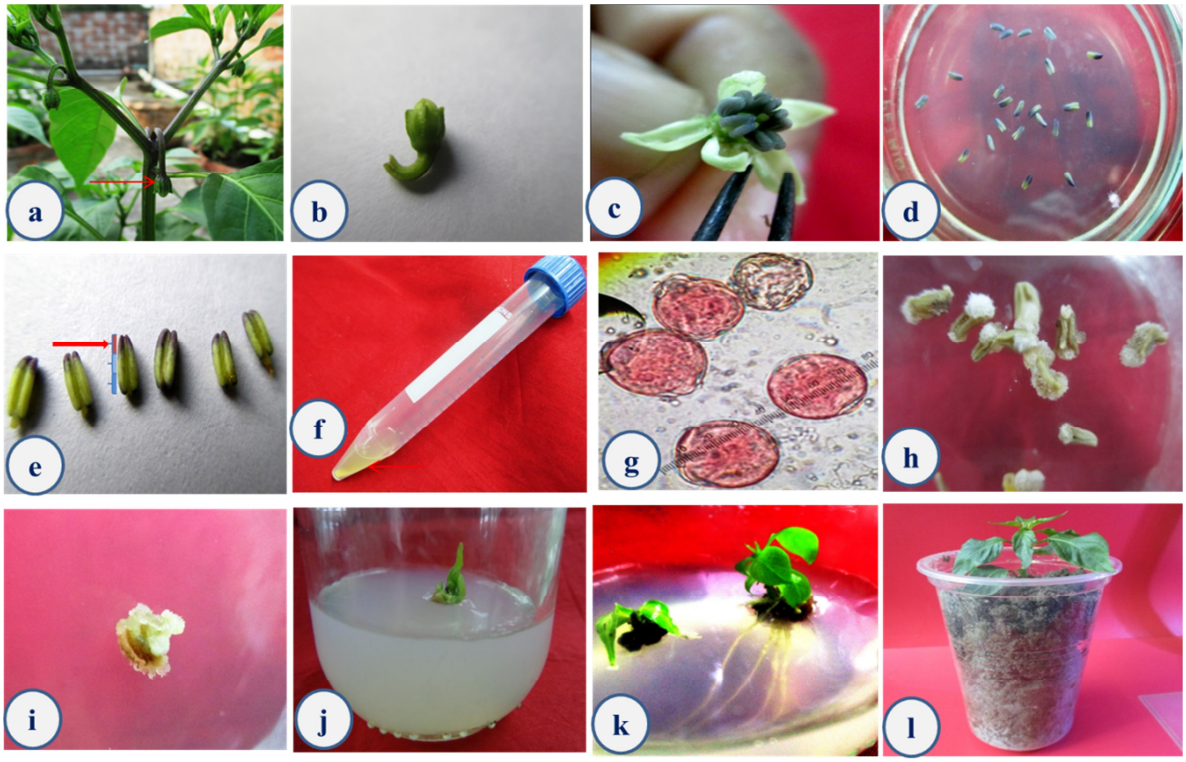

Fig. 24 (a-l): Callus induction and its subsequent regeneration from anther culture of chilli cultivars; (a) flower buds of first flowering, (b) appropriate size flower buds, (c) anther collection, (d-e) anther collected for inoculation, (f-g) uninucleate microspores, (h-i) callus initiation, (j) green plants regeneration, (k) well rooted green plants, and (l) plant transferred to pot.

5.1.4 Discussion

One of the main factors affecting success in pepper anther and microspore culture is genotype. Genotypic dependency is very important factor in getting the pepper haploid plants. Some previous reports on pepper androgenetic studies have emphasized that the most critical factors on obtaining haploid plants are genotypes (Rodeva et al. 2006, Nowaczyk et al. 2009). Because of the higher heterozygosity, hybrids are usually preferred as donor plants in order to increase the chance of producing androgenetic embryos (Niklas et al. 2012). It has been reported that some sweet and bell peppers are suitable for androgenetic responses than the hot ones (Liljana et al. 2013). In this case five Bangladeshi local hot chilli cultivars were used. Out of five cultivars Bullet showed highest response of

anther response, direct embryogenesis and plantlets formation, when cultured embryoids on C-R medium. It is reported that the ratio of embryoids formed in different anther culture studies of different *Capsicum* species is high but only a few of them are converted to plant. Although the number of study using the pepper anther culture technique is high, the efficiency of technique is still low (Trajkova and Gudeva et al. 2017). When the plant transformations of the embryos formed were examined, statistical differences were found in terms of genotype, media and genotype × media interactions. In this study out of three culture media, C-R showed highest response on embryogenesis and regeneration. In this sense, the best among the genotypes was Bullet, followed by other genotypes. It was observed that the medium C-R was the most successful in the plant transformation process and the medium MS was followed by it. The results of this study which is found different response between cultivars are in agreement with those Qin and Rotino (1995), who reported that anther donor genotype, had a strong effect on the androgenetic response in pepper. In the androgenesis method, which is a widely used technique for plants with commercially importance, the development program of anthers containing morphogenetic components can be converted from normal gametophytic to the sporophytic pathway (Kruglova et al. 2017). This is triggered by exposure to a number of physical and chemical stress factors, specific to each species (Shariatpanahi et al. 2006). Among them, temperature stress is the most affecting embryogenic development of pepper microspores. Although some researchers have reported that cold pre-treatment is also triggered, it has been suggested that high temperature pre-treatment is more effective (Popova et al. 2016). Pre-treatment of cultured anthers exposed to dark and high temperature conditions for the first few days, proving to be positive for previous embryogenesis studies in pepper anther and microspore culture studies (Lantos et al. 2012, Parra-Vega et al. 2013a) were also found to be having positive effect in present study. In this study 7 days heat shocked as stress pre-treatment factors showed highest result on embryoids induction as well as plant regeneration. Stress pre-treatment is the vital requirement for microspore embryogenesis

(Shariatpanahi et al. 2006). It has been reported that heat shock pre-treatment has been demonstrated to be an effective stimulating factor on microspore embryogenesis; it caused to change in microtubule and cytoskeleton (Hause et al. 1993, Cordewener et al. 1994, Simmonds 1994). The results from the present study demonstrate considerable genotypic differences in *in vitro* response of anthers from the studied Bangladeshi local chilli varieties. In this study, Bangladeshi local cultivars Bullet and Boltu gave the sufficient results to continue future works. Since androgenetic studies are first investigated of these local varieties from this study, additional research needed to improve embryo formation and plantlet regeneration that will be accelerated the breeding and advance biotechnological works for chilis improvement.

5.2 Effects of cold pre-treatment factors on anther culture responses in chilli pepper (*Capsicum annum* L.)

5.2.1 Introduction

Anther culture is one of the most important and useful tool to develop pure lines rapidly for plant breeding programs. However, some pepper genotypes are recalcitrant and embryogenic responses are not satisfactory in anther culture of chillis. There are some reports on low temperature (3-7°C) can facilitate switching the microspore to sporophytic developmental pathway. Regeneration in anther culture is dependent on various physical and chemical stress pre-treatment factors and the interaction between them as well: genotype, donor plants growth conditions, microspore developmental stage, pretreatment of flower buds and/or anthers, medium composition, etc (Germanà 2011, Seguí-Simarro et al. 2011, Parra-Vega et al. 2013a, Olszewska et al. 2014, Nowaczyk et al. 2015). The studies of androgenesis in pepper have proved that pre-treatment of flower buds or/and anthers with chemical and physical agents affect the androgenic responses very positively (Irikova et al. 2011). The aim of this experimental work was to investigate the influence of duration of low-temperature treatment and the genotypic effects on embryogenic responses as well as enhancement of regeneration efficiency of three chilli pepper genotypes in Bangladesh.

