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Enhancement of Abiotic Stress Tolerance in Rice (Oryza sativa L.) through Androgenesis and Transgenic Approache

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ENHANCEMENT OF ABIOTIC STRESS TOLERANCE IN RICE (*ORYZA SATIVA* L.) THROUGH ANDROGENESIS AND TRANSGENIC APPROACHES

THESIS SUBMITTED FOR THE DEGREE
OF
DOCTOR OF PHILOSOPHY
IN THE
INSTITUTE OF BIOLOGICAL SCIENCES
UNIVERSITY OF RAJSHAHI
BANGLADESH

By

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JUNE 2019

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Dedicated
To
My Parents &
Daughters

DECLARATION

I hereby declare that the research work embodied in this thesis entitled **"Enhancement of Abiotic Stress Tolerance in Rice (*Oryza sativa* L.) through Androgenesis and Transgenic Approaches"** has been carried out by me for the degree of Doctor of Philosophy under the guidance of Professor Dr. Md. Asadul Islam and Dr. Md. Khalekuzzaman, Dept. of Genetic Engineering and Biotechnology, University of Rajshahi, Bangladesh; Dr. Narendra Tuteja, Ex-Senior Scientist and Head, Plant Molecular Biology (PMB) group, International Center for Genetic Engineering and Biotechnology (ICGEB), Aruna Asaf Ali Marg, New Delhi, India. I also declare that the result presented in the thesis is my own investigation and any part of it has not been submitted for any degree or diploma of this University or elsewhere.

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This is to certify that **ISRAT ARA** worked under our supervision as a PhD Fellow, Session 2012-2013, Institute of Biological Sciences (IBSc), University of Rajshahi, Bangladesh. It is our great pleasure to forward her thesis entitled **"Enhancement of Abiotic Stress Tolerance in Rice (*Oryza sativa* L.) through Androgenesis and Transgenic Approaches"** which is a bonafide record of research carried out at Plant Genetic Engeneering Laboratory, IBSc, RU, Bangladesh and PMB- Plant Molecular Biology, ICGEB, New Delhi, India. This work is original and has not been submitted so far in part or in full, for the award of any degree or diploma by any other institute in home or abroad. It is to be mentioned that **ISRAT ARA** has fulfilled all the requirements of the regulations of research for submission the thesis for the award of the degree of **Doctor of Philosophy**.

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Training Certificate and CV of Israt Ara

ACKNOWLEDGEMENTS

All my gratitude and acknowledge to the **Almighty ALLAH** and all respects to the **Holy Prophet Hazrat Muhammad (S)** who is the symbol of guidance and fountain of knowledge.

I am highly delighted to express my cordial gratitude, sincere appreciation and profound regard to my Ph.D Supervisors Professor Dr. Md. Asadul Islam and Professor Dr. Md. Khalekuzzaman, Dept. of Genetic Engineering and Biotechnology, University of Rajshahi, Bangladesh; Professor Dr. Narendra Tuteja, Ex-Senior Scientist and Head, Plant Molecular Biology (PMB) group, ICGEB-International Center for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi, India for their valuable guidance, generous advice, constructive discussions and criticisms throughout this study. I appreciate very much for his excellent co-operation in the presentation of findings and preparation of this dissertation.

I am very much grateful to the Director, Professor Dr. Md. Firoz Alam, Institute of Biological Sciences, University of Rajshahi for his valuable suggestions and cooperation of this study. My sincere thanks to all Ex-Directors of the IBSc for providing fellowship and other research facilities during my working period at IBSc, RU. Special thanks to Prof. Dr. KAM Shahadat Hossain Mondal (Rtd.), Prof. Dr. M A. Bari Miah (Rtd.), Prof. Dr. Md. Wahedul Islam, Prof. Dr. Parvez Hassan, Prof. Dr. S. M. Shahinul Islam, Dr. Md. Ariful Haque for their cordial co-operation, inspiration and support of this study. Special thanks to the Head of Plant Genetic Engineering Lab., for providing me lab and other facilities to continue the work at IBSc, RU. Thanks to all respective Officers and Staffs of IBSc, RU for their continuous support and co-operation for my study.

Grateful appreciation and thanks to my research colleagues Dr. Abu Baker Siddike, Dr. Bakul Bhattacharjee, Dr. Mozidul Haque, Dr. Jamilur Rahman, Dr. Hurun-or-Rashid, Dr. Enayetus Saklain, Mr. TK Mondal, Md. Munir Hossain, Touhidul Islam, Mirza Fida Hassan, Nazrul Islam, Zannatul Ferdous, Saika Kabir Nitu and other research fellows of this institute for their co-operation during my Ph.D work. Grateful thanks also to Prof. Dr. S. K Sopory, Dr. Renu Tuteja, Dr. MK Reddy, Dr. Tanushree Kaul, Dr. Huda, Dr. Sandeep, Dr. Nishat, Dr. Neha, Dr. Ranjan, Dr. Marjan, Dr. SS Gill, Dr. Maryam, Dr. Ananda, Dr. Jamil and others members in Plant Molecular Biology Group, ICGEB, New Delhi, India for their inspiration and cordial cooperation during my work. Financial support provided by the CRP-ICGEB grant (CRP/BGD 10-01 RG, 2011-2014) for training at ICGEB-New Delhi, India and other facilities of this work is gratefully acknowledged.

I owe a debt of thanks to my family: I don’t think there are words to express my gratitude for your love and support. Heartiest thanks to my beloved husband Dr. S. M. Shahinul Islam and my two daughters Lamisha Islam and Tanisha Islam for their love and for the joy they bring into my life. I feel, the credit of my achievements also goes to my mother, mother-in-laws, sisters and brother-in-laws for their cordial co-operation and continuous support during this research work. It is their unshakeable faith into me that will help me to proceed further.

At the end, I would like to apologize for all those whom I could not accommodate in this note, but I would like to express my heartfelt gratitude to all those who went unmentioned in this note of acknowledgement.

The Author

ABSTRACT

The work was mainly focused on *in vitro* somatic and gametic embryogenesis for crop improvement, application of various stress pre-treatments factors to enhance androgenetic responses and increasing chromosome doubling efficiency in rice. Anther and microspore derived embryoids were used for *Agrobacterium*-mediated transformation system. For somatic embryogenesis mature seeds of a total number of twenty indica rice genotypes were considered out of twenty three and cultured them in four different media (MS, N6, SK3 and B5). Out of four media, N6 along with 3.0 mg/l 2,4-D + BAP 1.5 mg/l + Kin 1.0 mg/l showed best callusing in BRRI dhan29. The variety BRRI dhan29 showed maximum rooting (64-90.67%) in RRM (rice rooting medium). For anther culture we evaluated twenty three rice cultivars with four induction media where almost genotypes showed more or less callusing in all media but the SK3 showed better than others. An experiment was conducted for suitable regeneration system and reducing albino plant production by early transferring methods. Results indicated that transfer of anthers with or without embryo like structure (ELS) from induction to regeneration medium posed a significant impact on improving regeneration potentiality and reducing albinisms.

Another experiment was conducted to evaluate the effect of cold pre-treatment duration (4°C) to harvested spikes (1-15 days). It was observed that three days cold pre-treatment showed highest ELS, GPR (green plant regeneration) and reducing albinisms. Besides cold pre-treatment as physical stress pre-treatment factors drought was applied to excised anthers. Here three hours drought stress pre-treatment proved to be the more suitable for ELS and GRP. It may be concluded that application of drought stress pre-treatment for three hours to excised anthers prior to culture in liquid medium is very effective for embryoids induction as well as green plant regeneration. Combined effects with drought and heat stress pre-treatments were also found and interesting results obtained with 3h drought + 1h heat stress at 27°C anthers that enhanced embryo yield and GRP in rice.

Three antimitotic agents (azetidine, colchicine and pronamide) was considered in addition to the induction medium and observed that out of three dosages 150 mg/l azetidine and colchicine showed better performance on chromosome doubling efficiency. But the colchicine showed the most effective chemicals for chromosome doubling than azetidine and pronamide. For

chemical stress pre-treatments factors as osmoticum agent e.g sorbitol, PEG and mannitol with different dosages were applied to anther culture medium. Results indicated that 0.3 mM mannitol showed highest embryo yield (43.06%). An interesting experiment was also conducted using liquid and semi-solid induction medium. Here we found three days incubation period of anthers with mannitol showed best for both induction and regeneration of rice anther culture. For overall mean value on ELS and GRP the 60 g/l mannitol showed best performance than others. In other study different dosages of mannitol + heat pre-treatment at 30°C-33°C was also applied. It was observed that pre-treatment at 33°C showed the best results on ELS (43.12%) and GRP (22.86%) that was 2-3 times higher than control.

For isolated microspore culture six isolation procedures were evaluated on the basis of embryo yield and regeneration. Out of six isolation procedure the glass rod was much easier and simplest where 65-123% of embryoids were found from 100 anthers. In another study excised anthers were pre-treated by different dosages of mannitol before microspore isolation. The 0.4 M mannitol showed best results on embryoids induction, green plant regeneration and reducing albino plants. For another study various concentration of mannitol (0.2 M - 0.8 M) was directly used in addition to the induction medium (AMC) of microspore culture and incubated them for 2, 4 and 6 days. It was observed that the incubation period at 4 days to rice floret showed significantly higher embryo yield than Control and others durations.

The transformation work was done using anther and microspore derived-embryoids using Bangladeshi rice genotype of BRRI dhan29. Out of 17 plants only two showed positive response on putatively transgenic plants. Those plants were analyzed by PCR with gene specific primers. The transgenics (T_0) showed tolerance to NaCl stress at 200 mM level with leaf disk senescence assay. *Agrobacterium*-mediated transformation was attempted and regeneration of stable green haploid plants obtained that was confirmed by flow cytometry analysis. Under this study we have standardized the anther and isolated microspore culture procedure, optimized suitable media for both anther and microspore culture, plant growth regulators for somatic and gametic embryogenesis, culture conditions, etc. We have standardized the transformation protocol for rapid development of drought and salinity stress tolerant DHs transgenics that is very important methods to develop transgenic homozygous lines shortly to mitigate the challenges of climate change for sustainable crop development.

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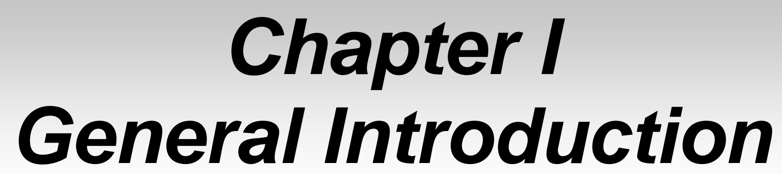
LIST OF ABBREVIATIONS

The following abbreviations have been used through the text.

ABA	:	Abscisic acid
AEC	:	Lysine S-2 aminoethyl L-cystein (analog of lysine)
$AgNO_3$	:	Silver nitrate
ANOVA	:	Analysis of variance
Azetidine	:	L-Azetidine-2-Carboxylacid (analog of proline)
BAP	:	6-Benzyl amino purine
bp	:	Base pair
BRRI	:	Bangladesh Rice Research Institute
CaMV	:	Cauliflower mosaic virus
cDNA	:	Complementary DNA
CGA	:	Gametocide
CH	:	Casein hydrolysate
cm	:	Centimetre
°C	:	Celsius
DH	:	Doubled haploids
DMRT	:	Duncan’s Multiple Range Test
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
dNTPs	:	Deoxynucleotide triphosphates
dS/m	:	deciSiemens per meter
EDTA	:	Ethylenediamine tetracetic acid
e.g.	:	exampli gratia = for example
et al.	:	Et alia = and others
etc.	:	et cetra = and other

GA3	:	Gibberellic acid
GFP	:	Green fluorescent protein
GUS	:	ß-glucuronidase
g/l	:	Gram per liter
HCl	:	Hydrochloric acid
$HgCl_2$	:	Mercuric chloride
IAA	:	Indole- 3-acetic acid
IBA	:	Indole-3- butyric acid
i.e	:	That is (to say)
IRRI	:	International Rice Research Institute
kb	:	Kilobase
Kin.	:	Kinetin
KOH	:	Potassium hydroxide
LB	:	Luria-Bertani medium
LS	:	Linsmaier and Skoog (1965) medium
LSD	:	Least Significance Difference
mg/l	:	Milligram per liter
mM	:	Millimole
MS	:	Murashige and Skoog (1962) medium
mRNA	:	Messenger ribonucleic acid
NAA	:	α-naphathalene acetic acid
NaOH	:	Sodium hydroxide
2-NHA	:	Hydroxy nicotinic acid
NptII	:	Neomycin phosphotransferase gene
N6	:	Chu et al. (1975) medium
OD	:	Optical density
PCR	:	Polymerase chain reaction

PGRs	:	Plant growth regulators
P^H	:	Negative logarithm of hydrogen ion (H^-) concentration
RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species
rpm	:	Revolutions per minute
Sig.	:	Significance
SE	:	Standard error/ Somatic embryogenesis
SK1	:	Basal medium Raina et al. (1989)
T-DNA	:	Transfer-DNA
Tris	:	Tris(Hydroxymethyl)aminomethane
w/v	:	Weight by volume
v/v	:	Volume by volume
viz.	:	Videlicet (L.); namely
0.1N	:	0.1 Normal solution
2,4-D	:	2,4-dichloro phenoxy acetic acid
%	:	Percent
µg	:	Micro gram
µl	:	Micro liter
µM	:	Micro mole



Chapter I
General Introduction

1. General Introduction

Rice (*Oryza sativa* L.) is a staple and major food crop and according to IRRI, Bangladesh is the fourth largets rice producing country in the world (IRRI 2016). Its cultivation playes a vital role in livelihood and maintenance of the huge population in Bangladesh. Rice is grown in three distinct seasons in Bangladesh: Aus (spring-summer), Aman (summer-fall), and Boro (winter-spring). A stable rice cultivation practice can ensure the food security for 135 million peoples in Bangladesh. More than 95% of population consumes rice and it alone provides 76% of calorie requirement of daily food intake in Bangladesh (Bhuiyan et al. 2002). In South-East Asia rice provides about 60% and in East and South Asia is about 35%. Peoples from Bangladesh, Cambodia, Indonesia, Laos, Myanmar, Thailand and Vietnam are taken highest calorie (55-80%) from rice and here yearly the highest level of consumption per capita is 130 - 180 kg. Rice contains low fat and protein compared with maize, wheat, barley and other cereal crops. Brown rice has a greater food value than white and it is a source of magnesium, thiamine, phosphorus, copper, zinc and vitamin-B6 and some varieties having iron, potassium and folic acid.

The most important sector of Bangladeshi economy in agriculture which contributes 19.60% to the national GDP and provides enrolement for 63% of the residents and the total arable land in Bangladesh is 61.2%. The United Nation's Population Division has estimated that the total population of Bangladesh will be 194.353 million in 2050 and the demand of rice will be 49.07 million tons which is about more than 30% compared to present rice production (Basak 2011). For rice production almost 10.5 million hectares land of this country are occupied which was stable over the past three decades (http://knowledgebank-brri.org). Three major types of rice varieties are cutivated traditionally by farmers in Bangladesh, those are Aman, Aus and Boro.

Bangladesh is a densely populated country and here the probability of increasing cultivable land is nearly zero. So, agricultural genetics is one of the easier parts for production of high yielding varieties and stress tolerant rice plants. The occurrence in rice sector happened

mainly due to genetic improvement in terms of high yielding rice varieties. But to cope up with the increasing population rate, rice production needs to be accelerated at a certain rate for ensuring food security in this country near future. In many countries rice production area is threatened due to high salinity and drought stress factors resulting in the failure to achieve self-sufficiency in food (Boyer 1982). Major biotic and abiotic stresses are adversely affect plant growth and productivity and trigger a series of morphological, physiological and biochemical changes in plants. However, there is a great need to exploit all genetic variability that can be used for stressful environments. Since plants are sessile, they have to perceive the changes in the environmental condition to survive and grow under stress conditions (Oleszczuk et al. 2006, Ochatt et al. 2009, Banu et al. 2014, Kalhori et al. 2017).

Global warming leads to various climate changes resulting in new abiotic stresses (drought, soil salinity, heat or high temperature, cold, heavy metal, water logging, wounding, ozone, etc) in many agriculturally used regions. In many countries, drought and soil salinity are the main contributors to food insecurity and poverty. Drought and salinity are becoming increasingly prevalent such that e.g. severe salinization is predicted to encompass more than 50% of all cultivable land by 2050 (Kasuga et al. 1999, Wang et al. 2003). To develop a suitable technology like biotechnology and genetic engineering that could be help to increase yield and productivity for sustainable agricultural and environmental development (Mahajan and Tuteja 2005, Purwoko et al. 2010, Kalhori et al. 2017). The application of biotechnology in combination with conventional breeding methods such as androgenetic technology for DHs production may help to increase food development and security in the world (Weeks et al. 1993, Otani et al. 2005, Shariatpanahi 2006a, Islam et al. 2013a).

It is to be mentioned that sustainable agricultural development and food security will not be achievable without new technologies for agricultural improvement such as genetic engineering and biotechnology. Plant embryogenesis is a process for the production of a fully developed embryo from a zygote after the fertilization of an ovule. Then the zygote

undergo various cellular divisions and differentiations to become a mature embryo and finally developed different organs of a plants e.g. shoot apical and root meristem, hypocotyl, root cap and cotyledons (Goldberg et al. 1994, Laux and Jürgens 1997, George et al. 2007*)*. Asexual reproduction through *in vivo* methods like parthenogenesis, adventitious embryos, somatic embryogenesis is also important for crop development. *In vitro* techniques like embryo culture (mature and immature) and gametic embryogenesis (DHs production) are also very efficient techniques for crop improvement. It's happen in plants in two important phases like morphogenesis and maturation. Somatic or gametic cells are able to differentiate into embryos *in vitro* following the application of plant growth regulators (PGRs) or various physical and chemical stress pre-treatment factors.

The *in vitro* somatic embryogenesis (SE) is the process where a plant embryo is developed from somatic cells (George et al. 2007). Major advances of somatic embryogenesis are- clonal propagation, elimination viruses causing diseases from plants, suitable explants source for advance biotechnological research, whole plants can be developed from single cells (e.g. protoplasts, microspores) and synthetic seed technology. A disadvantage of somatic embryogenesis is mutations, albino plant production. The *in vitro* production of doubled haploids through androgenesis (anther and microspore culture) is a powerful tool to generate homozygous lines much faster than conventional breeding methods. In case of conventional inbreeding procedures take 6-8 generations to achieve approximately complete homozygocity, whereas doubled haploidy it is one generation. Till now there are more than 200 species of angiosperms that produce haploid plants through *in vitro* androgenesis (Ma1uszyński et al. 2003, Dunwell 2010). In addition to crop improvement, DHs are an excellent source for gene mapping, cytogenetic research, and evolutionary studies (Campos et al. 2004). Evaluation of the DH lines and their hybrid products can provide an excellent opportunity to link phenotypic performance with the genotype. It has been reported that various abiotic stress pre-treatment factors may enhance somatic and gametic embryogenesis in cereal and higher crops also.

Haploid plants have the gametophyte number of chromosomes that is a single set of chromosomes in sporophyte. Niizeki and Oono (1968) was the first reporter who has done very successful in rice and culture and haploid plant production. Duncan and Heberle Bors (1976) reported that cold pre-treatment showed lows down degradation processes in the anther tissues thus protecting microspores from toxic compounds released in the decaying anthers.

Several researchers recognized the great advantage that a functional microspore transformation system would offer, and tried to transform microspores in several species such as barley (Jähne et al. 1994, Yao et al. 1997), tobacco (Stöger et al. 1995), and oil seed rape (Dormann et al. 1995 and 1998, Fukuoka et al. 1998) by micro-projectile bombardment and or *A. tumefaciens*-mediated methods. In this process, microspores become embryogenic by changing from the programmed gametophytic (pollen development) to sporophytic pathway (embryo development) and plant regeneration through *in vitro* culture.

Albinism is a great problem in androgenetic study by anther and microspore culture and for that some physiological factors may influence that need to be identified. To overcome the problem microspores needed a better environment so that the physiological limitations could be partially compensated for with readily available nutrient resources (Islam and Tuteja 2012). While stresses including reduced nutrient availability may be beneficial for the induction of androgenesis, some nutrients are needed for normal green plant formation at the very beginning of androgenesis induction (Islam 2013a).

Microspore embryogenesis is the most commonly used method and it is based on the ability of a single haploid cells. To dedifferentiate and regenerate microspore after being exposed to stresses like low (cold) or high temperatures (heat, starvation, drought), carbon starvation (osmotic stress) and as chemical (colchicines) may enhanced embryogenesis as well as regeneration (Islam 2010b, Islam and Tuteja 2012). Some stresses such as temperature treatments and carbon starvation have been used with success in many plant species whereas others such as colchicine had limited application in a few species (Saisingtong et al. 1996, Maraschin et al 2006, Islam 2010b).

Widely used stresses are cold, heat, carbon starvation and colchicine for the induction of microspore embryogenesis. As stress heat pre-treatment is usually carried out at 33°C to 37°C for a duration varying from several hours to several days, whereas cold treatment is carried out at 4°C - 10°C from some days to several weeks (Khatun et al. 2010 and 2012, Rukmini et al. 2013). Touraev et al. (2001) mentioned that incubation of microspores in media containing non-metabolisable carbon sources, i.e. in mannitol containing media enhanced gametic embryogenesis. Blakeslee and Avery (1937) were the first who made the sensational discovery of the induction of chromosome doubling by colchicine. The application of other antimitotic agents (trifuralin, nitrous oxide and oryzalin) in induction medium with various doses also showed very effective results on doubled haploid production. Colchicine is a commonly used microtubule-depolymerizing agent which actively binds to alpha and beta tubulin subunits of tubulin heterodimers of microtubules, thus, blocking the active sites of microtubules for binding to kinetochore protein (Shariatpanahi et al. 2006). Barnabás et al. (1996) reported the direct effect of colchicine on the mitotic divisions of microspores in culture by transmission electron microscopy and they found significant increase in the frequency of symmetrical divisions, induced by *in vitro* colchicine treatment. In addition, colchicine, a microtubule-depolymerizing agent, is being used as a stress pretreatment (Simmonds 1989, Zhao and Simmonds 1995, Saisingtong 1998). In maize colchicine pretreatment (250 mg/l) for 3-6 days produced the highest number of ELS-embryo like structures reported by Saisingtong et al. (1996). Burun and Emiroglu (2008) found that pretreatment of tobacco anther to 0.2% colchicine for one day resulted in the 60% diploidization of chromosomes. In maize the application of colchicine in the pretreatment media increased the number of fertile DH plants (Saisingtong et al. 1996, Antonie-Michard and Beckert 1997). As chemical stress pre-treatments factors by colchicine (250 mg/l) for 72 hrs in combination with physical stress factors as cold at 4ºC duration was effective in the production of doubled haploids (Motallebi-Azar and Panahadeh 2010).

Application of different stress pre-treatments factors may increase induction and regeneration efficiency in cereal crops (Touraev et al. 1997). Star-like microspores seem to be very effective on gametic embryogenesis reported in wheat and tobacco (Touraev et al. 1996 a and b, Touraev et al. 1997, Shariatpanahi et al. 2006). However, it was shown that depending upon the type of stress pre-treatment to explants (spikes, anthers or microspores) that showed symmetric and asymmetric first cell division that enhanced embryogenic development in wheat microspore culture (Hu and Kasha 1999). They also reported that the first division in embryogenic wheat microspores is usually symmetric when D-mannitol pre-treatment is used while it is asymmetric after cold pretreatment. Mannitol is the most suitable osmoticum for subsequent embryo development. Wojnarowiez et al. (2004) tested various dosages of sugars and mannitol at different steps on anther culture in barley to elucidate their influence on both the overall embryo yield and the structure of plastids in relation to albinism. They obtained best results using mannitol (364 mOsm/kg) and found 139.7 percentages of green plants per 100 plated anthers. Pre-treatment stresses with mannitol showed significant progress for microspore development in durum wheat (Slama-Ayed et al. 2010), rice (Raina and Irfan 1998) and maize (Pescitelli et al. 1990). Maraschin et al. (2005) reported that some genes are specifically expressed during embryogenic induction of microspores.

Microspore embryogenesis is an elegant system for genetic transformation and could provide a practical alternative for the production of transgenic doubled haploid plant species in which regeneration from somatic cells is difficult, especially in the recalcitrant cereals (Snape et al. 1986, Datta et al. 1990, Bikash and Mandal 2001, Kumlehn et al. 2004 and 2006, Karasawa et al. 2016). For genetic transformation of cereal crops have recalcitrant to recombinant techniques mainly because of problems in establishing regenerable cell and tissue cultures as well as efficient DNA delivery systems (Stöger et al. 1995, Obert et al. 2008, Shim et al. 2009, Chauhan and Khurana 2010 and 2011). This protocol may be used to overcome genotypic limitations of doubled haploid formation in cultivars that had previously been found to be recalcitrant in anther culture (Sopory and Munshi 1996, Zapata-Arias 1983a, Cegielska-Taras et al. 2008 and 2015).

Drought and high salinity stress causes the adverse effects on the plants growth and the development that resulted in significant economic losses worldwide and minimizing these losses is a major area of concern now. The responses to abiotic stresses are multigenic and the molecular mechanisms underlying these are not very well understood. Once the stress condition has been perceived, and signal transduction has been activated, downstream genes involved in response and tolerance mechanisms become activated. Overall, the physiologic response to the stress arises due to changes in cellular gene expression. Induction and repression of a number of genes are likely to play an integral role in stress acclimation or tolerance.

The RNA helicases are ubiquitous enzymes that catalyse the unwinding of energetically stable duplex RNA secondary structures. They play an essential role in basic cellular processes regulating plant growth and development, such as DNA replication, repair, recombination, transcription and translation (Tuteja 2003, Owttrim 2006, Vashist and Tuteja 2006). It seems therefore that helicase might be playing an important role in stabilizing growth in plants under stress by regulating stress-induced transcription and translation. A hallmark of most of the helicases (but not for all) is the existence of a set of highly conserved amino acid sequences called 'helicase motifs', which are clustered together for helicase function. Because of the presence of a DEAD motif, the family of these proteins is also called as DEAD box family of proteins. The *p68* (Ddx5) gene are one of the prototypic members of the DEAD box family of proteins and were one of the first proteins to be shown to exhibit RNA helicase activity *in vitro*. Expression of p68 was shown to be growth and developmentally regulated and to correlate with organ maturation and differentiation. Overall, *p68* is a multifunctional protein involved in RNA splicing, pre-rRNA processing, RNA-induced silencing, transcription initiation, transcriptional repressor, etc. However, very little is known about *p68* protein in plant system. The role of *p68* in stress tolerance has not been reported so far for other crops. In an attempt to permit growth of plants in drought and/or high salinity stress, genetic transformation will be carried out with stress-induced *p68* gene(s) using Bangladeshi rice cultivars combination

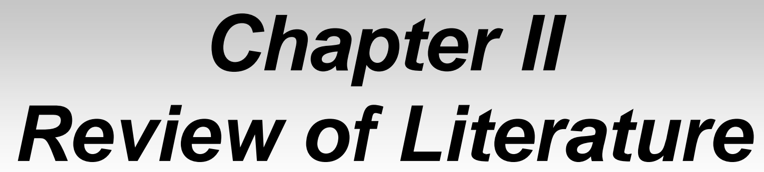
with androgenetic study. Under this study biotechnological approaches such as androgenesis and transgenic techniques have applied to ensure sustainable development and eco-friendly sustainable agriculture crops. An important strategy have been undertaken to develop homozygous fertile transgenic plants rapidly with enhance tolerant to drought and salinity. To resolve the urgent global issues, biotechnological research and the development of abiotic stress tolerant crops are very important for agricultural development. Here the targeted gene were transformd using callus derived from seeds and also anther and microspore derived-embryoids using Bangladeshi rice cultivars. The work mainly focused on the following objectives:

i) *In vitro* somatic (seed and embryo culture) and gametic embryogenesis (anther and microspore culture) for crop improvement.

ii) Application of various stress pre-treatments factors to enhance androgenetic responses and increasing doubled haploid (DH) plants in rice.

iii) To develop highly efficient gametic embryogenic system of getting haploid or doubled haploid plants through androgenesis (anther or microspore culture) using elite rice genotypes in Bangladesh.

iv) To develop anther and microspore derived embryoids for *Agrobacterium*-mediated transformation system in rice.



Chapter II
Review of Literature

2. Review of Literature

2.1 Background of rice

Rice (*Oryza sativa* L.) is one of the most important cereals crop worldwide and specially in Bangladesh. It is a monocotyledonous flowering plant of the family Poaceae providing the principal food source for half of the world's population. Rice (2n = 2X = AA = 24) is diploid and having chromosome number of 12, containing 370 Mb with 30,000 protein-coding genes. It is also having a size of 0.4 Gb, its genome is 40 times smaller than that of bread wheat (Ayres and Park 1994). Arumuganathan and Earle (1991) reported that rice is an important cereal crops and the first whole genome sequencing have been done.

Rice improvement has been conducted in what is now Bangladesh since 1911, when an economic botanist was appointed to do rice selection work at the Dhaka Research Station in East Bengal, India (Dalrymple 1986). In 1965 a set of 303 IRRI varieties and lines was brought to Bangladesh for an accelerated rice production program sponsored by the Ford Foundation. By 1972 the following varieties had been identified and recommended for farm production: IR5, IR8, IR20 (Irrisail), Chandina (IR532-1-176), Mala (IR272-4-1-2), and Purbachi (Chen-chu-ai from China). 11 Chandina and Mala subsequently were named BR1 and BR2. The Bangladesh Rice Research Institute (BRRI) was established in 1970 to develop varieties better suited to local growing conditions. BRRI introduced, in addition to BR1 and BR2: BR3. Biplab; BR4, Brrisail; BR6, IR28; BR7, BRRI Balam (IR2053-87-3-1); BR8, Asha; BR9, Sufala; BR10, Progati; and BR11, Mukta. Varieties scheduled for release in 1983 were: BR12, Monyna; BR14, Gazi; BR15, Mohini (IR2071-199-3-6); and BR16, Shahibalam (IR2793-80-1). BR5 and BR13 apparently have not been released. All releases selected from an IRRI line have IRRI ancestry (Dalrymple 1986).

Oryza sativa, commonly known as Asian rice, is the plant species most commonly referred to in English as rice. It is renowned for being easy to genetically modify, and is a model organism for cereal biology. *Oryza sativa* contains two major subspecies: the sticky, short-

grained *japonica or sinica* variety, and the nonsticky, long-grained *indica rice* (ja) variety. *Japonica* varieties are usually cultivated in dry fields, in temperate East Asia, upland areas of Southeast Asia, and high elevations in South Asia, while *indica* varieties are mainly lowland rices, grown mostly submerged, throughout tropical Asia.

The major achievements of BRRI has been the development of 72 high yielding modern variety (MV) along with six hybrid rice variety strains adaptable to different ecosystems since its establishment. Thirteen of these MV rice are suitable for cultivation in both the Boro and Aush seasons, seven in the Boro season and five in the Aus season, and twelve in the transplanted Aman (T Aman) season. With appropriate management, and under favorable soil conditions, these MV rice may yield 5-6 m tons/ha in the Boro, 3-4 m tons/ha in the Aus, and 4-5 m tons/ha in the T Aman seasons.

For rice improvement in Bangladesh the BRRI is working very sincerely and their varieties cover more than 80% of rice area and account for about 91% of the total annual rice production of the country. The BRRI developed modern varieties that covered 90% of winter rice (Boro), 25-30% of summer (Aus), and 50-55% of Aman (wet season) areas in Bangladesh. These varieties together cover 56% of the total rice area and account for about 74% of the total annual rice production of the country (BRRI 2016-2017).

Bangladesh is an agro-based developing country where rice is grown in three distinct seasons, namely Boro (January - June), Aus (April - August), and Aman (August - December) covering almost 11.0 million hectares of land, producing nearly 95% of the total food requirements. Our cultivable land is very insufficient, so that it is not possible to ensure food security without development of high yielding rice varieties. So why conventional breeding system and an emerging technologies based on molecular biology and genetic engineering technoloy is required to improve the productivity in the coming decades.

2.2 Taxonomy and classification of rice

The genus *Oryza* was named by Linnaeus (1753 a & b). According to Integrated Taxonomic Information System (ITIS 2006), the taxonomic position of rice is mentioned bellow:

Taxon		Name of taxon
Kingdom	:	Plantae
Sub-kingdom	:	Viridiplantae
Infra-kingdom	:	Streptophyta
Super-division	:	Embryophyta
Division	:	Tracheophyta
Sub-division	:	Spermatophytina
Class	:	Magnoliopsida
Super-order	:	Lilianae
Order	:	Poales
Family	:	Poaceae
Genus	:	*Oryza* L.
Species	:	*Oryza sativa* L.