5.2.2 Materials and Methods

5.2.2.1 Plant material

Seeds of three local chilli genotypes *viz*. Boltu, Bullet and Halda were used as explant sources for this study. Seeds were germinated and grown in winter time (from October to February) in the pot and in the experimental field of the Institute of Biological Sciences, University of Rajshahi, Bangladesh.

5.2.2.2 Harvesting stage and pre-treatment of buds

Initiation of flowering was observed around 7 weeks after sowing and young flower buds of all genotypes were harvested for about 7-8 weeks at their early flower production period for anther culture. Buds with equal sepal and petal in size that were containing microspores at mid-late uninucleate developmental stages were harvested at early morning. Flower buds of each genotype were pre-treated at 4°C in refrigerator. Five cold stress durations (h) were applied to induce cold stress to the anthers for better embryogenic response i.e. 12, 24, 36, 48 and 60 h.

5.2.2.3 Acetocarmine staining

Prior to culture, precise stage of microspore (mid to late uninucleate stage) was determined by squashing anthers from a flower bud. Microspore development was observed at the different days of culture initiation by 1% acetocarmin staining. For preparing acetocarmine squash, at first 11.25 ml acetic acid added to 13.75 ml distilled water which makes it 45% glacial acetic acid. It was boiled till all the pungent smell comes out then 0.5 g of carmium was added to and then it was brought to room temperature. 1% acetocarmine stain was prepared by adding 0.25 g carmine to 25 ml of 45% acetic acid.

5.2.2.4 Surface sterilization

The sterilization procedures of flower buds are briefly described in Chapter V (Section 5.1.2.4).

5.2.2.5 Anther culture process

The anther culture processes are briefly described in Chapter V (Section 5.1.2.4).

5.2.2.6 Data recording and statistical analysis

Statistical analysis procedures are briefly mentioned in Chapter V (Section 5.1.2.6).

5.2.3 Results

The anthers from three genotypes (Boltu, Bullet and Halda) were cultured on C-R induction medium and five cold stress durations (12, 24, 36, 48 and 60 h) were applied to induce cold stress to the anthers for better embryogenic response. In comparison of anther response from the three studied varieties, Bullet showed the highest percentage of direct embryogenesis (3.0%) followed by the variety Boltu (2.96%) when 24 h cold stress applied to anthers (**Table 23**). No response of direct embryo production was found at 60 h cold stress pre-treatment to anthers (**Table 23**). The genotype had a significant effect on indirect embryogenesis and callusing. The anthers from genotype Halda showed the highest rate of indirect embryo frequency of (1.12%) and callusing (22.63%), when treated with 24 h cold shock stress compared to other genotypes (**Table 23**). Though the frequency of regenerants of all studied varieties were very low, Both Bullet and Boltu showed highest regeneration (0.62%) when treated with 24 h cold shock stress (**Table 23**).

Table 23: Effect of genotype and cold stress duration on embryogenesis, callusing, indirect organogenesis and regeneration of *Capsicum annum* L.

Duration (hrs)	Genotype	Mean ± SE			
		Embryogenesis	Callusing	Indirect organogenesis	Regeneration
0 h (Cont.)	Boltu	0.04±0.03c	10.87±1.53efg	0.17±0.05b	0.01±0.01d
	Bullet	0.03±0.03c	10.54±1.37efg	0.19±0.05b	0.02±0.01d
	Halda	0.04±0.03c	11.25±1.50efg	0.20±0.04b	0.01±0.01d
12 h	Boltu	2.51±0.29ab	17.09±1.13bc	0.38±0.08b	0.49±0.11abc
	Bullet	2.69±0.48a	16.59±1.23bcd	0.35±0.08b	0.48±0.10abc
	Halda	2.70±0.26a	18.03±1.13ab	0.37±0.07b	0.51±0.11ab
24 h	Boltu	2.96±0.63a	22.13±2.98a	1.03±0.22a	0.62±0.22a
	Bullet	3.00±0.60a	21.52±3.07ab	0.96±0.19a	0.62±0.19a
	Halda	2.88±0.69a	22.63±3.27a	1.12±0.27a	0.61±0.17a
36 h	Boltu	1.55±0.25b	12.74±1.03cde	0.35±0.15b	0.19±0.09cd
	Bullet	1.57±0.24b	12.85±0.58cde	0.31±0.18b	0.18±0.06d
	Halda	1.53±0.29b	12.17±0.99cdef	0.38±0.16b	0.21±0.07bcd
48 h	Boltu	0.18±0.11c	11.65±1.07defg	0b	0.02±0.02d
	Bullet	0.19±0.11c	10.97±0.58efg	0b	0.01±0.01d
	Halda	0.19±0.10c	10.64±0.94efg	0b	0.02±0.02d
60 h	Boltu	0c	7.53±0.88efg	0b	0d
	Bullet	0c	6.53±0.81g	0b	0d
	Halda	0c	6.92±0.65fg	0b	0d

The mean values in a column followed by same letter(s) are not significantly different at $p \leq 0.05$ according to DMRT.

According to the results obtained from genotype effect, the highest percentage of direct embryo formation were found from the variety Bullet (1.65%), followed by the variety Boltu (1.11%) (**Fig. 25**). In these two genotypes it was observed also the highest frequency of callusing as 15.24 and 13.28% respectively (**Fig. 26**). The lowest percentage of direct embryogenesis (0.92%) and callus formation (11.92%) in comparison with the other genotypes were registered in the variety Halda (**Fig. 25 and Fig. 26**). The indirect organogenesis with the highest frequency was recorded in variety Bullet (0.49%), followed by the variety Halda (0.27%) and the lowest one in variety Boltu (0.21%) (**Fig. 25**). The regeneration with the highest frequency was recorded in variety Bullet (0.36%), followed by the varieties of Boltu (0.17%) and Halda (0.15%).

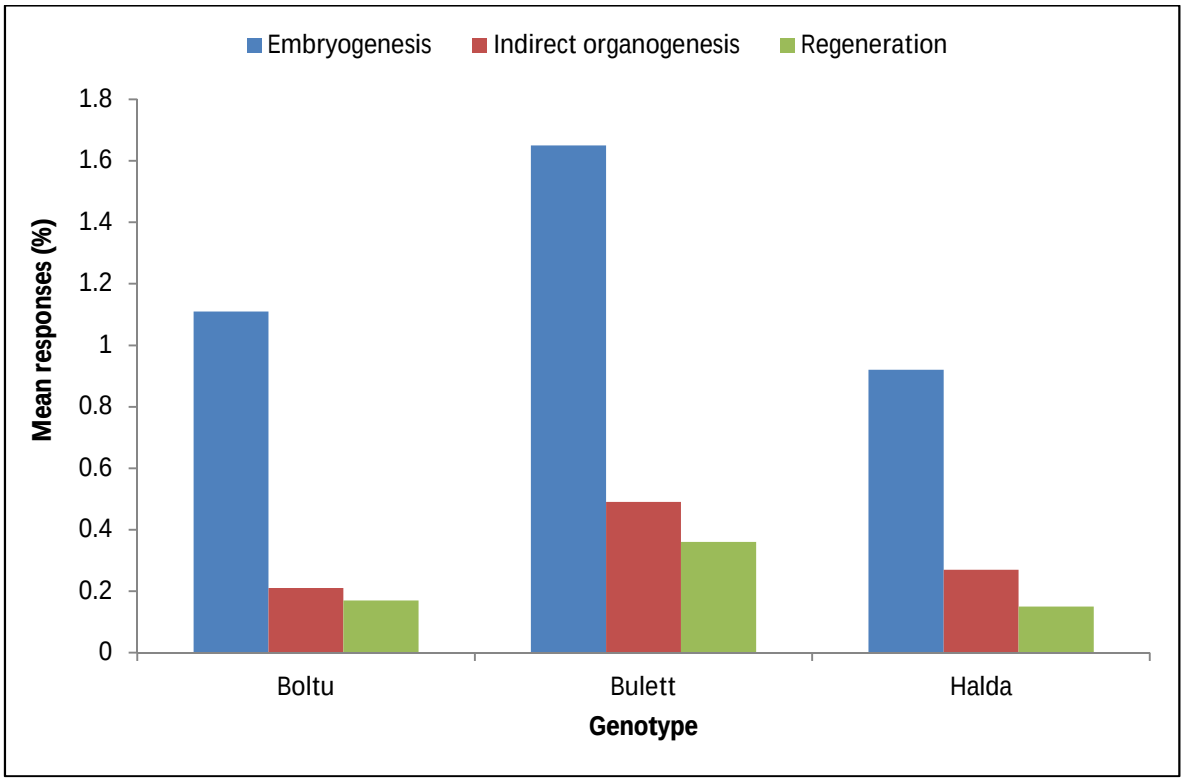

Fig. 25: Effect of genotype on embryogenesis, indirect organogenesis and regeneration of *Capsicum annum* L. on an average of six cold stress durations.

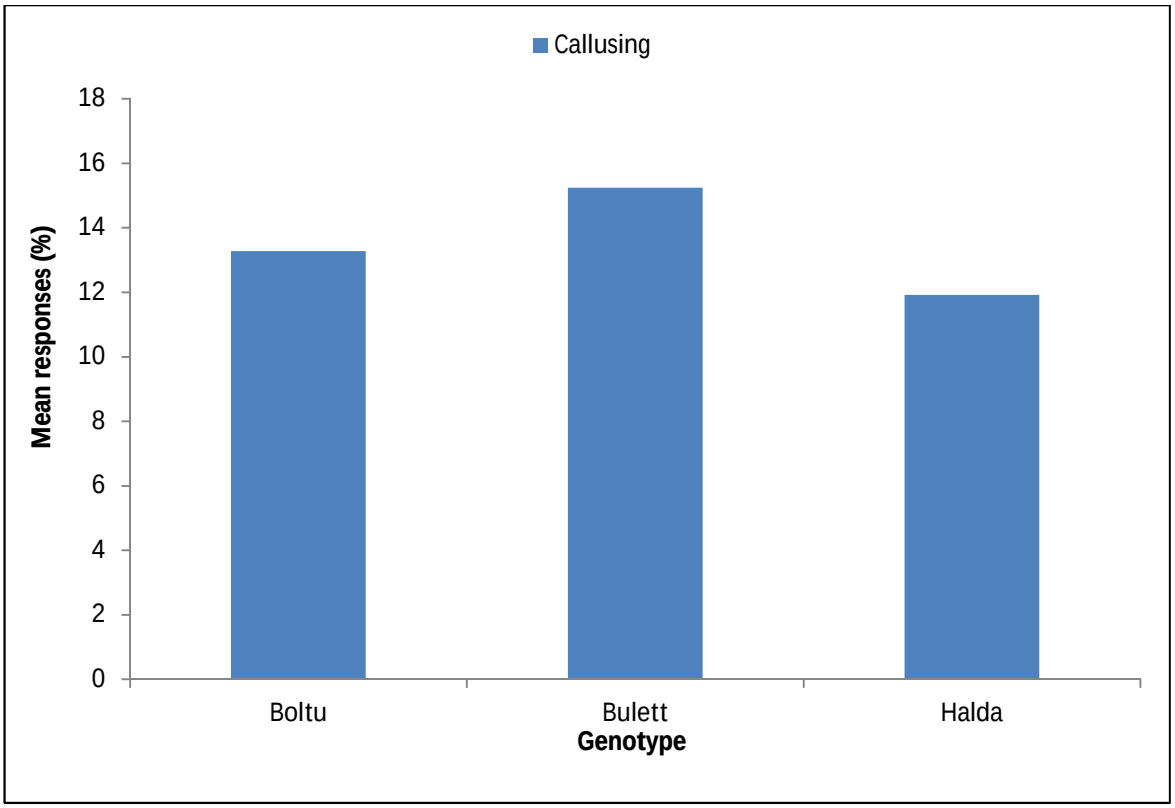

Fig. 26: Effect of genotype on callusing of *Capsicum annum* L. on an average of six cold stress durations.

According to the results obtained from cold stress duration effect, the highest percentage direct embryo production was found from the duration period 24 h (2.95%), followed by the duration period 12 h (2.63%) (**Fig. 27**). Out of two durations the highest frequency of callusing was recorded 22.09% and 17.24% respectively (**Fig. 28**). The indirect organogenesis with the highest frequency was recorded at 24 h cold stress duration (1.04%), followed by 12 h duration period (0.37%). No indirect organogenesis was found from 48 and 60 h cold shock stress to anthers. The regeneration with the highest frequency was recorded (0.62%) when anthers were pretreated with 24 h cold shock, followed by 12 h cold shock pre-treatment as 0.49% (**Fig. 28)**.

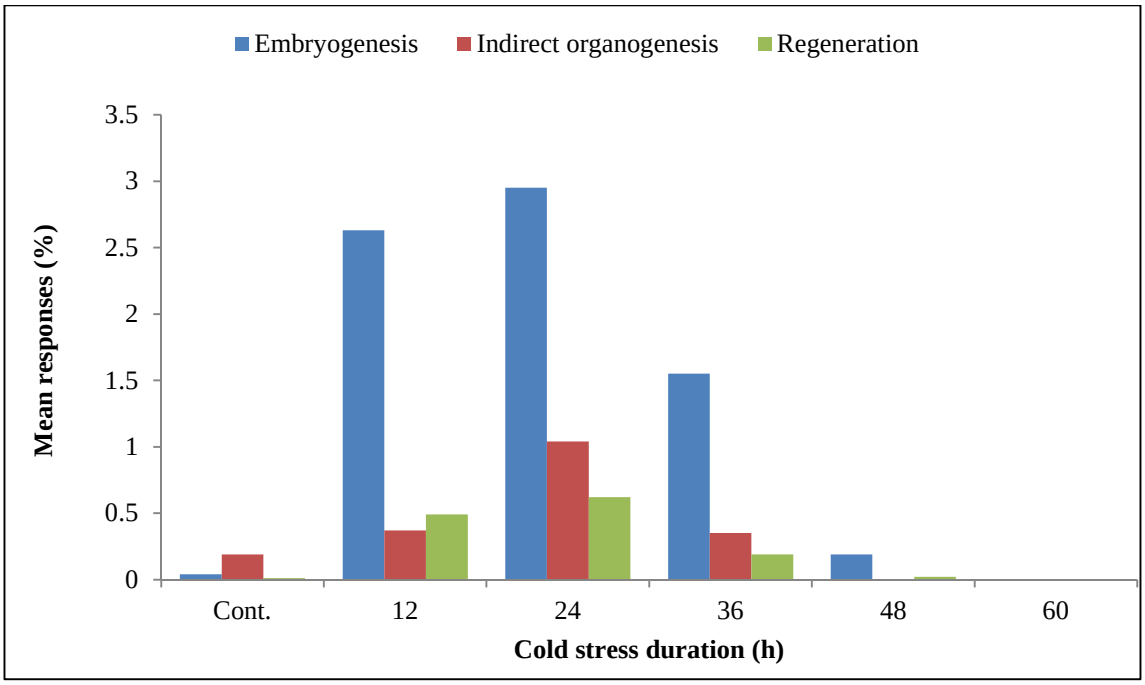

Fig. 27: Effect of cold stress duration (h) on embryogenesis, indirect organogenesis and regeneration of *Capsicum annum* L. on an average of three genotypes.