Oryza sativa L. Taxonomic Serial No. (TSN): 41976 http://www.itis.gov/servlet).

2.3 Rice genomes

The genomes size of rice (*Oryza sativa* L. japonica subspecies) is 420 Mb; and suggested the genome contain 32000 to 50000 genes reported by Goff et al. (2002). IRGSP (2005) mentioned that the size of rice genome is one sixth of the maize genome and 40 times smaller than the wheat genome. The species of the genus *Oryza* was grouped according to the compatibility of their genomes. *Oryza sativa* has an AA-type genome, which means

that its chromosomes can pair correctly at meiosis with other AA-type species (Vaughan et al. 2003). By definition, gene flow through conventional sexual hybridization is limited to *O. sativa* varieties and to the AA-type genome species within this genus. The other AA-type rice genome species are- *O. glaberrima*, *O. barthii, O. glumaepatula, O. longistaminata, O. meridionali, O. nivara* and *O. rufipogon.* The other BB/BBCC type rice genomes are *O. punctata, O. malampuzhaensis* and *O. minuta*. The CC and CCDD type rice genomes are- *O. eichingeri, O. officinalis, O. rhizomatis, O. alta, O. grandiglumis* and *O. latifolia.* Other rice genome species are (*O. australiensis*- EE type), (*O. brachyantha*- FF type), (*O. granulata* and *O. meyeriana*- GG type) and (*O. longiglumis* and *O. ridleyi*- HHJJ type).

2.4 *In vitro* development of rice

2.4.1 Somatic embryogenesis (SE)

Somatic embryogenesis is an important tool for plant cloning, looking toward the obtaining of improved plants by cell suspension culture or protoplast fusion. Suspension of plant cell cultures has several uses and applications for improving agronomical traits, and it is widely used in biotechnology for micropropagation, for the production of secondary metabolites or other substances, for obtaining somatic hybrids through protoplast fusion, and for modifying plants through genetic transformation (Gniech-Karasawa 2017). **Table 1** shows the *in vivo* and *in vitro* asexual embryogenesis (parthenogenesis, adventitious embryos, somatic embryogenesis) that is a powerful tool for mass production of artificial embryos (Steward et al. 1958).

Table 1: The processes of *in vivo* and *in vitro* asexual embryogenesis

Type of embryogenesis	Precursor cell	Mode of embryogenesis	Ploidy of embryo	Biological environment
Zygotic	Egg	Sexual	2n	*In vivo*
Parthenogenesis	Egg	Asexual	2n	*In vivo*
Adventitious embryos	Nucellar cell	Asexual	2n	*In vivo*
Somatic embryogenesis	Somatic cell	Asexual	2n	*In vivo/ in vitro*
Gametic embryogenesis	Egg/Sperm	Asexual	n	*In vitro*

Ref. Germanà and Lambardi (2016), n = haploid, 2n = diploid.

Success in breeding for better adapted varieties to abiotic stresses depend upon the concerted efforts by various research domains including plant and cell physiology, molecular biology, genetics, and breeding. Use of modern molecular biology tools for elucidating the control mechanisms of abiotic stress tolerance, and for engineering stress tolerant crops is based on the expression of specific stress-related genes (Amudha and Balasubramani 2011). There are many methods of crop improvement e.g. cross breeding; mutation assisted breeding (gamma radiation, X-rays); chemical mutagen; genetic engineering/transgenics approaches; molecular biology; molecular assisted breeding; *in vitro* techniques- doubled haploid (DHs), somatic embryogenesis, micro propagation, etc.

The International Rice Research Institute (IRRI) is employing *in vitro* tissue culture techniques to develop rice varieties (Bajaj and Rajam 1996). Regeneration from callus was achieved long back in japonica varieties (Nishi et al. 1973). The totipotent character of plant cells that retains its nucleus has the ability to regenerate entire new plant by somatic embryogenesis (SE) or organogenesis (Fortes and Pais 2000). Protocol of SE has been

reported to regenerate plants *in vitro* for indica rice (Wang et al. 1987, Rance et al. 1994, Seraj et al. 1997, Islam et al. 2005, Khalequzzaman et al. 2005, Makerly et al. 2012, Haque et al. 2013, Mostafiz et al. 2018) and also for japonica rice (Lee et al. 2002, Sah et al. 2014). Some author have experimented the potentiality of Bangladeshi indica rice for plant regeneration via SE (Hoque et al. 2007, Haque et al. 2013, Zinnah et al. 2013, Fook et al. 2015, Hossain et al. 2015, Din et al. 2016). Siddique et al. (2014) reported that Bangladeshi indica rice *viz*. BR10, BRRI dhan32 and BRRI dhan47 with high frequency through somatic embryogenesis. Furthermore, Bangladeshi indica rice has also been regenerated through anther and microspore culture (Khatun et al. 2010, Islam and Tuteja 2012). Regeneration of *in vitro* plants has been successfully done through organogenesis and somatic embryogenesis in rice (Sahrawat and Chand 2001, Khatun et al. 2003, Ramesh et al. 2009, Ghobeishavi et al. 2014, Vennapusa et al. 2015, Sankepally and Singh 2016, Yaqoob et al. 2016, Repalli et al. 2017).

Regeneration efficiency is affected by a number of factors including the genotype, the type and physiological status of the explants, media composition, plant growth regulators (PGRs) and culture conditions (Rueb et al. 1994, Mostafiz et al. 2018). Efficient plant regeneration has been widely reported in japonica and indica rice by optimizing several factors influenced such as sugars (Geng et al. 2008, Feng et al. 2011), amino acids (Pons et al. 2000), micronutrients (Rueb et al. 1994) and hormones (Zuraida et al. 2011). Aananthi et al. (2010) tested two basal media MS and N6 for induction in five indica rice cultivars *viz.*, ASD 16, White Ponni, Pusa Basmati 1, Pusa Sugandh 4 and 5. Till now reports on *in vitro* plant regeneration as well as on advance biotechnological research in Bangladeshi indica rice is limited. However, due to various abiotic stresses like drought, submergence tolerance and salinity, production of rice is decreased; therefore, development of abiotic stress-tolerant rice cultivars is the main priority (Grover and Minhas 2000). Against the increasing population, production of rice at the rate of our expectation is hampered due to many threats around the environment, such as drought, salinity, heat, cold etc.

Rice is a staple and major cereal crop in Bangladesh and also in the world. Many countries rice production area is threatened due to high salinity and drought (abiotic) stress factors resulting in the failure to achieve self-sufficiency in food (Boyer 1982). However, there is a great need to exploit all genetic variability that can be used for stressful environments. Biotechnologies could provide us with useful tools in sectors such as agriculture, fisheries, food production and industry (Purwoko et al. 2010). Crop production will have to cope with rapid increasing demand while ensuring environmental sustainability in the world and also in Bangladesh. The application of biotechnology in combination with conventional breeding methods may help to increase food production properly (Datta et al. 1990, Stöger et al. 1995). The *in vitro* production of doubled haploid plants through androgenesis (anther and microspore culture) is an efficient system for the production of fully homozygous plants rapidly (Fukuoka et al. 1998, Bikash and Mandal 2001). Traditionally, plant breeders can achieve homozygosity by using self-fertilization or backcrossing, which is a time consuming process (Snape et al. 1986, Sopory and Munshi 1996, Pauk et al. 2003).

2.4.2 Gametic embryogenesis (GE)

Gametic embryogenesis is a convenient alternative in plant breeding because it makes possible the development of homozygous lines, increasing efficiency and speed in conventional breeding programs (Gniech-Karasawa 2017). 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.

2.4.2.1 The induction of haploids

Haploids may be grouped into two broad categories: i) monoploids (monohaploids) - which possess half the number of chromosomes from a diploid species, and ii) polyhaploids (gametophytic set) - which possess half the number of chromosomes from a

polyploid species. The general term 'haploid' is applied to any plant originating from a sporophyte and containing half the number of chromosome (single set of chromosomes). However, till now various approaches such as parthenogenesis, genomic elimination, gynogenesis and androgenesis are employed in order to obtain haploid plants in many cereal and other crop plants.

2.4.2.1.1 Parthenogenesis

Through ovary or ovule culture that produced haploids (n) as an alternative way where the female megaspore, rather than the male microspore, is induced to develop into an entire plant is called parthenogenesis. It has been possible to induce parthenogenesis by *in vitro* culture of unpollinated ovaries and ovules (Yang and Zhou 1982). Attempts have been made to culture unfertilized ovules of ovaries in different plants species but growth of most of them stopped at the callus stages; only some crop species developed into haploid green plants namely barley, wheat, maize, sugar beet, apple, rose, gerbera, carnation, onion, tef, *Cucurbita pepo* L., mandarin orange, niger- *Guizotia abyssinica*, cucumber and cotton.

2.4.2.1.2 Genome elimination

Genome elimination occurs in intergeneric and interspecific crosses due to selective elimination of one of the parental genomes during the process of development after fertilization (Gernard et al. 2005). Consequently, the embryo is formed with only one genome and the plant arising from such embryo is expected to be a haploid e.g. crosses between *Hordeum vulgare* and *H. bulbosum* results in the production of haploid *H. vulgare* (Jensen 1977). In wheat, doubled haploids were produced through wheat × maize crosses (Comeau et al. 1992, Lefebvre and Devaux 1996), with emphasis on the time of pollination and age of embryos.

2.4.2.1.3 Gynogenesis

Development of haploid plants from the female gametophyte by culture of unfertilized ovules or ovaries is called gynogenesis. Since the first report of gynogenesis in barley by San Noeum (1976), this approach has been successfully applied to raise haploid plants of

Helianthus annuus, Beata vulgaries, Nicotiana tabacum, Oryza sativa, Triticum aestivum, and *Zea mays* (Yang and Zhou 1982, Yang et al. 1986, Zhou et al. 1986). *In vitro* induction of haploid plants from unpollinated ovules has been applied to several other crops and considered to be an alternative to anther culture technique in cases where anther culture has not been successful (Yang and Zhou 1982). Very recently El-Mahrouk et al. (2018) reported *in vitro* regeneration and production of haploid plants through ovule cultures and identification of the regenerated haploids using flow cytometry in black cumin (*Nigella sativa*). The reported results can facilitate breeding programs of black cumin in which haploids can be produced in a short time frame to generate homozygous lines. The introduction of *in vitro* techniques for the induction of androgenesis or gynogenesis has significantly facilitated the production of doubled haploids (DHs) as a support to plant breeding programmes, resulting in the early release of varieties.

2.4.2.1.4 Androgenesis

Haploid plants derived from anther or isolated microspore culture is termed androgenesis. Androgenesis in flowering plants is a unique biological process (**Fig. 1 & 2**). Androgenesis is defined as a development route, alternative to zygotic embryogenesis whereby a haploid individual is obtained from a male derived haploid (reduced) nucleus. The *in vitro* production of doubled haploids (DHs) through androgenesis is an efficient method for production of fully homozygous lines rapidly and this plant is very useful in the field of plant biotechnology and breeding (Redha and Islam 2010). Use of unconventional techniques such as doubled haploid lines through androgenesis is becomming more useful in the speeding up of the application of conventional breeding programme (**Fig. 1**). However, genotype dependency is a critical factor for androgenetic study in cereal and other crops (Seraj et al. 1997, Islam et al. 2001, Islam et al. 2013b).

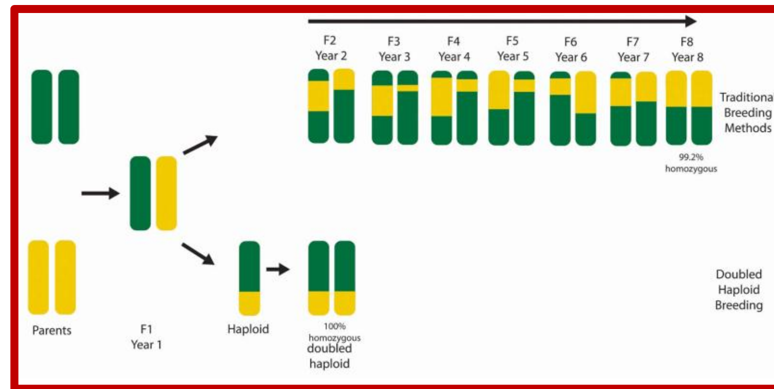


Fig. 1: In comparison of conventional breeding methods and doubled haploid breeding for pure line development (Figure adapted from Colodra wheat 2013, https://coloradowheat.org).

Androgenesis rarely occurs in nature, but is relatively easy to induce several plant species under *in vitro* conditions (**Fig. 2**). Till now there are more than 200 species of angiosperms that produce haploid plants through *in vitro* androgenesis (Ma1uszyński et al. 2003, Dunwell 2010). Through androgenesis complete homozygous plants can be produced within a year as compared to the conventional lengthy inbreeding methods, which may take 5-10 years. Significant advantage of androgenesis systems are is that these systems not only speed up the process of homozygosity, but also increase the selection efficiency (Germanà 2011).

Last few years there are some report on doubled haploid production in many economically important crops such as rice (Reddy et al. 1985, Suriyan et al. 2009), sunflower (Coumans and Zhong 1995), *Nicotiana* (Touraev et al. 1996a), apple (Höfer 1999), *Corchorus* (Ali and Jones 2000), rye (Guo and Pulli 2000), wheat (Kunz et al. 2000, Cistué et al. 2009, Slama-Ayed et al. 2010), asparagus (Wolyn and Nichols 2003), maize (Obert and Barnabás 2004), flax (Obert et al. 2004 and 2009), *Brassica* (Abdollahi et al. 2007), *Datura* (Iqbal and Wijesekara 2007), tomato (Seguí-Simarro and Nuez 2007), barley (Shim et al. 2009), *Capsicum* (Lantos et al. 2009), *Medicago* (Ochatt et al. 2009), oat (Sidhu and Davis 2009), carrot (Górecka et al. 2010), Horse chestnut (Dragosavac et al. 2010). Till now androgenic lines through anther and microspore culture have been developed in a number of agricultural crops (Sopory and Munshi 1996). Novel androclones of rice developed through anther culture, which showed higher quality and more yield as reported by Chen et al. (2001).

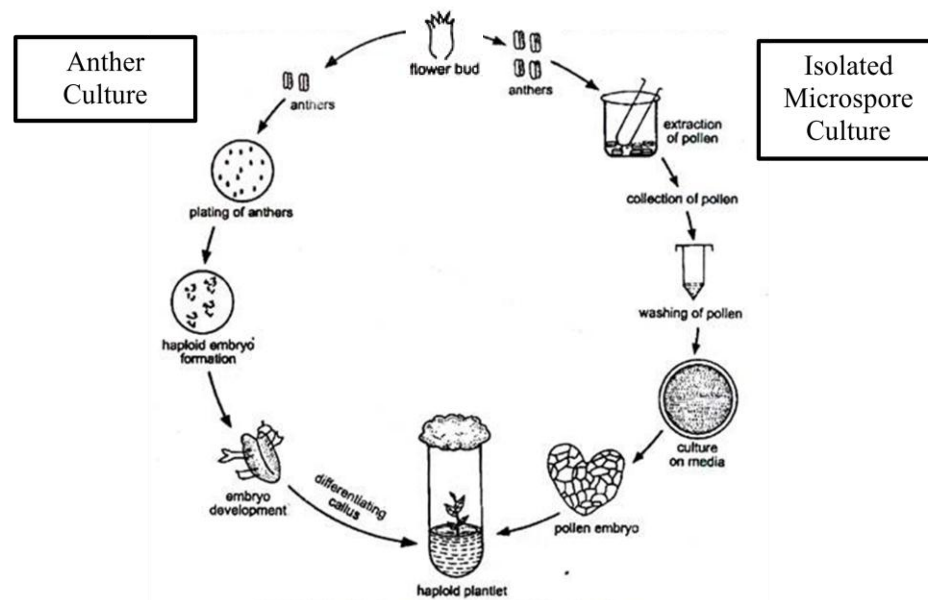


Fig. 2: Androgenesis (anther and microspore culture) process in plant species (http://www.biologydiscussion.com/essay/plant-breeding-essay/essay-on-plant-tissue-culture-history-methods-and-application/17639).