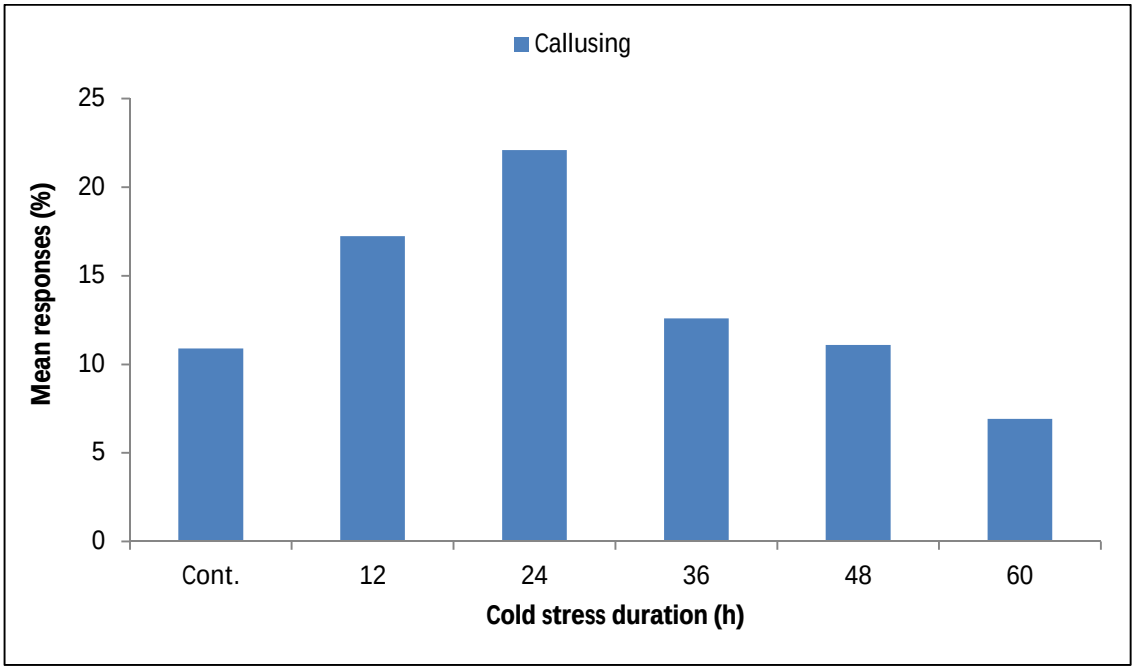

Fig. 28: Effect of cold stress duration (h) on callusing of *Capsicum annum* L. on an average of three genotypes.

Table 24: Analysis of variances of chilli pepper genotypes and cold stress duration for embryogenesis, callusing, indirect organogenesis and regeneration from anthers

Category	Source of Variation	Sum of Squares	df	Mean Square	F-value	Sig.
Embryogenesis	Duration + Genotype	81.409	17	4.789	15.455	0.000
	Error	11.154	36	0.310	-	-
	Total	92.564	53	-	-	-
Callusing	Duration + Genotype	1302.868	17	76.639	9.957	0.000
	Error	277.080	36	7.697	-	-
	Total	1579.949	53	-	-	-
Indirect organogenesis	Duration + Genotype	6.684	17	0.393	8.925	0.000
	Error	1.586	36	0.044	-	-
	Total	8.270	53	-	-	-
Regeneration	Duration + Genotype	3.287	17	0.193	7.001	0.000
	Error	0.994	36	0.028	-	-
	Total	4.281	53	-	-	-

df = degrees of freedom, Sig. = Significance

5.2.4 Discussion

Temperature stress pre-treatment is considered to be the most effective treatment to induce microspore embryogenic development in chilli pepper. The optimal temperature (low or high) and duration of pre-treatment were determined by Morrison et al. (1986 a&b), Supena and Custers (2011) and Parra-Vega et al. (2013b). Out of three studied genotypes, Bulett performed the best result concerning direct embryo formation. Experimental results in the present study showed that microspore embryogenesis depends on duration of cold treatment and genotype. Studied three genotypes reacted with direct embryo formation up to 48 h cold treatment. There was no direct embryo production found from any genotype, when 60 h cold treatment was applied. Cold pre-treatment stimulates the process of callusing in some of the studied pepper genotypes, but plants were not obtained may be due to the difficulties in the regeneration from this tissue and inappropriate culture conditions. Cold shock treatment of anther cultivations from 0 to 60 h stimulated androgenesis response and showed the best results at 24 h. In all the treatments, tested genotype showed varied number of direct and indirect embryogenesis, callusing and regenerants production. Cold shock stress for 24 h showed highest percentage of direct embryogenesis, callusing, indirect organogenesis and regeneration. Increasing the duration of cold shock by more than 24 h had a detrimental effect and suppressed microspore embryogenesis as 60 h decreased callusing dramatically. Low-temperature shock was applied with success in pepper anther culture by González-Melendi et al. (1996), shed-microspore culture (Supena and Custers 2011, Ari et al. 2016b).

Chapter VI

General Discussion

6. General Discussion

Bangladesh is a large producer and consumer of chilli, has a vast potentiality to increase the production in order to meet domestic necessities and promote export. Low productivity of chilli is caused by some factors. Plant tissue culture is an important system for plant regeneration. But *in vitro* culture of chilli is most difficult for its recalcitrant nature. The present investigation was conducted to develop a suitable regeneration of six chilli varieties. These are most popular for its attractive colour and pungency. The first experiment (**Exp. 1, Section 4.1**) was conducted to find out the effect of varieties, explants and phytohormones for callus induction and regeneration. For callus induction in chilli, explants *viz.*, cotyledon, hypocotyls, petiole, stem, leaf, fruit and root segments are widely used. As explants hypocotyls, cotyledons and shoot tips (cotyledonary node) were used in this study. The observation was done following the methods of Kale (2005), where he used hypocotyl as explants and selected them after 10 to 11 days of culture inoculation. The most important factor that influence is the growth regulators which influence the rate of callus induction. The watery nature of callus produced through on low concentration (0.5 mg/l) of 2,4-D with BAP but in higher concentration of those growth regulators white compact cottony type of callus was produced. Hard greeninh callus was observed in 2.5 mg/l BAP concentration. For regeneration, MS medium supplemented with 0.50 mg/l kinetin showed embryogenesis in significantly higher number of explants (71.40%). Lowest response was recorded at 0.25 mg/l BAP (14.40%). Without using any growth regulators, the callus showed no responses. The interaction effect of MS medium supplemented with 0.5 mg/l kinetin showed in significantly higher number of regeneration (71.40%) of cotyledon explants. Only 14.40% regeneration showed from hypocotyle explants produced on MS medium with 0.25 mg/l BAP. The interaction effect of variety and PGRs, Bullet with kinetin (0.50 mg/l) showed best regeneration in significantly higher percent embryo induction variety was Bullet (71.40%)

followed by Boltu (70.80%), Yellow wonder (68.00), Halda (64.60%), Kulali (63.60%) and California wonder (58.80%). The lowest embryods (14.40%) produced on MS medium with 0.25 mg/l BAP of variety California wonder. From the present study it is conclude that auxins with higher doses of cytokinins induce compact non-embryogenic green callus. Similar types of results were found in chilli embryogenesis using BAP which is non-embryogenic and similar type of results found by Fari (1986). Therefore, in present study, MS with fixed levels (0.5 mg/l) of 2,4-D and different concentration and combination varying levels of kinetin and BAP were used and tried to observed the effect of callus formation nature of callus, days to callus initiation and callus quantity. Similar types of calli were obtained when the explants cultured on medium with different levels of 2,4-D (Mathew 2002). It was observed that, when the concentration of 2,4-D was increased than the rate of callus induction showed higher results. MS basal medium with 0.5 mg/l kinetin and 1.0 mg/l GA_3 showed highest shoot elongation of cotyledonary explants for Yellow wonder (76.40%), Kulali (71.60%), California wonder (70.80%), Boltu (70.80%) and Bulet (66.00%). On the other hand shoot elongation was observed in lowest (5.80%) on Kulali in MS medium. The media supplemented with GA_3 showed positive interaction for shoot elongation of chilli which is similar to Wang et al. (1991). Highest frequency of rooting was observed on MS medium containing with 0.5 mg/l IAA and 0.1 mg/l kinetin followed by on Halda (98.0%) and Boltu (98.0%). It was observed that MS with 1.0 mg/l NAA and 0.1 mg/l kinetin showed higher results (95.00%). Low concentration of IAA and NAA played a significant role on rooting derived from chilli (Christopher et al. 1986). After ten days of culture shoot produce root. It was short and thick media containing NAA and long thin root was initiated on IAA. Similar observation was found by Klukarni (1997). The effect of the component was for the first time investigated here in the *in vitro* regeneration of four popular local chilli varieties.

In the present study an experiment (Exp. 2) was set up to optimizetion the effect of phytohormones, explant and genotype for adventious shoot induction, elongation and rooting using four local chilli genotypes of Bangladesh. Direct regeneration is a rapied shoot multiplication system, most often it occurs through shoot proliferation from pre-exting mesristem. Cotylodonary leaf is the commonly used explants for shoot produce (Gunay and Rao 1978). Chaudhari (2004) described that direct somatic embryogenesis is better than callus mediate regeneration because of genetic and cytological variations often associates. In the present staudy it was tried to regenerate plants from various explants. The axillary shoot-tip explants proliferated to produce the maximum number of shoot buds on a medium containing 5-10 mg/l BAP single uses. Thidiazuron induced high frequency shoot bud formation and plant regeneration from cotyledonary node explants of *Capsicum annuum* L. The interaction effect of explant and growth regulator combination given significantly higher number in cotyledonary nodal region explants around 69.60% of the explants showed shoots at the cultivar of Boltu. And irrespective of growth regulators TDZ at 0.5 mg/l induced shoots on nearly 65% of cotyledonary nodal region explants at the cultivar Boltu, significantly highest number of explants showed by cotyledonary nodal region explants at 10 mg/l BAP (69.60%). It was observed that BAP has induced shoots derived from cotyledon and cotyledonary nodal region but not used hypocotyls of this study. The results are in agreement with previously reported by Gunay and Rao (1978), Manoharan et al. (1998) and Ashajyothi (2004) usng BAP for shoot induction. The response of successful shoot regeneration also observed under this study using growth regulators as TDZ. Using hypocotyls as explants and cutured them in medium + TDZ (0.5 mg/l) showed significantly higher number of shoots (45.0%) for Boltu.

Callus induction from explants is most important step for successful plant regeneration. In the present experiment (Exp. 3) a suitable protocol was developed for *in vitro* callus induction and plant regeneration. Two popular local chilli cultivars *viz.* Bullet and Boltu were grown in *in vitro* and explants cotyledon and hypocotyl were excised form *in vitro* grown seedlings used for the present investigation. For all cases MS medium was used that supplemented with different concentration of phytohormones and $AgNO_3$. $AgNO_3$ influence positively to improve plants through *in vitro* culture as well as callus induction and subsequent plant regeneration. Among the hormone combinations, callus formation potentiality from hypocotyls explants was the highest in BAP 4 mg/l + $AgNO_3$ 3.0 mg/l in both Bullet and Boltu. Here, Bullet (51.74%) showed better callusing than Boltu (41.26%). However, in the present investigation, 10.60 to 86.40% callus induction was recorded. The wide range of variability might be occurred due to genotypic effect along with the different levels of BAP, Kin and $AgNO_3$. Simillar results were obatained in *Capsicum chinense* by Bora et al. (2018). The prolonged culturing of calli on the same media failed to differentiate into shoots, but sub-culturing of calli on respective fresh media developed the shoots considerably. In this current study the organogenesis frequency was increase dgradually by increasing the concentration of $AgNO_3$ up to 3.0 mg/l but decreased rapidly by further increase of $AgNO_3$ with BAP 5.0 mg/l. All the genotypes and explants responded in a different way to diverse concentrations of $AgNO_3$. The highest *in vitro* shoot regeneration frequency (75% and 64.60%) and from hypocotyls and cotyledon derived calli, respectively were recorded in Bulett and Boltu cultivar. The present results clearly indicate that BAP had harmonious effect with $AgNO_3$ in the enhancement of *in vitro* shoot regeneration. Results of this experiment was simillar with Cruz de Carvalho et al. (2000) who reported 63.8% shoot development in *Phaseolus vulgaris* using BAP only but it was increased up to 100% by applying BAP in combinationwith $AgNO_3$. For induction of strong and determined root system, BAP and NAA was the best combination for promoting root formation. The maximum roots (9.40) were formed in MS

supplemented with 2.0 mg/l BAP + 0.1 mg/l NAA from hypocotyl explants in Bullet. Simillar findings were observed by Anantasaran and Kanchanapoom (2008) in *Zinnia* cultivars. Some earlier reports on root initiation prefer NAA and IAA over IBA (Siddique and Anis 2006, Sanatombi and Sharma 2008) and IBA over NAA and IAA (Otroshy et al. 2011, Kumari et al. 2012 and Hedgre et al. 2017). On the contrary, Sunandakumari et al. (2004) reported that 2.67 mg/l of $AgNO_3$ gave the improved frequency of rooting in *Rotula aquatica*. Conversly, the positive effect of $AgNO_3$ for root formation in barley genotypes was observed by Kumar et al. (2009). Ptak et al. (2010) reported that necrosis was observed in callus cultures due to ethylene biosynthesis. Vikrant and Rashid (2002) conducted a study on minor millet (*Paspalum scrobiculatum*) for somatic embryogenesis and concluded that $AgNO_3$ at 10.0 mg/l increased the frequency of embryogenesis by 76% as compared to 53% in control. Sharma et al. (2008) reported that $AgNO_3$ inhibited ethylene action in plant growth and development. The exact mechanism of $AgNO_3$ mediated ethylene production and its activity regulation is unclear but it has been explained by an interference of ethylene perception or stress exerted by silver ion (Sharma et al. 2008). It has been proved beyond doubt that polyamines play crucial roles in plant growth and development as well as basic biological process (reviewed by Kumar and Rajam 2004, Kumar et al. 2009). There have been reports confirming the significance of $AgNO_3$ on *in vitro* processes in *C. annuum* (Ashrafuzzaman et al. 2009) and *C. frutescens* (Sharma et al. 2008).