2.4.2.1.4.1 Anther culture (AC)

In the case of anther culture, plants may originate from anther somatic tissue other than microspores. Haploids obtained with anther culture are very potential breeding material in crop improvement (Collins and Genovesi 1981). Anther culture allows a rapid production of appropriate genotypes for breeding purposes in an effort to identify promising homozygous lines within shortening of time (Redha et al. 2000, Lazaridou et al. 2005, Islam et al. 2013b). Production of haploid plants in rice through anther culture was first reported by Niizeki and Oono (1968). Spectacular progress has been made in rice anther culture in the recent past (Afza et al. 2000, Ramakrishnan et al. 2005, Roy and Mandal 2006, Wijesekera et al. 2007, Bagheri and Jelodar 2008, Niroula and Bimb 2009, Pauk and Simon-Kiss 2009, Suriyan et al. 2009, Gueye and Ndir 2010, Khatun et al. 2010, Naik et al. 2017).

The success in the anther culture depends on first genotype and then duration of cold pretreatment, genotypes and growth medium (Ponitka and Ślusarkiewicz-Jarzina 2007, Niroula and Bimb 2009, Kahrizi et al. 2011, Ślusarkiewicz-Jarzina and Ponitka 2015). It appears that optimum media and pretreatment conditions vary from genotype to genotype.

Other important factor appears to be the cold pretreatment to androgenesis in the development of callus from anthers (Oleszczuk et al. 2006, Żur et al. 2009, Islam and Tuteja 2012). In cereals, production of haploids through anther culture has major obstacle with low regeneration rate and albino plant production. However, application of different methods and treatments may improve induction and regeneration efficiency. Ball et al. (1992) mentioned that the anther culture process is still under strong genetic control, but improved analyses of the functions of media components may reduce differential responses and improve efficiency of culture methods.

2.4.2.1.4.2 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 (Jähne and Lörz 1995). 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 (Guo and Pulli 2000, Islam et al. 2013b). 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 (Coumans and Zhang 1995, Ferrie et al. 2011). Microspore embryogenesis is an important technique also for genetic transformation and could provide a practical alternative for the production of doubled haploid plant species in which regeneration from somatic cells is difficult, especially in the recalcitrant cereals (Islam and Tuteja 2012). It has been reported that most of the genotypes, the production of embryos per anther obtained in isolated microspore culture is generally higher than in anther culture (Islam 2010b).

For most of the genotypes, the production of embryos per anther obtained in isolated microspore culture is generally higher than in anther culture (Islam et al. 2013b). Selection of single cells is more preferable than the multicellular structures. Anther culture, as technically simplest method to carry out DH production, is used for breeding purposes in

some species. However, the isolated microspore offers the following advantages compared to anther culture on some aspects are mentioned below:

i) Gametic origin: Isolated microspore culture avoids (fusion of microspores could occur) potential confusion regarding the origin of the obtained plant since it is a single cell system.

ii) *In vitro* selection: Without the anther wall interaction, microspores are more amenable to research because they can be directly exposed to selection treatments (cell sorting).

iii) Mutagenic studies: Microspore is ideal for up-taking mutagenic agents, as microspore may be evenly exposed to chemicals or physical mutagens.

iv) Response: In vegetable crops most genotypes responded better to isolated microspore culture and embryo yield is generally higher than anther culture (Cao et al. 1994 and 1995, Chuong and Beversdorf 1985).

v) Transformation: Isolated microspores are very promising targets for genetic manipulation, because they are uninucleate and can be regenerated to homozygous plants providing less chance for chimera formation.

vi) Observation: Microspores *in vitro* developmental pattern can be followed easily by visual observation.

vii) Statistics: In isolated microspore culture the microspore population can be distributed to several dishes, with each dish receiving a similar homogenous microspore fraction. In anther culture, such a randomized distribution is difficult to realize.

2.5 Advantages of androgenetic/haploids and doubled haploids (DHs) research

A stable homozygous (or as much as possible) plant is defined as a true breeding line. Haploids provide beneficial tools for plant breeding and for genetic studies. Haploid production is attractive because it can only provide an opportunity to select at the haploid

level *in vitro* for desirable agronomic traits and seed quality characteristic, but also to provide a means of producing genetically stable homozygous lines, fixed by chromosome doubling (Kott and Beversdorf 1990). Haploids possess only one set of alleles at each locus, so it is possible for recessive genes/mutants to be detected. The recessive traits are easily expressed at the haploid level, which facilitates the *in vitro* selection of recessive monogenic mutants, and is valuable for mutation breeding (Attanasov et al. 1995).

The main advantage of using doubled haploid techniques is the rapid homozygosity of the descendants, results a time saving procedure for the development of new varieties (Islam 2010a, Rukmini et al. 2013). Till now some rice cultivars namely Tanfeng 1 (*O. sativa*), Hu Yu No. 1 and No. 2 (*O. sativa*), and Xin Xiu (*O. sativa*) (Yin et al. 1976, Hu and Zeng 1984) has been developed. Zatapa-Arias (2003) developed new rice varieties that originated from anther derive embryoids and exhibited higher quality and yield.

Androgenetic system is also an elegant system for genetic transformation (Stöger et al. 1995, Kunz et al. 2000), and could provide a practical alternative for the production of transgenic plants (GMOs) in species in which regeneration from somatic cells are difficult or not possible, especially in the recalcitrant cereals (Touraev et al. 1996a). Microspore derived embryos have been used for example in studies of lipid biosynthesis and storage (Taylor et al. 1993). Under this study various stresses have been applied to enhanced androgenetic responses and rapid development of double haploid plant by anther and microspore culture.

2.6 Disadvantages and problems associated with of androgenetic research

2.6.1. Genotype dependency

Genotype of donor plants plays an impotent role in callus induction in rice anther culture (Lee et al. 2004). Culture media also play key role in induction and development of doubled haploid plantlets (Bishnoi et al. 2000). Literature reveals allow the choice of any anther culture medium for general use i.e culture response is highly genotype and media specific.

2.6.2 Mixoploidy

Mixoploidy is a great problem also for androgentic studies specially in cereal crops. It is often encountered in androgenetic research is the emergence of plants of mixed ploidy due to the fusion of haploid nuclei at the initial stages of androgenesis (Sangwan-Norreel 1983). De Paepe et al. (1983) growth depression effects and the appearance of abnormalities with consecutive cycles of androgenesis have been observed in *Nicotiana sylvestris*. This is accompanied by the increase in total DNA and increasing proportion of highly repeated DNA sequences (Dhillon et al. 1983).

2.6.3 Effect of albinism

For androgenetic research still a great lacking of embryo induction and high percentage of albinos among regenerated plants (Islam 2010c). Albino plants cannot survive in nature and it has no agronomic value and, consequently, warrant studies to increase the frequency of green plants in anther cultures of cereals. Albino plants are very often produced during the regeneration of microspore-derived plants in cereals (Olmedilla 2010). The frequency of albinos may vary from 5-100% in rice anther culture (Talebi et al. 2007). Till many researchers have done on several experiments in different aspects for influencing of androgenesis. But there is not enough report on rice and some other crops about the suitable microspore isolation procedure. This work has been undertaken to develop an efficient embryoids induction and regeneration methods using different isolation techniques of microspore using Bangladeshi rice cultivars.

2.7 Enhancement of androgenesis by various abiotic stress pre-treatment factors

Some stress agents had a positive effect on androgenesis from the treated microspores (**Table 2**). To develop varieties resistant to abiotic and biotic stresses through conventional breeding is being employed for many crop plants, but the progress is still very slow. Abiotic stress enhances androgenesis from isolated microspores of some legume species recently reported by Ochatt et al. (2009). This method offers unusual potential for reducing the time required for the production of homozygous progeny. Application of different

stress pretreatment such as heat, starvation, pH, heavy metal, ethanol, osmotic stress, gamma irradiation, hypertonic shock, feminizing agents, centrifugal treatment, ABA and atmospheric pressure enhanced androgenetic response mainly in cereal and other crops.

Table 2: Application of different stress pre-treatment factors to rice for enhancement of androgenesis

Abiotic stress pre-treatments factors	Microspore stages	References
Cold	Mid UC- early BC	Zatapa-Arias (2003)
Heat shock	Late UC	Xie et al. (1997)
Starvation (sugar)	Late UC	Raina and Irfan (1998), Ogawa (1994)
CHA-Feminizing agents	Late UC-early BC	Hu et al. (1978)
Gamma irradiation	Mid-late UC	Zhao et al. (1982)

UC: uni-cellular, BC: bi-cellular.

Touraev et al. (1996b) mentioned that during the plant life cycle stress in general seems to be common trigger for phase change. First, stressed microspores undergo a transition towards a more dedifferentiated state. Second, the degradation of cellular components and certain proteins takes place and is considered a prerequisite for developmental re-programming in many organisms (Maraschin et al. 2005). Third, in tobacco and rapeseed the stress-treated microspores undergo a cell cycle arrest, which is relieved when microspores are transferred to non-stress conditions (Binarova et al. 1997).

Stress acts as a trigger to induce microspore embryogenesis and represents a key point where the reprogramming of microspores takes place. Some reports described that stress pretreatment is not only needed for switching the development fate, but also influence of divisions and embryos, green plant production and spontaneous doubling efficiency (Kasha et al. 2001, Oleszczuk et al. 2006).

2.7.1 Physical stress pre-treatment factors

Some physical stress factors i.e. cold, heat shock, drought, starvation, gamma radiation, osmotic pressure, photoperiod, temperature, supply of water and nutrients growth regulators and pesticides applying to donor plants have a certain influence on the induction of androgenesis reported by Bajaj (1983). Those pre-treatments factors have a great influence in androgenesis to produce a high number of embryos and green plantlets. It was reported that high light intensity before meiosis and low temperature, which lengthen the vegetative period, can enhance the induction of androgenesis.

2.7.1.1. Cold

In some cases, especially after low temperature pretreatment, symmetrically dividing microspores were found to be the main sources of androgenic haploids in rice (Reddy et al. 1985). It was reported that low temperature (12-18ºC) treatments favour explants survival for long period and resulting in a more homogenously developed population of microspores (Duncan and Heberle-Bors 1976, Jähne and Lörz 1999).

Lazar et al. (1985) showed that variation in intensity and duration in cold pretreatment lead to significant differences in callus formation frequency. Ouyang et al. (1987) concluded that the most suitable culture temperature for anther culture of field-grown material was about 2°C higher than that of the greenhouse-grown material, no matter what genotype was used. They also noticed that the anthers of the greenhouse grown material did not develop as well as those of the field grown material. Marsolais and Kasha (1983) also reported that cold pretreatment is not always necessary to obtain the higher percentages of plants derived from anthers or isolated microspores.

Pande (1997) observed that cold pre-treatment was found to be essential for androgenesis in anther culture of indica cv. IR43, and 10°C for 10 days was most conducive environment for maximum culture response. He mentioned that pretreatments longer than 11 days resulted in albino production. However, regeneration of green plants is the most

important factor to be considered to improve the efficiency of androgenesis. The present investigation was undertaken to find out the highly anther culture responsive genotypes, to standardize the induction media and cold pretreatment period for anther culture of rice in Bangladesh.

2.7.1.2 Heat

It has been reported that thermal shock may influence induction and regeneration potential in both, anther and microspore culture. Keller and Armstrong (1979) applied an elevated temperature of 30-40°C for 1 h in *B. campestris* and in *B. napus* followed by 40°C for 3 h prior to anther culture and found very effective results on embryoid yield and regeneration. Touraev et al. (1996b) applied heat shock treatment along with starvation stress on wheat and observed very efficient induction and regeneration. Ho et al. (1978) obtained high embryo yield and plantlets in wheat anther culture following heat stress pretreatment at 28°C-32°C.

2.7.1.3 Drought

Islam (2010a) obtained highest percentage of embryo yield in wheat when spikes that explants subjected to drought stress pre-treatment for three hours. In the reported findings drought stress treatment was applied on excised anthers prior to culture and observed very efficient induction and regeneration for optimum duration (3 hrs) stress. Bueno et al. (1996) reported that increasing of embryo yield that subjected to starvation and a mid heat shock stress pre-treatment at 33°C for five days in anther culture of *Quercus*.

2.7.1.4 Starvation

As abiotic stress pre-treatment factors starvation and heat shock at 33°C or 37°C either single along or combined application influenced the efficiency of callus induction in tobacco (Touraev et al. 2009). Two processes were reported to be involved in the switch to sporophytic pathway: the dedifferentiation of the pollen during the period of starvation and its re-differentiation after the resupply of necessary nutrients (Wang et al. 1999). Touraev et al. (1996a) investigated in tobacco the possible reversibility of the process of

embryogenic induction and concluded that the stress treatments (starvation alone or combined with a heat shock) applied to isolated microspores irreversibly blocked normal gametophytic development.

2.7.1.5 Osmotic stress

Osmotic stress improves anther culture responses were reported by Keller and Armstrong (1983). Zhou et al. (1991) mentioned that the osmotic potential of media influences to improve embryogenesis as well as green plant regeneration. Kyo and Harada (1986) reported that the effect of oxidative stress on pre-culture medium anthers could modify and influences androgenic microspore development and cell viability controlling by metabolic pathway.

2.7.1.6 Oxidative stress

Oxidative stress was applied to cultured anthers and determined the possible involvement of ROS in the machinery on microspore embryogenesis in triticale (Żur et al. 2009). They reported high level of ROS responsible to decreased microspores viability which helped a great numbers of survival microspores triggered switch towards sporophytic development. It was observed that many ROS progenitors influenced the microspore developmental pathway that ultimately influences to improve callus induction in maize. The majority of induced structures (68%) developed on the control induction medium were compact or friable.

2.7.1.7 Combined effect of heat and cold

There are many physical factors such as cold, heat, drought, starvation applied either single or in combination enhances embryogenesis in many cereal crops are reported by several scientist. George and Rao (1982) reported that a combined treatment of cold at 4°C + high temperature shock at 31-37°C is essential for pollen embryogenesis in *Brassica juncea.* Obert et al. (2009) reported that the combined effect of cold (3 d) + heat (33°C, 1 day) pre-treatment to cultured anthers of flax enhanced the androgenenetic responses up to 25 percent.

2.7.2 Chemical stress pre-treatment factors

Chemical treatments such as colchicine, heavy metal, ABA, CGA, AEC, Azetidine, 2-NHA, either individually or sometimes combined effect of more than one stress factors may positively influence androgenetic efficiency (Islam 2000). Some important factors influence the development of microspore, including different chemicals. Bennett and Hughes (1972) reported that such chemical substances enhance haploid production in wheat. They demonstrated that plants of *Triticum aestivum* cv. Chinese spring wheat sprayed with Ethrel (2-chloro-ethylphosphonic acid) just before meiosis in the pollen mother cell undergo additional mitosis to give rise to multinucleate pollen. It is possible that multinucleate pollen isolated from Ethrel- treated anthers might be induced to form embryos when cultured, and in this way the yield of haploids could be enhanced. Lower and Miller (1962) reported that 2 chloro-ethylphosphonic acid (commercially known as Ether or Ethephon) acts as a gametocide and causes male sterility (Rowell and Miller 1971). Picard et al. (1987) used fenridazone-potassium as a chemical hybridization agent (CHA) for inducing anther culture. They sprayed it on plants prior to harvest of spikes for androgeneic studies and obtained very significant results on induction, regeneration and improvement of doubled haploids (DH) lines.

Chemical substances, like gametocide (CGA) influence androgenic response very strongly (Schmid and Keller 1986). For further observation of androgenic potentials in wheat other chemical substances such as Azetidine and AEC (analog of amino acid) were chosen by Schmid (1996). Miao et al. (1988) applied lysine and proline e.g. S-2-aminoethyl-L-cysteine (AEC) and azetidine-2-carboxylic acid (A2C) to improve maize callus culture. Grewal et al. (2006) reported that androgenic response enhanced by application of cysteine in medium in rice.

Colchicine is the most important chemical agent for chromosome doubling which is used very frequently. It disrupts mitosis by binding to tubulin, the protein sub unit of microtubles, thus inhibiting the formation of microtubules and the polar migration of

chromosomes, which results in a cell with a doubled chromosome number (Blakeslee et al. 1922, Wan et al. 1991). Several research have already been done by treating anthers using colchicine in the induction media with different plant species e.g. wheat (Barnabás et al. 1991, Navarro-Alvarez et al. 1994), maize (Michard and Beckert 1997, Saisingtong 1998), rice (Alemanno and Guiderdoni 1994) and in *Brassica napus* (Chen et al. 1994). Hassai and Liang (1991) mentioned that chromosome doubling of plants produced from microspores, using colchicine by *in vivo* and *in vitro*. Redha (1998) used colchicine in the induction medium for 3 days and observed embryo production with higher percentage of doubled haploids (DH).