Anther culture of chilli is difficult for its recalcitrant nature. One of the main factors affecting success in pepper anther and microspore culture is genotype. Genotypic dependency is very important factor in getting the pepper haploid plants. In this study (Exp. 4) five Bangladeshi local chilli cultivars were used and out of them Bullet showed highest response of anther culture. The anthers from five genotypes (Boltu, Bullet, Halda, Zeha and Baromashi) were cultured on three induction media (MS, C-R and N). In

comparison of anther response from the five studied varieties, Bullet showed the highest percentage of reacted anthers (20.81%), when cultured on C-R medium, followed by the same variety (18.70%) cultured on MS medium. The variety Baromashi showed no response to anthers culturing with N medium. The genotype had a significant effect on direct embryogenesis and callusing. The anthers from genotype Bullet showed the highest rate of embryoids induction (3.28%) and callusing (16.54%), when cultured them on C-R medium compared to other genotypes. In case of indirect embryogenesis, Halda showed as the most responsive genotype (1.43%) among tested ones when cultured on C-R medium. Though the frequency of regenerants of all studied genotypes were very low, Bullet showed highest regeneration (0.35%) culturing on C-R medium among all varieties. Zeha and Baromashi showed no regeneration culturing on any type of medium. According to the results obtained from genotype effect, the highest percentage reacted anthers were found from the variety Bullet (14.67%), followed by the variety Boltu (12.81%). It was observed that the highest frequency of callusing (12.29 % and 10.05%, respectively) was recorded for two studied genotypes. The percentage of reacted anthers (1.11%) and the lowest frequency of callus formation (0.97%) in comparison with the other genotypes were registered in the variety Baromashi. The direct embryogenesis with the highest frequency was recorded in variety Boltu (2.0%), followed by the varieties Bullet (1.98%), Halda (1.67%), Zeha (0.88%) and the lowest one in variety Baromashi (0.19%). The regeneration with the highest frequency was recorded in variety Bullet (0.2%), followed by the varieties Boltu (0.13%) and Halda (0.04%). Zeha and Baromashi showed no response of green plant regeneration. Wang et al. (1973) described 3 to 5% embryoids and 24 to 29% microsporial callus induction on *C. annuum* var. *Annuum* cv. Yeo Hsien. Gudeva et al. (2007) gaind highest 17% androgenesis analyzeing 21 pepper cultivar. The reported results are in agreement with the present findings. The results of this study which is found different response between cultivars are in agreement with those Qin and Rotino (1995), who reported that anther donor genotype, had a strong effect on the androgenetic response in

pepper. In this study 7 days heat as stress pre-treatment factors showed highest result on embryoids induction as well as plant regeneration. Six different durations of heat pre-treatment e.g. 3, 5, 7, 9, 11 and 13 d were applied to anther of variety Bullet culturing on C-R medium for embryogenesis and regeneration. Cold stress pre-treatment of anther cultivations from 3 to 13 days stimulated androgenesis response and showed the best results at 7 days. In all the treatments, tested genotype showed varied number of direct and indirect embryogenesis, callusogenesis and regenerants production. Heat shock for 7 days showed highest percentage of direct embryogenesis, callusogenesis, indirect organogenesis and regeneration as 5.28, 17.42, 1.26 and 2.99% respectively. Increasing the duration of cold stress pre-treatment by more than 7 days had a detrimental effect and suppressed microspore embryogenesis as 13 days decreased callusing dramatically.

The final experiment was conducted to examine the effect of cold shock pre-treatment to enhanch embryoyogenesis and regeneration of capsicum anther culture (Exp. 5). The anthers from three genotypes (Boltu, Bullet and Halda) were cultured on C-R induction medium and five cold stress durations (12, 24, 36, 48 and 60 h) were applied to induce cold stress to the anthers for better embryogenic response. In comparison of anther response from the three studied varieties, Bullet showed the highest percentage of direct embryogenesis (3.00%) followed by the variety Boltu (2.96%) when 24 h cold stress pre-treatment applied to anthers. No response of direct embryo production was found at 60 h cold stress pre-treatment to anthers. The genotype had a significant effect on indirect embryogenesis and callusing. The anthers from genotype Halda showed the highest rate of indirect embryo frequency of (1.12%) and callusing (22.63%), when treated with 24 h cold shock stress compared to other genotypes. Though the frequency of regenerants of all studied varieties were very low, Both Bullet and Boltu showed highest regeneration (0.62%) when treated with 24 h cold shock stress. Out of three genotypes Bullet performed best concerning direct embryoids formation. Experimental results in the present study

showed that microspore embryogenesis depends on duration of cold treatment and genotype. Studied three genotypes reacted with direct embryo formation upto 48 h cold treatment. There was no direct embryo production found from any genotype, when 60 h cold treatment was applied. Cold shock treatment of anther cultivations from 0 to 60 h stimulated androgenesis response and showed the best results at 24 h. In all the treatments, tested genotype showed varied number of direct and indirect embryogenesis, callusing and regenerants production. Cold shock stress for 24 h showed highest percentage of direct embryogenesis, callusing, indirect organogenesis and regeneration.

Chapter VII

Summary

7. Summary

The present study was conducted to improve chilli cultivars considering the features related to abiotic stress tolerance through *in vitro* somatic and gametic embryogenesis. For this purpose ten different cultivars of *Capsicum annum* L. were considered as plant materials. Out of ten, five popular local cultivars are Boltu, Bullet, Halda, Zeha and Baromasi were considered because of much popularity in coastal region for its pungency, flavour and attractive colour. The other five cultivars *viz*. Hybrid-1, Shimla, Kulali, Yellow wonder, California wonder were chosen also for the study because of much popularity in our country and abroad for its flavour pungency, mild or less pungency and attractive colour. In this study, the experiments were done on somatic embryogenesis (SE), e.g., *in vitro* improvement of calli and its subsequent regeneration, direct organogenesis from different explants, effect of PGRs and silver nitrate on callus induction and regeneration and gametic embryogenesis (GE), e.g., embryoids induction and plant regeneration via anther culture and effects of cold pre-treatment factors on anther culture responses in chilli cultivars.

The first experiment (**Exp. 1, Chap. IV**) was conducted to develop a simple, efficient and reliable *in vitro* regeneration of chilli. In the present experiment, seeds of selected varieties were cultured on MS medium without any plant growth regulators. Among the ten studied genotypes Bullet showed highest percentage (97.10%) for seed germination in *in vitro* culture whereas in the field condition the Bullet showed 67.20% germination. For callus initiation hypocotyls, cotyledons, shoot tips and root were used as explants. The variety Yellow wonder showed higher percent of callus induction (98.60%) followed by Boltu (97.80%) and Bullet (96.20%) when cultured on MS medium supplemented with 0.5 mg/l 2,4-D + 2.0 mg/l kinetin. The nature of calli showed different with various concentration and combination of growth regulators. It was compact and whitish in nature when grown in MS medium supplemented with 0.5 mg/l 2,4-D +1.0 mg/l BAP. The watery nature of

callus produced through on low concentration (0.5 mg/l) of 2,4-D with BAP but in higher concentration of those growth regulators produced whitish compact callus. Hard green callus was observed from 2.5 mg/l BAP concentration. For regeneration, Bullet produced the highest regenerated plants (71.40%) from cotyledonary explants when cultured on MS medium supplemented with 0.50 mg/l kinetin followed by Boltu (70.80%), Yellow wonder (68.00) and Halda (64.60%). Without using any growth regulators, the callus showed no responses. The highest number (76.40%) of shoot elongation was observed on Yellow wonder from cotyledonary explants followed by Kulali (71.60%) and California wonder (70.80%). The least response to shoot elongation (5.80%) was recorded from MS without any growth regulators. On the other hand shoot elongation was observed in lowest (5.80%) on Kulali in MS medium. Highest frequency of rooting was observed on Halda (98%) and Boltu (98%) culturing on MS medium containing with 0.5 mg/l IAA and 0.1 mg/l kinetin.