The induction of p-pollen (pollen competent for embryogenesis) occurs early in flower development during the meiotic prophase (Heberle-Bors 1982). In barley, a mannitol pretreatment improved response (Ziauddin et al. 1992), whereas the inclusion of culture media macronutrients in the mannitol pretreatment were needed to obtain the microspore division and sporophytic development in wheat (Heberle-Bors 1982), and rice (Ogawa et al. 1994). Hansen and Andersen (1998) applied colchicine treatment to microspore culture, which caused an increase in the frequency of fertile plants up to 53%. Barnabás et al. (1991) mentioned that the frequency of symmetrical divisions of microspores was significantly increased after *in vitro* colchicine treatment.

2.8 Physiology of donor plants

The physiological state of the donor plants is very effective to improve androgenetic responses in rice, wheat and other cereal crops. The growth conditions of the donor plants have significant effect on the embryo yield and regeneration in many cereal crops. Using light and day/night temperature regime to anther donor plants a remarkable androgenic response was found in indica rice cultivar (Raina and Zapata 1997). The environmental conditions under which donor plant greatly affect the microspore embryogenesis (Maheshwari et al. 1980). It is reported that plants grown under controlled environment conditions usually are more responsive than field grown plants. Generally vigorous and

healthy plant exhibits anthers with high androgenic potential. The new inflorescence of older plant of *Brassica* has more potential to undergo embryogenesis as compared to inflorescence of new plant (Takahata et al. 1991). Whereas in citrus anthers harvested from old and senescent branches had reduced embryogenic response than the anthers harvested from young tree (Chen 1985). Grapes plants grown under controlled glass house conditions were more uniform and give better results as compared to that grown in natural field conditions (Mullins and Rajasekaran 1981). Pandey (1973) examined the effect on plants grown under natural light condition during the normal season have been found to be better than those grown under artificial light during off season.

2.9 Culture media

Media composition is one of the most important factors in the success of androgenesis. The available literature does not allow recommendation of any anther culture medium for general application. The most common basal media used for androgenetic studies in cereal crops are MS, N6, He2, MO19 (Raina and Irfan 1998), R2 (Chaleff 1978), B5, Potato- 2 (Chuang et al. 1978) and LS. Raina (1997) examined in addition of maltose with different concentration to the induction medium embryos and regeneration frequency was increased in rice. Herath et al. (2007) reported that high frequency of callus induction and plant regeneration found in both indica and japonica rice cultivars. They also got good responses in Fl hybrids rice plant also. However, most cases semi-solid medium used for anther culture and for isolated microspore culture liquid medium is preferable.

2.9.1 Effect of amino acids and ammonium nitrate in medium

In liquid induction medium, amino acids play an important role for the production of embryoids yield and regeneration was reported many scientists specially for wheat, rice and barley. Ku et al. (1978) reported that several amino acids, vitamins and myo-inositol stimulate androgenesis. Higher percentage of green plants was obtained in barley anther culture when induction medium was supplemented with amino acids (Muyan et al. 1990). Single amino acid supplemented in the induction medium may increase embryo yield in

wheat and maize anther culture was reported by Schmid et al. (1996). In addition to the medium amino acids (L-proline and L-glutamine) played an important role and promoted callus induction for microspore cultures of a japonica cultivar were reported by Cho and Zapata (1988).

Chu (1975) found a very positive effect on embryos induction as well as regeneration in addition of ammonium nitrogen with different concentration in the rice anther culture medium. Raina (1989) found that a medium with high concentration of KNO_3 and NH_4 + ions completely replaced by 50 mg/l casein hydrolysate showed very significant results for indica × indica F1 hybrids rice.

2.9.2 pH effect on medium

Datta et al. (1998) mentioned that the pH of the liquid media changes dramatically with time at the onset of embryo development. The pH is an important factor that greatly influenced on induction and regeneration efficiency in higher plants (Forster et al. 2007).

2.9.3 Effect of gelling agents

Generally various solidifying (gelling) agents are used in plant tissue culture media. Anther culture media are generally solidified by agar, but there are some other solidifying agents e.g. clerigel, starch (potato, wheat, corn or barley starch), gelrite, agarose, phytagel and ficoll, have also been reported in many crop plants. Semi-solid media provide a fixed anchorage that determines polarity and vertical growth for plant tissue and in the culturing of anthers, solid and liquid media can be used widely and instead of agar, which often contains impurities, the use of agarose is proposed by Dunwell (1986). George and Sherrington (1984) reported that advantages of using solidifying agents are their stability at all feasible incubation temperatures, their inability to be digested by plant enzymes and the absence of reaction with media constituents. Kohlenbach and Wernicke (1978) examined that anther culture of *Nicotiana tabacum* in liquid medium showed significantly better results on induction than semisolid medium.

2.10 Androgenesis and genetic transformation for rice improvement

There are many abiotic stress pre-treatment such as drought, cold, heat, starvation and salt are the major factors (**Fig. 03**) that adversely affect plant growth and productivity in the world (Kasuga et al. 1999, Mahajan and Tuteja 2005, Chauhan and Khurana 2010). Minimizing these looses is a major concern to workers and modern biotechnology has provided us with means to improve plants by generating genetically engineered plants with new and improved characteristics (Shariatpanahi et al. 2006b). It is generally agreed that in order to meet future challenges in food production, multi-disciplinary, multi-faceted approaches are needed.

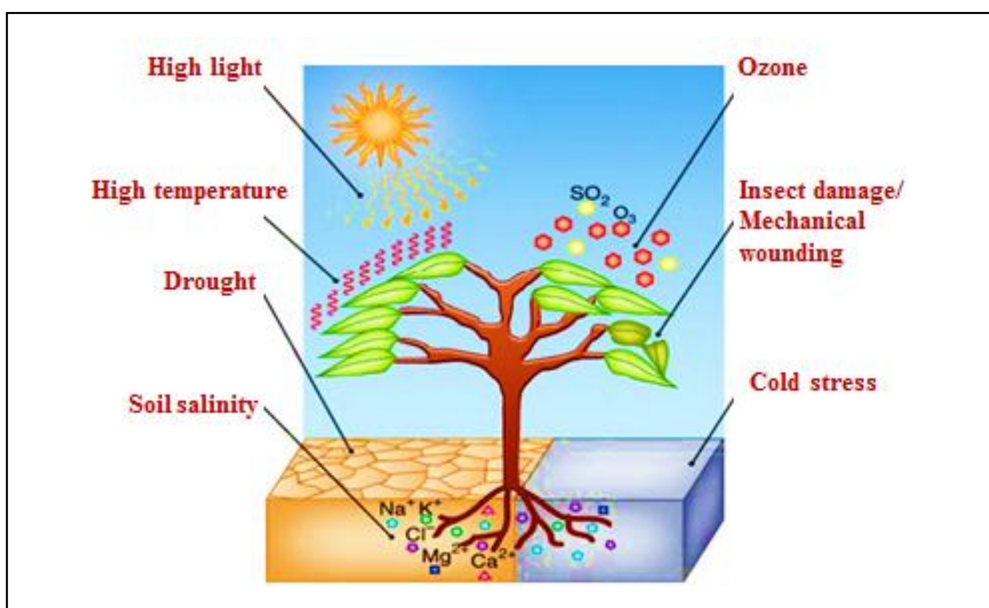


Fig. 03: Various abiotic stress factors that affects to crop yield

(Source: Preshobha KP, KIT, Germany, 2015).

The application of biotechnology in combination with conventional breeding methods such as androgenetic methods may help to increase food production properly (Weeks et al. 1993, Kumlehn et al. 2004, Otani et al. 2005, Shariatpanahi 2006a, Islam et al. 2013a). An endeavor for genetic improvement of this crop, with respect to disease resistance, drought, heat and salinity tolerance with high yielding cultivars, may be helpful to boost up the rice production for developing countries. In order to respond quickly to their changing

environment plants use an elaborate system of hormonal signals such as JA (Jasmonic acid), GA (gibberellins), abscisic acid (ABA) and auxins (Chini et al. 2007, Creelman and Mullet 1995). The use of genetic engineering technology could lead to simpler and more effective gene-based approaches for improving crop tolerance (Fukuoka et al. 1998, Tuteja 2007, Chauhan and Khurana 2011).

Plants are sessile, they have to perceive the changes in the environmental condition to survive and grow under stress conditions (Banu et al. 2014). In many countries, drought and soil salinity are the main contributors to food insecurity and poverty. Drought and salinity are becoming increasingly prevalent such that e.g. severe salinization is predicted to encompass more than 50% of all cultivable land by 2050 (Wang et al. 2003, Kasuga 1999). To counterbalance the predicted increase in the world population up to nine billion by 2050, and the related implication of climate change, science has to develop technologies that increase yields and productivity in a sustainable way and adapting crops to match the effects of changes in the environment (Mahajan and Tuteja 2005, Fenning et al. 2008).

The use of genetic engineering technology could lead to simpler and more effective gene-based approaches for improving crop tolerance (Tuteja 2007). An endeavour for genetic improvement of this crop, with respect to disease resistance, drought, heat and salinity tolerance with high yielding cultivars, may be helpful to boost up the major cereal crop production for developing countries (Weeks et al. 1993, Shim et al. 2009). However, application of biotechnology is important to ensure sustainable development of agriculture-food and other crops, nutrition, health and environment. Considering evidence for global climate change, it is clear that improving crop ability to grow in adverse environmental conditions remains the greatest challenge to ensure that the demand side for agricultural production. The p68 (Ddx5) is a multifunctional protein and has a role in abiotic stresses such as salinity and drough was reported in plant system (Tuteja 2010).

2.11 Inducing chromosome doubling by antimitotic agents

Induction of doubled haploids (DHs) by the application of antimitotic agents such as colchicine, trifuralin, nitrous oxide and oryzalin is very effective methods in the field of plant breeding and biotechnology. Blakeslee and Avery (1939) were the first who made the sensational discovery of the induction of chromosome doubling by colchicine. It is a commonly used microtubule-depolymerizing agent which actively binds to alpha and beta tubulin subunits of tubulin heterodimers of microtubules, thus, blocking the active sites of microtubules for binding to kinetochore protein reported by Shariatpanahi et al. (2006a). Barnabás et al. (1996) observed the direct effect of colchicine on the mitotic divisions of microspores in culture by transmission electron microscopy and they found significant increase in the frequency of symmetrical divisions, induced by *in vitro* colchicine treatment.

In few crops, spontaneous haplo-diploidization results in the formation of doubled haploids, but spontaneous doubling frequency is very low (Aleza et al. 2009). Castillo et al. (2000) reported that the chromosome doubling frequency increased significantly in low androgenic wheat cultivar 'Caramba' by application of colchicine during anther culture initiation. The application of colchicine during the isolated microspore culture of *Brassica*, results in high degree of embryo induction with the synchronized embryogenesis (Zaki and Dickinson 1995, Zhao et al. 1996). In case of *B. napus* they found that DH plants from trifluralin treatment was 85.7%, from colchicines 74.1% and 66.5% in the case of oryzalin, while only 42.3% was found in the untreated control.

Beneficial effect of colchicine as chemicals in anther and microspore culture has been reported for several species such as wheat (Barnabás et al. 1991, Hansen and Andersen 1998, Islam 2010b), in maize (Wan et al. 1991, Saisingtong et al. 1996, Michard and Beckert 1997), rape seed (Möllers et al. 1994, Iqbal et al. 1994, Zaki and Dickinson 1995, Hansen and Andersen 1998) and in rice (Alemanno and Guiderdoni 1994, Hu and Liang 1979).

Motallebi-Azar and Panahandeh (2010) reported that in tomato anther culture maximum number of callus induction and chromosome doubling frequency was observed by the application of 250 mg/l colchicine for 72 hrs at 4°C. In maize colchicine pretreatment (250

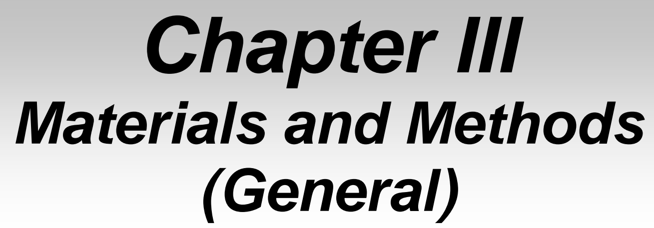
mg/l) for 3-6 days produced the highest number of ELS-embryo like structures reported by Saisingtong et al. (1996). Burun and Emiroglu (2008) found that pretreatment of tobacco anther to 0.2% colchicine for one day resulted in the 60% diploidization of chromosomes. In maize the application of colchicine in the pretreatment media increased the number of fertile DH plants (Antonie-Michard and Beckert 1997). As chemical stress pre-treatments factors by colchicine (250 mg/l) for 72 hrs in combination with physical stress factors as cold at 4ºC duration was effective in the production of doubled haploids (Motallebi-Azar and Panahadeh 2010). However, they also found that colchicine concentrations higher than 250 mg/l had negative effect on androgenesis response of anther cultures.

2.12 Significance of the research work

There is a great need to exploit all genetic variability that can be used in breeding for stressful environments. An important strategy is to improve yield stability and increase production to develop crop varieties with enhanced tolerance to environmental stresses such as drought, salinity, heat, starvation and cold by genetic engineering and androgenetic study for development of homozygous transgenic plants rapidly. Use of unconventional techniques such as doubled haploid (DH) breeding through anther and microspore culture will become more useful in the speeding up of the application of conventional plant breeding methods. Application of different physical and chemical pretreatments had a positive effect on microspore development and embryogenesis and influencing doubling efficiency (Islam and Tuteja 2012). But still applications of stress pretreatments have not showed complete success to major crop plants. It is very important to know about the activity of stress factors and its pathways for further research in cereal and other crops.

For genetic transformation of cereal crops have recalcitrant to recombinant techniques mainly because of problems in establishing regenerable cell and tissue cultures as well as efficient DNA delivery systems (Stöger et al. 1995). Microspore embryogenesis is an elegant system for genetic transformation and this protocol may be used to overcome genotypic limitations of doubled haploid formation in cultivars that had previously been

found to be recalcitrant in anther culture. Under this research project targeted gene (p68) were transformd using anther and microspore derived-embryoids using Bangladeshi rice cultivars for rapid development of transgenic pure lines. This research project has been considered to find out the combination of *in vitro* androgenesis and *Agrobacterium*-mediated genetic transformation for the production of drought and/or salinity tolerant wheat cultivars in Bangladesh. The outcome of this research is very much helpful to develop stress tolerant fertile transgenic plants which may contribute for food security and development of our human resources in Bangladesh.



Chapter III
Materials and Methods
(General)

3. Materials and Methods (General)

Under these study three major steps e.g. *in vitro* somatic embryogenesis, androgenesis (anther and isolated microspore culture) and genetic transformation works have been done and for each steps some selected rice varieties, media, plant growth regulators (PGRs), stress pre-treatments and for each work different protocols/methods were employed and optimized.

3.1 Plant materials

Ten (10) Boro-BR3, BR14, BR19, BRRI dhan28, BRRI dhan29, BRRI dhan47, BRRI dhan50, BRRI dhan55, BRRI dhan58 and BRRI dhan59; six (06) Aus- BRRI dhan24, BRRI dhan27, BRRI dhan42, BRRI dhan48, Chinigura and Kalijira, four (04) Aman- BR4 (BRRI Shahil), BR11, BRRI dhan30, BRRI dhan31 and three (03) IRRI rice varieties *viz.* IR-43, IR-54 and Jaya (Total = 23) were used for this study (**Table 3**). For somatic embryogenesis only 20 rice genotypes were considered for this study. Mature seeds of all of these varieties were collected from Bangladesh Rice Research Institute (BRRI), Gazipur and also Regional Rice Research Center, Shyampur, Rajshahi, Bangladesh. Some local varieties were also collected from Rajshahi, Bogra, Dinajpur, Chapai Nawabgonj and Khulna, Bangladesh. Plants were grown in the research field of the Institute of Biological Sciences, University of Rajshahi during the growing seasons of 2014-2016. Spikes were also collection from the Regional Rice Research Center, Shyampur, Rajshahi. Planting time of Aman is April - May and harvested in July-August. The planting time of Aus in April - May and harvesting period is November-December. The planting time of Boro rice in December - February and harvesting perios is April to May.

Table 3: Important features of selected rice varieties under this study

Seasons	Sl.	Variety name	Short description	Plant (cm)	Life (days)	Average yield/per h (tons)	Resistance/ Tolerance
Boro (Sl. No. 1-10)	1	BR3*	BR3 rice is the most cultivated and high yielding crop. This variety is usually suitable for transplant Boro, Aus and Aman area. Grain medium bold and white marked in belly. Planting time is mid to late November and harvesting time is mid April to early May. The popular name of BR3 rice is Biplob.	95	170	6.5	Tolerant to blast and sheath blight.
	2	BR14	BR14 rice is usually suitable for Boro and Aus season and it is the most cultivated and high yielding crop. Medium bold and white grain type. Planting time is mid June to late July and harvesting time is Late November. The popular name of BR14 rice is Gazi. Its leaf is erect and strength of stem is strong.	120	160	6.0	Moderately tolerant to leaf blight
	3	BR19	The popular name of BR19 rice is Mangal. Clean rice medium bold. Planting- late October to mid November and harvesting - mid April to early May. It cultivated in low land area. Stem length is quite long.	110-115	165-170	6.0	Lodging stress and moderately resistance to blight
	4	BRRI dhan28	BRRI dhan28 is the most cultivated and high yielding crop. Grain nature is clean rice medium slender and white. Planting - mid to late November and harvesting early to mid April. It's a suitable variety for cultivation in flood affected regions of Bangladesh.	90	140	5.5 - 6.0	Moderately resistance to blast and lodging stress

Seasons	Sl.	Variety name	Short description	Plant (cm)	Life (days)	Average yield/per h (tons)	Resistance/ Tolerance
	5	BRRI dhan29	BRRI dhan29 is early and high yielding rice cultivars. Grain nature is medium slender and white. Planting- late October to mid November and harvesting- mid April to early May.	95	160	7.5	Moderately resistance to leaf blight, sheath blight
	6	BRRI dhan47	BRRI dhan47 is a suitable variety for cultivation in salt regions of Bangladesh. Clean rice medium bold. Planting - mid to late November and harvesting - early to mid April.	105	152	6.0	Salt tolerance
	7	BRRI dhan50	BRRI dhan50 is a suitable and high yielding variety for cultivation in high land of Bangladesh. Grain nature is long slender, white scented. Planting - late October to early November and harvesting- April. The popular name of BRRI-50 is Banglamoti and it is an aromatic rice variety.	82	155	6.0	-
	8	BRRI dhan55	BRRI dhan55 rice is the most cultivated and high yielding earlier variety. Rice medium slender, long, 1000grain weight 23.5 g. Seedling in seed bed 18-30 April. Harvesting time is 14 August - 4 September. Protein 8.3% in rice.	100	100	4.5	Medium salt tolerant (8-10ds/m up to 3 week, drought tolerant.
	9	BRRI dhan58	BRRI dhan58 was developed by *in vitro* somaclonal variation. It's early and high yielding rice cultivars. In vegetative stage size and shape taller than BRRI dhan 29, grain as like BRRI dhan 29 but slight slender,1000 grain weight 24 g, ripe grain colour as like straw colour. Seedling in seed bed late November to mid December. Harvesting time is mid April to early May.	100 - 105	150 - 155	7.0 - 7.5	Medium wilt disease tolerant.

Seasons	Sl.	Variety name	Short description	Plant (cm)	Life (days)	Average yield/per h (tons)	Resistance/ Tolerance
	10	BRRI dhan59	BRRI dhan59 rice is a high yielding rice variety that can be cultivated upland area in Bangladesh. Stout, rice clean and medium size, 1000 grain weight 24.6 g. Seedling in seed bed 15-30 November and harvesting: 15-30 April. Protein 7.5% and amylose 24.6% in rice.	83	153	7.1	-
Aus (Sl. No. 11-16)	11	BRRI dhan24	BRRI dhan24 (Rahmant) is clean rice with long, slender and white. Planting time is mid March to late April and harvesting- mid June to late July.	105	105	3.5	Resistance to tungro and tolerant to blast
	12	BRRI dhan27	BRRI dhan27 is most cultivated and transplanted nature and mainly cultivated in the southern part of Bangladesh in salt dominant area. It's an earlier rice variety. Clean rice medium bold. Planting- mid March to mid April and harvesting- late June to mid August.	140	115	4.0	Salt tolerance. Moderately resistance to sheath blight, BPH and GLH and resistance to blast and white back plant hopper
	13	BRRI dhan42	BRRI dhan42 is the most cultivated and earlier variety of rice. Clean rice long slender medium bold white. Planting- mid March to late April and harvesting- late June to mid August.	100	100	3.5	Drought tolerance. Tolerant to leaf blight and sheath blight
	14	BRRI dhan48	BRRI dhan48 is the most cultivated and high yielding crop. This variety is usually suitable for transplant Aus area. Clean rice medium bold. Planting- late March- early April and harvesting- late July.	105	110	5.5	-

Seasons	Sl.	Variety name	Short description	Plant (cm)	Life (days)	Average yield/per h (tons)	Resistance/ Tolerance
	15	Chinigura**	Chinigura is an aromatic, transplanted and photoperiod sensitive rice variety that cultivated to the field as mid to late September. The most popular aromatic variety of all is high-yield BRRI dhan-34 strain developed by the BRRI. With high sales prices and suitable geography, aromatic rice farming across the north is flourishing.	116	78	2.47	Adaptive to our environment (Bangladesh)
	16	Kaijira**	Kalijira is also an aromatic, transplanted and photoperiod sensitive rice variety. Its yield is low but its price is high. The rice cultivars varied consideration in terms of crop growth characteristics as well as yield and yield contributing characters.	116	78	2.15	Adaptive to our environment (Bangladesh)
Aman (Sl. No. 17-20)	17	BR4	The popular name of BRRI-4 rice is BRRI Shaile. This rice is the most cultivated and high yielding earlier variety. Medium bold and white grain type. Planting- mid June to late July and harvesting- late November.	125	145	5.0	Photoperiod sensitive. Moderately tolerant to leaf blight.
	18	BR11	BR11 or Mukta is a very popular and high yielding rice variety in Bangladesh. Clean rice medium bold. Cultivated in low lands areas with low light. Planting- mid June to mid July and harvesting- late November.	115	145	6.5	Photoperiod sensitive. Moderately resistant to Tngro & tolerant to yellow stem borer.

Seasons	Sl.	Variety name	Short description	Plant (cm)	Life (days)	Average yield/per h (tons)	Resistance/ Tolerance
	19	BRRI dhan30	BRRI dhan30 is a photoperiod sensitive and high yielding rice cultivars in Bangladesh. Medium slender grain. Planting time is mid June to mid July and harvesting- 1st week of November to 1st week of December.	120	145	5.0	Weakly photosensitive
	20	BRRI dhan31	The characteristic of BRRI dhan31 is quite similar with BR11. It is earlier cultivars of rice. Clean rice, medium bold and white. Planting time is mid June to mid July and harvesting- early to mid November.	115	141	5.0	Weakly photosensitive. Resistance to BPH and moderately resistance to leaf blight and Tungro.
Other rice varieties (Sl. 21-23)	21	IR-43	This rice variety was released on 1978 by IRRI. It is a high yielding rice variety and cultivated in upland areas in dry season.	Medium short	131-140	5.9	Drought resistance
	22	IR-54	This rice variety was released on 1980 by IRRI. It is high yielding and cultivated in upland areas in dry season.	Medium short	121-130	5.5-6.0	Drought resistance
	23	Jaya	Jaya rice, a cross between TN-1 and TN141, high yielding photoperiod-insensitive, semi-dwarf plant stature.	Semi-dwarf	120-125	5.5-6.0	Moderately resistant and photoperiod insensitive

*= Boro, Aus and Aman; **Local variety; BR4, BR11, BRRI dhan30 and BRRI dhan31 = Ropa Aman; BRRI dhan24 and BRRI dhan42 = Bona Aus; BRRI dhan27 and BRRI dhan 48 = Ropa Aus. Sources of information = Adhunik Dhan Chash 2011, www.dhcrop.bsmrau.net/.

3.2 Methods (*In vitro* micropropagation)

3.2.1 Preparation of stock solutions

At first, five types of stock solutions were prepared using various constituents of the medium. As the different constituents were required in different concentrations separate stock solutions for macro and micro nutrients and vitamins were prepared as follows:

3.2.1.1 Stock solution A

It was prepared using all the macronutrients [KNO_3, NH_4NO_3, KH_2PO_4, $CaC1_2.2H_2O$, $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, $Ca(NO_3)_2.H_2O$, NaH_2PO_4, etc.) at strength 20 times to that of the strength required for the medium. All macronutrients were weighted accurately with the help of a 4-digit electric balance and dissolved in 400 ml distilled water taken in a one liter glass beaker. For better mixing the solution was stirred placing it on a magnetic stirrer with mild heating. The solution was transferred into 500 m1 volumetric flask and more distilled water was added to make the final volume (500 ml). The stock solution were taken in a clean reagent bottle and stored in a refrigerator (4°C).

3.2.1.2 Stock solution B

It was prepared using the seven micronutrients *viz*. $MnSO_4.4H_2O$ H_3BO_3, $ZnSO_4.4H_2O$, KI, Na_2MoO_4, $CuSO_4.5H_2O$, $CoC1_2.6H_2O$, etc.) at a concentration 500 times to that of final strength needed for the medium. The stock solution was prepared by well mixing these nutrients in glass-distilled water as done in case of preparation of stock A. The solution was taken in a reagent bottle and preserved in a refrigerator at 4°C.

3.2.1.3 Stock solution C

It was prepared using two micronutrients *viz*. $FeSO_{4.}7H_2O$ and Na_2-EDTA at 100 times strength to that of the final strength needed for the medium.

3.2.1.4 Stock solution D

Stock D was prepared using the vitamins- Glycine, Nicotinic acid. Pyridoxine, Thiamine HC1 at 500 times concentration to that of the final strength needed for the medium. The procedure was same as that followed in case of Stock A. The solution was preserved in a refrigerator at 4°C.

3.2.1.5 Stock solution E

It was prepared at 20 times the final strength of myo-inositol in 500 ml glass distilled water as described for stock solution A and stored in a refrigerator at 4°C.

3.2.1.6 pH of media

The pH of the solution was adjusted to 5.8 by a digital pH meter with the help of 1N NaOH or 1N HCl. The culture vessels (test tubes/flasks/bottles) containing medium were autoclaved at 1.9 kg/cm^2 of pressure and a temperature of 121°C for 15 minutes for medium and for any glassware 20 min to ensure sterilization.

3.2.2 Media preparation and sterilization

The macro and micro-nutrients, vitamins and myo-inositol were taken from the stock solutions according to the requirement of plant tissue culture medium. As carbon sources sucrose (30 g/l) was added and mixed well. The pH of the media was adjusted to 5.6 - 5.8 using 0.1N NaOH or 0.1N HCl. As solidifying agent 0.8% agar was added to the medium and autoclaved at 15 lbs pressure at 121ºC for 15 min.

3.2.3 Culture media and maintenance of culture materials

The MS (Murashige and Skoog 1962), N6 (Chu et al. 1975), B5 (Gamborg et al. 1968) and SK3 (Raina 1989) media were used for this study (**Table 4**). The N6 and MS media for callus induction, which supplemented with 2 mg/l 2,4-D, 500 mg/l Casein hydrolysate, 3% sucrose and 0.7% agar in addition with 200 mg/l BAP, 300 mg/l L-proline and 500 mg/l L-glutamine with other components. For this study only embryogenic calli were transferred to callus induction medium for multiplication. The pH was adjusted to 5.6 - 5.8 before autoclaving. The working area of the laminar airflow cabinet was first surface sterilized with 70% ethanol. The petri dishes and essential tools (forceps, scalpel, Whatmann 1 filter paper, glassware, etc) which were used during inoculation of the seeds were sterilized in autoclave at 15 lbs 121ºC for 20 min. The ultra violet (UV) light was switched on for 15 min before inoculation for surface sterilization in laminar air flow cabinet. The petri dishes were sealed with parafilm and incubated at 27±2°C in the dark for three to four weeks. Then the vessels were kept under 36 Watt white fluorescent lamps with light intensity of

5000 lux at 16/8 hours cycle of light/dark. The temperature of growth chamber was maintained at 25±1°C. The surface sterilized seeds were carefully inoculated into various media as mentioned in **Table 1**. Different modes of incubation were assessed based on the germination percentage using the following formula.

$$\text{Germination (\%)} = \frac{\text{Number of germinated seeds}}{\text{Total number of inoculated seeds}} \times 100$$

3.2.4 Hardening of plants

Well rooted plantlets were put out of vessel and washed the root portions under running tap water to clear the entire agar medium to avoid the chances of contamination in soil. Then the plants were transferred to small plastic pot diameter of about 5 cm containing pre-soaked sterilized vermiculite. Afterwards pots were kept inside the moist chamber. To avoid evapo-transpirational losses of water the plants covered with polyethylene bag for 1-2 weeks. Then the plants were transferred under the shade conditions for hardening the transferred to larger pot diameter of about 20 cm containing the mixture of compost, sand and loamy soil with ratio of 1:1:1. For field culture the plants were kept under open light maintaining the natural environmental conditions.

3.2.5 Data recording and statistical analysis

To determine the frequencies of callus induction and plant regeneration data were recorded as number and calculated using the formulae followed by Zaidi et al (2006). The average values as well as the means were determined from 5 (five) replicates. To analyze the variances (ANOVA) the software SPSS 17.0 were used along with DMRT and test of homogeneity were done.

$$\text{Frequency of callus induction (\%)} = \frac{\text{No. of explants induced callus}}{\text{No. of cultured explants}} \times 100$$

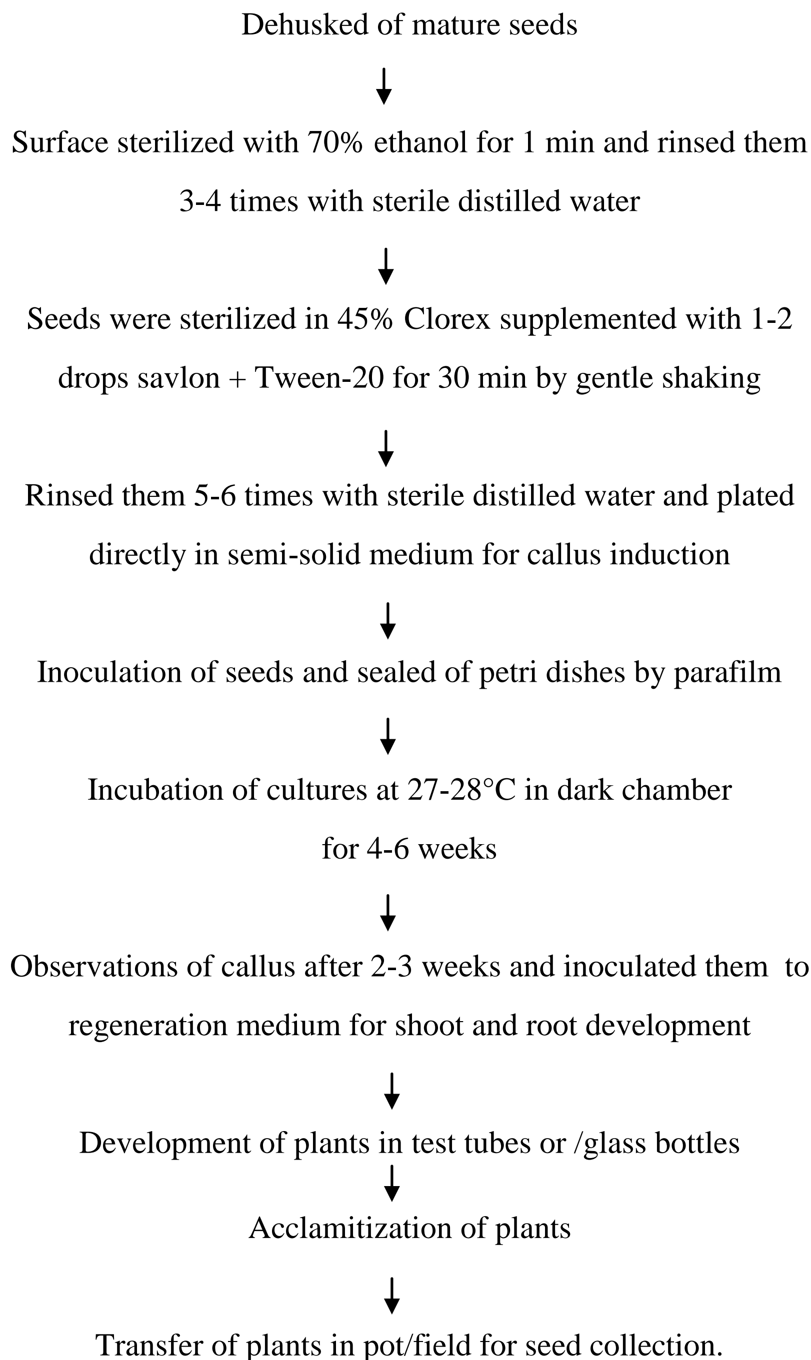
$$\text{Frequency of plant regeneration (\%)} = \frac{\text{No. of callus regenerated shoot}}{\text{No. of cultured callus}} \times 100$$

$$\text{Frequency of root induction (\%)} = \frac{\text{No. of shoot induced root}}{\text{No. of cultured shoot}} \times 100$$

Table 4: Various basal media used for callus induction and plant regeneration derived from seeds for studied rice varieties.