The second experiment **(Exp. 2, Chap. IV)** has been considered to develop a simple, efficient and reliable *in vitro* regeneration of chilli using cotyledon, hypocotyl and cotyledonary nodal region as explants. Four local chilli cultivars *viz*. Boltu, Bullet, Halda and Zeha were considered for this experiment. For each cases 4-6 explant segments were placed horizontally in each vessel and gently placed into the surface of the sterilized MS culture medium with various combinations of growth regulators like BAP (2.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 14.0 mg/l) and TDZ (0.10 , 0.5 and 1.0 mg/l). In case of explants used in the experiment cotyledonary nodal region showed significantly highest number of shoot induction (69.60%) followed by cotyledon (67.20%). Highest number of multiple shoots was formed using BAP from cotyledon and cotyledonary nodal region but no shoot was formed from hypocotyl explants using BAP at any concentration. From hypocotyl explants only TDZ produced multiple shoots. In this perspective 10 mg/l BAP induced shoots in significantly higher number (69.60%) from cotyledonary nodal region followed by TDZ at 0.5 mg/l (65.00%) from hypocotyl. In respect of all explants, Boltu produced highest

number of shoots as 69.60% for cotyledonary nodal region, 67.20% for cotyledon and 45% for hypocotyl. For shoot elongation BAP + GA_3 (8.0 + 0.5, 8.0 + 1.0, 8.0 + 2.0, 8.0 + 2.5, 8.0 + 3.0 mg/l) were used. Well elongated shoots were transferred to MS medium supplemented with BAP + IAA (2.0 + 0.1, 2.0 + 0.5, 2.0 + 1.0, 2.0 + 1.5, 2.0 + 2.0 mg/l) and BAP + NAA (2.0 + 0.1, 2.0 + 0.5, 2.0 + 1.0 mg/l) for root formation. Highest number (82.6%) of shoot elongation was observed in Boltu followed by Bullet (82.4%) and Zeha (82.0%). Among the growth regulators and its combinations shoot elongation found significantly higher (82.6%) in MS + 8.0 mg/l BAP + 1 mg/l GA_3 after 45 days of inoculation. Well elongated shoots were transferred to MS medium supplemented with BAP + IAA (2.0 + 0.1, 2.0 + 0.5, 2.0 + 1.0, 2.0 + 1.5, 2.0 + 2.0 mg/l) and BAP + NAA (2.0 + 0.1, 2.0 + 0.5, 2.0 + 1.0 mg/l) for root formation. Highest (99.2%) rooting was observed on MS medium containing 0.5 mg/l NAA and 2.0 mg/l BAP in Bullet. Boltu also showed quite higher rooting as 97.4% on the same medium.

Another investigation **(Exp. 3, Chapter IV)** was carried out to examine the effect of PGRs and silver nitrate on callus induction and regeneration of two local chilli cultivars. Two local chilli cultivars *viz.* Bullet, Boltu and two types of explants *viz*. cotyledon and hypocotyl were used for the experiment. Twenty different treatments were used for callus induction which prepared on MS medium containing various hormonal combinations. In which four various concentrations of BAP (1.0, 2.0, 3.0 and 4.0 mg/l) and four various concentrations of kinetin (1.0, 2.0, 3.0 and 4.0 mg/l) were used singly for callus induction and another twelve treatments were the same combinations of BAP + $AgNO_3$ and Kin + $AgNO_3$ (3.0 + 2.0, 3.0 + 3, 3.0 + 4.0, 4.0 + 2.0, 4.0 + 3.0 and 4.0 + 4.0 mg/l). The highest frequency of callusing was recorded from hypocotyl explant in 4.0 mg/l BAP + 3.0 mg/l $AgNO_3$ for Bullet (86.40%); followed by Boltu (73.80%) and the cotyledon explants was in Bullet (81.20%) and Boltu (67.30%) in the same combination. No callus was observed in PGRs free medium. Among the cultivars higher callusing was recorded in Bullet

(51.74%) than Boltu (41.26%). On the other hand it was also observed that hypocotyl showed better callusing (48.34%) than cotyledon (44.66%). For regeneration twelve treatments with three combinations- i) BAP (5.0 mg/l) + $AgNO_3$ (1.0 - 4.0 mg/l), ii) BAP (5.0 mg/l) + IAA (0.1 - 1.5 mg/l) and iii) BAP (5.0 mg/l) + NAA (0.1 - 1.5 mg/l) were evaluated. The best shoot regeneration (75%) was recoded from the hypocotyl that cultured in MS medium supplemented with BAP (5.0 mg/l) $AgNO_3$ + (3.0 mg/l) for Bullet. For cotyledon explant the same combination produced highest shoot regeneration (67.20%) in case of Bullet. For another genotype Boltu, the same combination i.e. MS medium supplemented with BAP (5.0 mg/l) $AgNO_3$ + (3.0 mg/l) also produced highest shoot regeneration from both explants such as hypocotyl (64.40%) and cotyledon (52.40%). The elongated shoots derived from hypocotyl and cotyledonary callus were transferred to rooting medium that supplemented with different combinations of fixed level of BAP (2.0 mg/l) with different levels of NAA (0.1 - 1.0 mg/l) or IAA (0.1 - 1.0 mg/l). Number of roots per shoot varies from 5.0 - 9.8 in Bullet and 3.4 - 6.9 in Boltu. The number of roots per shoot was higher in the medium supplemented with 2.0 mg/l BAP + 0.1 mg/l NAA. The maximum number (9.40) of roots per shoot was observed in hypocotyl callus derived shoot and in case of cotyledonary callus derived shoot it was 8.20. However, no root was induced in PGRs free MS medium. Hormonal combination 2.0 mg/l BAP + 0.1 mg /l NAA was the most effective for root formation.

Exp. 4, Chapter-V was conducted to determine the androgenetic response especially for anther culture of some chilli including some Bangladeshi genotypes. Seeds of five local chilli genotypes *viz*. Boltu, Bullet, Halda, Zeha and Baromashi were used as explant sources for this study. Three basal media were considered for embryos induction in this study *viz.* MS, N and C-R. All media were supplemented with 2.0 mg/l 2,4-D, 1.0 mg/l kinetin, 8.0 g/l agar and 30 g/l sucrose. In comparison of anther response from the five studied varieties, Bullet showed the highest percentage of responded anthers (20.81%),