Nutrients/ Others	Constituents	Media (mg/l)			
		MS	N6	B5	SK-3
Macro	KNO_3	1900.00	2830.00	2500.00	2830.00
	NH_4NO_3	1650.00	-	-	-
	KH_2PO_4	170.00	400.00	-	641.00
	$CaCl_2.2H_2O$	440.00	166.00	113.23	166.00
	$MgSO_{4.}7H_2O$	370.00	185.00	121.56	280.00
	$(NH_4)_2SO_4$	-	463.00	134.00	314.50
	$Ca(NO_3)_{2.}\ H_2O$	-	-	-	-
	NaH_2PO_4	-	-	130.44	-
Micro	$MnSO_4.4H_2O$	22.30	4.40	10.00	4.40
	H_3BO_3	6.20	1.60	3.00	1.60
	$ZnSO_{4.}4H_2O$	8.60	1.50	2.00	1.50
	KI	0.83	0.80	0.75	0.80
	Na_2MoO_4	0.25	-	0.25	-
	$CuSO_{4.}\ 5H_2O$	0.025	-	0.025	-
	$CoCl_2.6H_2O$	0.025	-	0.025	-
	$AlCl_2$	-	0.03	-	-
	$NiCl_2$	-	0.03	-	-
Iron	$FeSO_4.7H_2O$	27.80	27.80	-	48.10
	$Na_{2}.EDTA$	37.30	37.30	-	64.40
Organics/ Vitamins	Glycine	2.00	2.00	-	10.00
	Nicotonic acid	0.50	1.00	1.00	2.50
	Pyridoxine HCl	0.50	0.50	1.00	0.50
	Thiamine HCl	0.10	0.50	10.00	0.50
	Myo-inositol	100.00	100.00	100.00	-
	Yeast extract	-	-	-	1000.00
Carbon	Sucrose	30000.00	30000.00	30000.00	30000.00

3.2.6 Steps of sterilization and culture procedure of rice seeds



3.3 *In vitro* Androgenesis (Anther and Microspore culture) of rice

3.3.1 Anther culture

Anther culture allows a rapid production of appropriate genotypes for breeding purposes in an effort to identify promising homozygous lines within shortening of time.

3.3.1.1 Plant materials

Screening out of 23 rice varieties *viz*. BR-03, BR-04, BR-11, BR-14, BR-19, BR dhan27, BRRI dhan28, BRRI dhan29, BRRI dhan30, BRRI dhan31, BRRI dhan42, BRRI dhan47, BRRI dhan48, BRRI dhan50, BRRI dhan55, BRRI dhan56, BRRI dhan58, BRRI dhan58, IR-43, IR-54, Chinigura, Kalijira and Jaya for their responses in androgenesis (**Chapter-V, Table 18**).

3.3.1.2 Conditions of success in androgenesis

3.3.1.2.1 Genotype

The genotype dependency is a critical factor affecting the variability of response in anther, microspore and tissue culture system. Success in androgenesis is predominantly dependent on the genotype of the donor plant. So screening of androgenetic responsive cultivars is very important. For this study agronomically important rice variety were considered from Bangladesh for gametic embryogenesis and to evalute their regeneration effeciency.

3.3.1.2.2 Growth condition of anther donor plants

The physiological conditions of donor plants are very important for the anther development and that affect success in androgenetic study. In case of cereal crops if donor plants grown in unfavourable conditions and due to bad physiological conditions enhanced to the unwanted production of albino plants. For better resposes in androgenesis donor plants must be grown under controlled environments or in the field where light, temperature, water and nutrition supply is optimum and sufficient. The age of the spikes/inflorescence, the position of anthers in spikes is also very immporatnt for embryogenic responses.

3.3.1.2.3 Harvesting stage of spikes

In cereal crops the early or mid-uninucleate stage of microspore development gave the best results in androgenesis (Bajaj 1983, Wenzel and Foroughi-Wehr 1984, Dunwell 1986).

The best stage for the initiation of androgenesis is just before the first pollen mitosis, from mid-uninucleate pollen. Prior to culture, precise stage of microspore (mid to late-uninucleate stage) was determined by squashing anthers from a floret close to the middle of the spikes. Microspore development was observed at the different days of culture initiation by aceto-carmin staining.

3.3.1.2.4 Pre-treatment of spikes

Certain pretreatment of the inflorescences can have a positive effect on the development of microspores. For cereals, cold pretreatment was found most effective. Therefore, the selected tillers containing spikes with microspore at the mid to late-uninucleate stage were wrapped in foil and kept at 3°C chamber for 7-10 days (De Buyser and Henry 1980). The upper and lower anthers of the spikes were discarded and only those at the appropriate in the middle were used in culture.

3.3.1.2.5 Major steps of anther culture

i. Plants were grown in the experimental field of the Institute of Biological Sciences, University of Rajshahi and spikes were also harvested from the BRRI Regional Rice Resaerch Center, Shyampur, Rajshahi when anthers contained early to mid uni-nucleate stages. Microspore conditions were observed by 1% aceto-carmine staining under microscope.

ii. Pick the proper stage of the spikes and wrap them in sterilized tissue paper with aluminium foil, and place at 4ºC chamber for cold pre-treatment.

iii. Dip the selected spikes in 70% alcohol for few seconds, then wash with sterile distilled water 3-4 times and transfer them to 0.1% mercuric chloride solution for 2-3 minutes. Finally rinsed them again for 4-5 times with sterile water.

iv. Precised anthers were cultures in MS medium containing 30% sucrose, 7% agar (Difco-Bacto), amino acids (L-proline and L-glutamine) and various auxins such as 2,4-D, IAA, NAA as well as cytokinins, like BAP and Kinetin, either single or in combination. The pH of all media was adjusted at 5.8 before autoclaving. Then maintained the cultures at 26-28ºC in dark for embryos induction.

v. After 3-4 weeks suitable embryoids were transferred to regeneration medium and cultures were incubated under continous cool, white light (approx. 22.5 μmol^{-2}s^{-1}), produced by Philips florescent tubes of 40 Watts at 27±2ºC and 45-55% relative humidity.

vi. The induction of secondary embryogenesis from gametic embryos and the efficient regeneration of the secondary embryos into doubled haploids plants were also observed.

3.3.2 Microspores culture

Under these study microspores was isolated mechanically into the liquied medium by squeezing the precise anthers through homogenizer following the standared protocol of Kunz et al. (2000). Petri dishes containing microspores will be sealed with parafilm and incubated at 28°C chamber in dark for embryo induction. After 3-4 weeks in culture the target embryos will be removed weekly and transferred to regeneration medium (M-019).

3.3.2.1 Major steps for microspore culture

Plant growth condition : Donor plants were grown in the field of IBSc, RU and spikes were also collected from the Regional Rice Research Center, Shyampur, Rajshahi.

Harvest of spikes : Spikes were harvested from plants when the flag leaf has just emerged and the microspores at the late uni-nucleate to early bi-nucleate stage.

Cold treatment : Harvested spikes were stored at 4°C chamber in dark for 3-21 days.

Sterilization of spikes : Treated spikes were sterilized with 70% ethanol (EtOH) in Laminar air flow cabinet.

Release of microspores	:	Anthers were removed from the central part of the sterilized spike using a fine tweezers (forcep) and kept in 3 ml washing medium (WM). Microspores were released into the medium by squeezing the anthers with a sterile glass rod/slide.
Filtration, centrifugation and distribution in PD	:	The suspension was further diluted with 10 ml WM and filtered through a sieve with a 100 μm mesh. The sediment was carefully re-suspended in 2 ml AMC and transferred to a petri dish (30 mm). Each dish contained approximately 5.10^4 microspores isolated from 50 anthers.
Incubation of Culture and transfer of embryos	:	The cultures were incubated in the dark at 28°C without shaking. Formed embryos were transferred to solidified regeneration medium (MSR, Henry and De Buyser 1990, plus 0.3% Phytagel, Sigma) and regenerated to haploid or spontaneously doubled haploid plants (26°C, 16 h light).
Transfer of plantlets	:	Regenerated plantlets were transferred to PM (plant medium) for good root and shoot formation.
Ploidy level	:	Analysis of ploidy levels (staining of nuclei isolated from green young leaves with DAPI solution) by flow cytometry.
Transfer of plants to soil and colchicine treatment	:	For chromosome doubling regenerated plants with root system were immersed in 0.2% colchicine and 2% DMSO (dimethylsulfoxide) for 5 h and then transferred to soil.
Seed harvest (DH)	:	Doubled haploid plants were grown in greenhouse and DH seeds were harvested in time.

3.3.2.2 Recorded parameters

Data were recorded on the basis of embryogenesis, regeneration and diploidization of anther and microspore cultures on the following traits: embryo like structures (ELS) production rate, expressed as the number of embryos per 100 anthers (ELS/100 anthers); green plant regeneration (GPR); expressed as the number of green plantlets per 100 embryos (GPR/100 ELS); albino regenerated plants (ARP); expressed as the number of albino plantlets per 100 embryos (ARP/100 ELS); and total plants regeneration (TRP); expressed as the number of green and albino plantlets per 100 embryos (TRP/100 ELS). Doubling index (DI) = doubled haploids / total number of regenerated plants × 100, and the ratio of experimental output to input from the success index (SI) = doubled haploids / total number of anthers × 100. In certain experiments the number of inoculated anthers or microspore was used as basis parameters.

3.3.2.3 Determination of ploidy level

The ploidy level of regenerated green plants were determined using a flow cytometry (Partec CAII) and to study the morphological status in the field specially in flowering.

3.3.2.4 Statistical analysis

Analysis of variance was performed on the following traits: embryo induction rates, embryo regeneration rates and expressed as the number of green and albino plantlets. The significance of cold pretreatment, medium and genotype effects on frequency of callus formation and also medium and pretreatment, genotype and medium, and genotype and pretreatment interactions were analyzed using three-way analysis of variance (ANOVA). For all cases 3 replications were undertaken and in a column the mean values followed by same letter (s) are not significantly different at $p < 0.05$ according to DMRT.

3.3.3 Genetic engineering to develop abiotic stress tolerance homozygous plants

Brief materials and methods are explained in **Chepater-VI.**

3.3.4. At a glance complete research outline of this study are shown in **Fig. 4.**

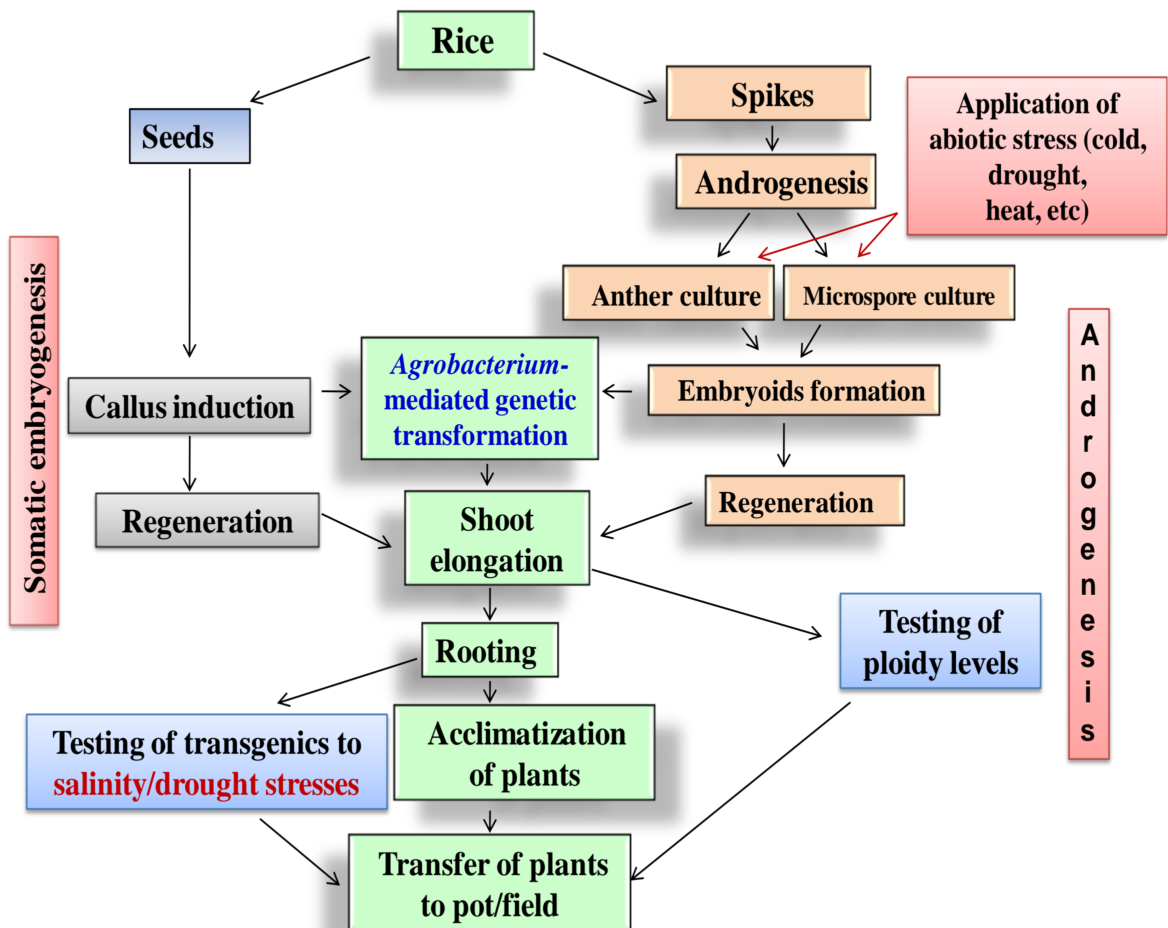
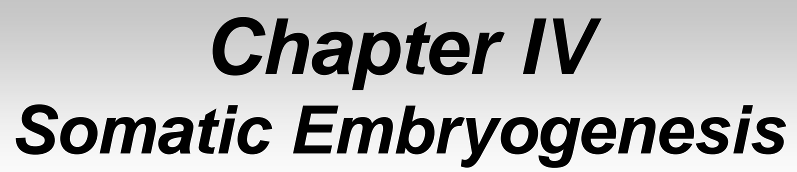


Fig. 4: At a glance schedule research works of this study.



Chapter IV
Somatic Embryogenesis

4. Somatic Embryogenesis

4.1 Effect of PGRs on callus induction and regeneration

4.1.1 Introduction

In vitro regeneration through somatic embryogenesis (SE) is an artificial process in which a plant or embryo is derived from a single somatic cell or group of cells which are not normally involved in the development of embryos (George et al. 2007). SE has served as a model to understand the physiological and biochemical events that occur during plant developmental processes as well as a component to biotechnological advancement (Quiroz-Figueroa et al. 2006). The first documentation of somatic embryogenesis was reported in carrot cell suspension cultures by Steward et al. (1958). An efficient results obtained in rice through somatic embryogenesis was reported by Wang et al. (1987), George et al. (2007), Reddy et al. (2013) and Kalhori et al. (2017).

Cells derived from competent sources of tissues are cultured to form an undifferentiated mass of cells is called callus. Plant growth regulators (PGRs) in the tissue culture medium can be manipulated to induce callus formation and subsequently changed to induce embryos from the callus (Laux and Jürgens 1997, Khalequzzaman et al. 2005, Mostafiz et al. 2018). The ratio of different PGRs required inducing callus or embryo formation varies with the type of plant. SE is mainly produced *in vitro* and for laboratory purposes, using either solid or liquid nutrient medium which contain plant growth regulators, carbohydrates, amino acids and for the culture many factors are required in rice and other crops (Sankepally and Singh 2016, Yaqoob et al. 2016).