when cultured on C-R medium, followed by the same variety (18.70%) cultured on MS medium. In comparison of anther response from the five studied varieties, Bullet showed the highest percentage of responded anthers (20.81%), when cultured on C-R medium, followed by the same variety (18.70%) cultured on MS medium. In comparison of anther response from the five studied varieties, Bullet showed the highest percentage of reacted anthers (20.81%), when cultured on C-R medium, followed by the same variety (18.70%) cultured on MS medium. The variety Baromashi showed no response to anthers culturing on N medium. The genotype had a significant effect on direct embryogenesis and callusing. The anthers from genotype Bullet showed the highest rate of embryoids induction (3.28%) and callusing (16.54%), when cultured them on C-R medium compared to other genotypes. In case of indirect embryogenesis, Halda showed as the most responsive genotype (1.43%) among tested ones when cultured on C-R medium. Though the frequency of regenerants of all studied varieties were very low, Bullet showed highest regeneration (0.35%) culturing on C-R medium among all varieties. Zeha and Baromashi showed no regeneration culturing on any type of medium. According to the results obtained from genotype effect, the highest percentage reacted anthers were found from the variety Bullet (14.67%), followed by the variety Boltu (12.81%). In these two genotypes it was observed also the highest frequency of callusing (12.29% and 10.05%, respectively). The direct embryogenesis with the highest frequency was recorded in variety Boltu (2.0%), followed by Bullet (1.98%), Halda (1.67%), Zeha (0.88%) and the lowest response was recorded in Baromashi (0.19%). The regeneration with the highest frequency was recorded in Bullet (0.2%), followed by Boltu (0.13%) and Halda (0.04%). Zeha and Baromashi showed no response of green plant regeneration. According to the results obtained from culture media, the highest percentages of responding anthers were found from the medium C-R (12.47%), followed by MS (10.32%). In these two culture media it was observed the highest frequency of callusing (9.69% and 8.67%, respectively). The direct embryogenesis with the highest frequency was recorded in the medium C-R (2.0%), followed by MS

(1.32%) and the lowest one in medium N (0.71%). The regeneration with the highest frequency was recorded in the medium C-R (0.14%), followed by MS (0.07%). There was no regeneration found on N culture medium.

To determine the effect of heat stress to anthers, six different durations of heat shock stress (3, 5, 7, 9, 11 and 13 d) were applied to anther of variety Bullet culturing on C-R medium for embryogenesis and regeneration. Heat shock treatment of anther cultivations from 3 to 13 days stimulated androgenesis response and showed the best results at 7 days. Heat shock stress for 7 days showed highest percentage of direct embryogenesis, callusogenesis, indirect organogenesis and regeneration as 5.28, 17.42, 1.26 and 2.99% respectively. Increasing the duration of heat shock by more than 7 days had a detrimental effect and suppressed microspore embryogenesis as 13 days decreased callusing dramatically.

Other attempts of this study were taken in **Exp. 5, Chapter-V** to investigate the influence of duration of low-temperature treatment and the genotypic effects on embryogenic responses as well as enhancement of regeneration efficiency of three chilli genotypes in Bangladesh. The anthers from three genotypes (Boltu, Bullet and Halda) were cultured on C-R induction medium and five cold stress durations (12, 24, 36, 48 and 60 h) were applied to induce cold stress to the anthers for better embryogenic response. In comparison of anther response from the three studied varieties, Bullet showed the highest percentage of direct embryogenesis (3.0%) followed by the variety Boltu (2.96%) when 24 h cold stress applied to anthers. No response of direct embryo production was found at 60 h cold stress pre-treatment to anthers. The anthers from genotype Halda showed the highest rate of indirect embryo frequency (1.12%) and callusing (22.63%), when treated with 24 h cold shock stress compared to other genotypes. Though the frequency of regenerants of all studied varieties were very low, both Bullet and Boltu showed highest regeneration (0.62%) when treated with 24 h cold shock stress. According to the results obtained from genotype effect, the highest percentage of direct embryo formation were found from the

variety Bullet (1.65%), followed by the variety Boltu (1.11%). In these two genotypes it was observed also the highest frequency of callusing as 15.24% and 13.28% respectively. The indirect organogenesis with the highest frequency was recorded in variety Bullet (0.49%), followed by the variety Halda (0.27%) and the indirect organogenesis with the highest frequency was recorded in variety Bullet (0.49%), followed by the variety Halda (0.27%). According to the results obtained from cold stress duration effect, the highest percentage direct embryo production was found from the duration period 24 h (2.95%), followed by the duration period 12 h (2.63%). Out of two durations the highest frequency of callusing was recorded 22.09% and 17.24% respectively. The indirect organogenesis with the highest frequency was recorded at 24 h cold stress duration (1.04%), followed by 12 h duration period (0.37%). No indirect organogenesis was found from 48 h and 60 h cold shock stresses to anthers. The regeneration with the highest frequency was recorded (0.62%) when anthers were pretreated with 24 h cold shock, followed by 12 h cold shock pre-treatment as 0.49%.

Throughout the above mentioned study results revealed that on the basis of various characteristics on callus induction, regeneration, physical and chemical stress pre-treatments factors that enhanced somatic and gametic embryogenesis. Some promising genotypes of chilli peppers has been undertaken to evaluate their efficiency on androgenetic (gametic embryogenesis) responses in Bangladesh. Here successfully screening out some chilli genotypes showed a better result than others. To develop high yielding and stress tolerant genotype through anther culture is an essential technique to build up a homozygous line. In this case anther culture procedures, optimization of media, plant growth regulators and culture conditions have been optimized nicely. Very less number of plants was obtained from anther derived embryoids and due to time limitation plants was not tested in their ploidy levels. However, very thin types of plants and less growth it could be imagined its haploid characteristics in nature. It was the first attempts

on anther culture studies in Bangladesh and for further advance research on anther culture and these protocols will be very much helpful for scientist in Bangladesh as well as in the world. Calli derived from the way of somatic and gametic embryogenesis and evaluation of their effects on various abiotic stress factors such as cold, heat shock and $AgNO_3$ to enhance callus induction and regeneration and the message is very much helpful for biotechnologist and plants breeders for further advance research on chilli peppers. Consequently, further research is needed to establish the anther and microspore culture procedure using more recent and local chilli genotypes in Bangladesh. The information obtained from this investigation should be considered in designing future breeding program also.

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	Division/Class
SSC	1981	Jessore Board	First
HSC	1984	Jessore Board	Second
B.Sc (Hons) in Botany	1988	University of Rajshahi	Second Class
M.Sc (Thesis group)	1989	University of Rajshahi	Second Class

C. List of Publications

i) Published articles (01)

1. Islam SMS, Islam T, Bhattacharjee B, **Mondal TK** and Sreeramanan Subramaniam S (2015). *In vitro* pseudobulb based micropropagation for mass development of *Cymbidium finlaysonianum* Lindl. Emir. J. Food, Agric. 27(6): 469-474.

ii) Abstracts (Conference/Workshop/Symposium)- 02

1. **Tushar Kanti Mondal**, Bakul Bhattacharjee, Touhidul Islam and S. M. Shahinul Islam (2014). Direct regeneration through cotyledonary nodal explants of chilli peppar (*Capsicum annuum* L.). Annual PTC&B Conf., March 28, Abst. 23, p 36.
2. M. Touhidul Islam, Bakul Bhattacharjee, **Tushar Kanti Mondal** and S. M. Shahinul Islam (2014). *In vitro* Plant regeneration in Soybean [*Glycine max* (L.) Merr.] from Cotyledonary and Nodal Explants. 7th Int. Plant Tiss. Cult. & Biotech. Conf., 1-3 March, 2014, Org. by BAPTC&B, University of Dhaka, Bangladesh. Abst. 24, p 24.

iii) MSc Thesis

1. Synecological Studies on Some Moist and Dry Habitat with their Natural Flora of Rajshahi University Campus. MSc Thesis, Supervised by Prof. Dr. M. Samsur Rahman, Dept. of Botany, University of Rajshahi, Bangladesh, Exam. 1989.