In culture condition potentiality of callus induction and plantlets regeneration depends on a number of factors, like genotype, physiological and biochemical status of explants, media, PGRs, concentration and composition of chemical components, etc (Raghavan 1976, Raina 1989, Ghobeishavi et al. 2014, Vennapusa et al. 2015). Among the mentioned factors, genotypic difference is one of the most important things are reported by Abe and Futsuhara (1986), Rueb et al. (1994) and Ge et al. (2006). Several authors are reported that the

genotypic dependency is an important factor through *in vitro* tissue culture systems in rice (Hoque and Mansfield 2004, Ramesh et al. 2009, Vega et al. 2009, Ghobeishavi et al. 2014). In particular genotype and explants are important factors for a successful embryogenic callus induction and regeneration in rice was reported by Rueb et al. (1994). Puhan et al. (2012) studied on different methods to regenerate plant using a set of twelve rice accessions representing indica, japonica, aromatic and wild groups. Differences in the production of embryogenic calli and the regenerated plantlets depends on a suitable genotype, PGRs, tissue sources of explants, media, culture conditions and various stress pre-treatment factors (O'Toole 1982, Seraj et al. 1997, Wang et al. 2003, Lutts et al. 2004, Mahajan and Tuteja 2005, Khalequzzaman et al. 2005, Islam and Tuteja 2012, Vennapusa et al. 2015, Sankepally and Singh 2016, Yaqoob et al. 2016, Repalli et al. 2017, Mostafiz et al. 2018, Mostafiz and Wagiran 2018). Basically the yield of callus induction and somatic embryos depends on the composition of the medium and explants sources are reported by Ozias-Akings and Vasil (1982) and Fennel et al. (1996). He et al. (1988) expressed that the yield depends on genotype and age of embryos in wheat. Aananthi et al. (2010) and Khatun et al. (2012) reported that induction and proliferation of callus varies with genotype, auxin source and its concentration in rice. The effect of various factors on carbon sources were reported in banana by Hossain et al. (2009) and in barley by Haque et al. (2015). Light effect also influenced callus induction and regeneration significantly in tobacco tissue culture system was reported by Yanjie (2004) and Siddique and Islam (2015). Enhancements of somatic embryogenic by silver nitrate and callus age and size are efficient for regeneration in barley (Haque and Islam 2015, Haque et al. 2015).

There are some reports on embryogenic and non-embryogenic calli through *in vitro* culture in rice (Yaqoob et al. 2016). It has been reported that non-embryogenic calli was not be able to regenerate plant; whereas, embryogenic callus develop granular form and produce plants *in vitro* after developmental stages (Raghavan 1976, Raina 1989). For somatic embryogenesis MS medium most widely used as a basal medium for indica and japonica rice varieties. In basmati rice cv.370 obtained highest callus by Zafar et al. (1992) on MS + 2.0 mg/l of 2,4-D. Bano et al. (2005) achieved somatic embryos using either N6 or MS

medium with 2.0 mg/l each of 2,4-D and kinetin. Sah et al. (2014) reported a protocol to produce embryogenic callus and plant regeneration rapidly in *Oryza sativa* cv. Kitaake as japonica rice cultivar. For callus induction they used MS + 2,4-D (3.0 mg/l) + BAP (0.25 mg/l) that showed an efficient regeneration in rice. It was reported that N6, LS and SK1 media gave better response for rice tissue culture than others. In addition to the composition of culture media, the concentrations of plant growth regulators also influence the process of callus induction, multiplication and somaclonal variation. Aananthi et al. (2010) tested two basal media e.g. MS and N6 for callus induction in five indica rice cultivars. They found highest regeneration in Pusa Basmati 1 and the lowest in White Ponni. There are some reports mentioned that N6 and LS media gave better response also for some japonica rice cultivars. Ge et al. (2006) reported that indica sub-species are more efficient than japonica cultivars. In several reports it has been shown that most of the indica lines are less responsive to callus induction and regeneration as compared to japonica lines (Abe and Futsuhara 1984, Mikami and Kinoshita 1988, Reddy et al. 1994). The indica sub-species had not shown similar responses for *in vitro* culture (Seraj et al. 1997, Khanna and Raina 1998). Morel and Wetmore (1951) reported that tissue culture was difficult in monocots than dicotyledonous plants. Jain (1997) expressed embryogenic cell cultures and regeneration often difficult from the cultured cells specially those belonging to indica sub-species of rice.

Mannan et al. (2013) worked with two Bangladeshi traditional aromatic rice varieties *viz*. Kalijira and Chinigura and found better results on callus induction and regeneration using various growth regulators with different concentration and combination with MS medium. They found for callus induction and regeneration both cultivars showed better responses when 2.0 mg/l of 2,4-D added in MS medium along with other components. Hoque (2002) reported that efficient plant regeneration is essential for the successful utilization of biotechnology for rice improvement. Hoque et al. (2007) reported that somatic embryogenesis for six elite Bangladeshi indica rice cultivars such as BR14, BRRI dhan28, BRRI dhan29, BRRI dhan38, BRRI dhan39 and BRRI dhan40. However, in Bangladesh many rice cultivars having various expected features known as high yielding (Upshi) and

some of them are aromatic, salt and drought resistant in nature (BRRI Annual Report 2016-2017). For *in vitro* micropropagation and advance biotechnological research a suitable protocol is very essential, particularly for callus induction, multiplication and plantlet regeneration of rice in Bangladesh. As far as it is known, till now there is not enough report on efficient regeneration system for Bangladeshi indica rice cultivars. Therefore, the present study have been considered on the following objectives- i) to screen the suitable indica rice cultivars for *in vitro* culture systems as well as for callus induction and plant regeneration for advanced biotechnological research, and ii) to investigate the effect of various media and PGRs for efficient callus induction and regeneration with stress pre-treatments factors.

4.1.2 Materials and Methods

4.1.2.1 Plant materials

For callus induction and regeneration out of 23 only 20 (*Oryza sativa* L.) rice genotypes *viz*. Boro- BR3, BR14, BR19, BRRI dhan28, BRRI dhan29, BRRI dhan47, BRRI dhan50, BRRI dhan55, BRRI dhan58 and BRRI dhan59; Aus- BRRI dhan24, BRRI dhan27, BRRI dhan42 and BRRI dhan48; Aman- BR4, BR11, BRRI dhan30, BRRI dhan31 and two local variety (Chinigura and Kalijira) were considered for this work. The mature seeds of all the varieties were collected from Bangladesh Rice Research Institute (BRRI), Joydebpur, Gazipur and Regional Rice Research Center, Shyampur, Rajshahi, Bangladesh. Seeds of some local varieties were also collected from different regions (Rajshahi, Dinajpur, Chapai Nawabgonj and Khulna) of Bangladesh under this study.

4.1.2.2 Methods

4.1.2.2.1 Sterilization of seeds and media used under this study

Mature seeds of studied varieties were dehusked and surface sterilized for 1 min with 70% (v/v) ethanol. Then 0.1% (v/v) mercuric chloride ($HgCl_2$) treatment for 5 min was done for seed sterilization and washed them with 4-5 times by autoclaved distilled water. The compositions of the used media containing inorganic and organic nutrients were followed by the basal media of MS, N6, LS and SK-3 as shown in **Table 04**. The pH of all media

was adjusted to 5.8. The media were solidified with 0.7% agar (Sigma-Aldrich) and autoclaved at 15 pound square inch (psi) for 20 minutes at 121°C. Seed sterilization procedure and inoculation steps are described in **Chapter III, Chapter 3.2.5.**

4.1.2.2.2 Sterilization and callus induction of rice

For callus induction sterilized seeds were inoculated in N6 medium supplemented with six hormonal concentration and combinations e.g. Cont. = 2,4-D (2.0 mg/l) + BAP (0) + Kin (0), T_1 = 2,4-D (1.0 mg/l) + BAP (0.5 mg/l) + Kin (0.5 mg/l), T_2 = 2,4-D (1.5 mg/l) + BAP (0.75 mg/l) + Kin (0.5 mg/l), T_3 = 2,4-D (2.5 mg/l) + BAP (1.0 mg/l) + Kin (0.5 mg/l), T_4 = 2,4-D (3.0 mg/l) + BAP (1.5 mg/l) + Kin (1.0 mg/l), T_5 = 2,4-D (3.5 mg/l) + BAP (2.0 mg/l) + Kin (1.0 mg/l) and T_6 = 2,4-D (4.0 mg/l) + BAP (2.5 mg/l) + Kin (1.0 mg/l). Here, 300 mg/l casein hydrolysate (CA) + 7.0 g/l agar were added for all treatments (T). Then petri dishes were sealed with paraflim and incubated them at 25±1ºC in dark chamber for callus induction. Three weeks old calli were considered to determine the frequency of callus induction induced in different media for the studies rice genotypes. Under this study for one week old calli were transferred onto the same fresh medium for sub-culturing for shoot proliferation. The pH of all the media was adjusted at 5.8.

4.1.2.2.3 Effect of media on callus induction

In this case an experiment was conducted to test the effect of media on callus induction. To prepare callus induction as PGRs 2,4-D (2.0 mg/l) along with other constituents like CA (300 mg/l) + sucrose (30 g/l) + agar (7.0 g/l) was added in four basal media *viz*. MS, N6, B5 and SK-3.

4.1.2.2.4 Effect of media on regeneration

To test the effect of media the suitable concentration and combination of plant growth regulators e.g. BAP (1.0 mg/l) + Kin (1.0 mg/l) + NAA (0.5 mg/l) + CA (300 mg/l) + sucrose (30 g/l) + agar (7.0 g/l) was considered in addition with the mentioned basal media in **Section 4.1.2.2.3**.

4.1.2.2.5 Regeneration media and plant growth regulators

For plant regeneration N6 basal medium was employed that supplemented with four different concentration and combinations of PGRs: i) = BAP (1.5 mg/l) + Kin (0.5 mg/l) + NAA (0.5 mg/l), ii) = BAP (2.0 mg/l) + Kin (1.0 mg/l) + NAA (0.5 mg/l), iii) = BAP (2.5 mg/l) + Kin (1.5 mg/l) + NAA (0.5 mg/l) and iv) = BAP (3.0 mg/l) + Kin (2.0 mg/l) + NAA (0.5 mg/l). For all cases 300 mg/l casein hydrolysate (CA), sucrose (30 g/l) and 7.0 g/l agar were added.

For plant regeneration 4 weeks old calli were transferred in N6 medium (T4) that supplemented with 2,4-D (3.0 mg/l) + BAP (1.5 mg/l) + Kin (1.0 mg/l). After transferring plantlets culture vessels were sealed with paraflim and kept under the 36 watt white fluorescent lamp for 16/8 h light/dark cycle. In this case the intensity of light was 5000 lux. The temperature of the culture room or growth chamber was maintained at 25±1°C. The calli which produced the shoots were counted and considered to determine the regeneration frequencies.

4.1.2.2.6 Root induction

For rooting half strength of MS, N6 and RRM (modified MS) medium was used that supplemented with BAP (2.0 mg/l) + Kin (0.5 mg/l) + NAA (1.0 mg/l) + sucrose (30 g/l) + agar (7.0 g/l). In addition, five types of hormonal combinations were added to RRM. In this case, the induced shoots length of 3 - 5 cm was placed into culture vessels or test tubes containing RIMs. Culture incubation and related other procedures are described in **Section 3.2.5.**

4.1.2.2.7 Acclimatization and hardening of *in vitro* grown plants

Well rooted plants were carefully removed from the culture vessels and washed the root portions under running tap water to clear the entire agar medium to avoid the chances of contamination in soil. Then the plantlets were transferred to small plastic cups diameter of about 5 cm containing pre-soaked sterilized vermiculite. After that the pots were kept inside the moist chamber. To avoid evapotranspirational losses of water the plants covered

with polyethylene bag for 1-2 weeks. After one week the plants were kept under the shade conditions for hardening. Then the plants were transferred to a larger pot diameter of about 20 cm containing the mixture of compost, sand and loamy soil with ratio of 1:1:1. For field culture the plants were kept under open light maintaining the natural environmental conditions.

4.1.2.2.8 Data recording and statistical analysis

Previously described in **Section 3.2.4**.

4.1.3 Results

4.1.3.1 Effect of various media on callus induction

Four types of media *viz*. MS, N6, SK-3 and B5 were tested to observe its effect on callusing of rice. The most effective PGRs as 2,4-D (2.0 mg/l) along with CA (300 mg/l) + sucrose (30 g/l) + agar (7.0 g/l) was considered to all of the tested media under this study. The results showed that N6 medium performed the best callusing (78.67%) for BRRI dhan29 (**Table 05**). On an average of the genotypes N6 showed 61.00% callus induction which was the maximum value among the tested media. However, the medium B5 performed with the lowest value of callusing (37.33%) for BRRI dhan56 and BRRI dhan59. ANOVA showed the significant difference within the media tested for callus induction at $p \leq 0.001$ (**Table 06**). The studied varieties were selected from four groups; such as 10 Boro, 6 Aus and 4 Aman rice cultivars (**Table 5**). However, the varieties of Aus produced the highest value of callus induction (78.67%) when they were cultured in N6 medium (**Table 05, Fig. 05**). Furthermore, the varieties of the same group Aus gave maximum production of callusing in all the media tested.

Table 05: Effect of four basal media on callus induction for 20 rice varieties in Bangladesh

Season	Variety	Media				Mean ± SE
		MS	N6	SK3	B5	
		CI (% ± SE)	CI (% ± SE)	CI (% ± SE)	CI (% ± SE)	
Boro Rice	BR3	46.67 ± 2.98	52.00 ± 2.49	48.00 ± 3.27	40.00 ± 2.98	46.67 ±2.49
	BR14	44.00 ± 1.63	46.67 ± 2.98	41.33 ± 2.49	38.67 ± 2.49	42.67 ±1.72
	BR19	58.67 ± 2.49	58.67 ± 2.49	50.67 ± 1.63	48.00 ± 3.27	54.00 ±2.75
	BRRI dhan28	53.33 ± 2.98	60.00 ± 2.98	52.00 ± 2.49	46.67 ± 2.11	53.00 ±2.74
	BRRI dhan29	74.67 ± 4.90	**78.67** ± **3.89**	69.33 ± 3.40	64.00 ± 3.40	71.67 ±3.19
	BRRI dhan47	54.67 ± 5.33	56.00 ± 4.52	49.33 ± 4.99	44.00 ± 4.52	51.00 ±2.74
	BRRI dhan50	61.33 ± 3.27	57.33 ± 2.67	52.00 ± 2.49	50.67 ± 1.63	55.33 ±2.46
	BRRI dhan55	49.33 ± 4.52	54.67 ± 3.27	45.33 ± 2.49	37.33 ± 2.67	46.67 ±3.65
	BRRI dhan58	52.00 ± 2.49	56.00 ± 3.40	45.33 ± 2.49	42.67 ± 4.00	49.00 ±3.05
	BRRI dhan59	45.33 ± 2.49	50.67 ± 1.63	40.00 ± 2.11	**37.33** ± 2.67	43.33 ±2.96
Aus Rice	BRRI dhan24	72.00 ± 3.89	77.33 ± 3.40	68.00 ± 3.89	64.00 ± 2.67	70.33 ±2.85
	BRRI dhan27	66.67 ± 2.98	70.67 ± 2.67	61.33 ± 3.27	57.33 ± 3.40	64.00 ±2.93
	BRRI dhan42	68.00 ± 3.27	73.33 ± 2.98	62.67 ± 2.67	58.67 ± 2.49	65.67 ±3.19
	BRRI dhan48	54.67 ± 5.33	54.67 ± 3.89	49.33 ± 3.40	48.00 ± 2.49	51.67 ±1.75
Aman Rice	BR4	65.33 ± 2.49	65.33 ± 2.49	61.33 ± 3.89	61.33 ± 2.49	63.33 ±1.15
	BR11	64.00 ± 2.67	66.67 ± 3.65	62.67 ± 3.40	52.00 ± 3.27	61.34 ±3.22
	BRRI dhan30	45.33 ± 2.49	50.67 ± 4.99	46.67 ± 2.11	41.33 ± 3.89	46.00 ±1.93
	BRRI dhan31	60.00 ± 2.98	61.33 ± 2.49	56.00 ± 3.40	49.33 ± 2.67	56.67 ±2.69
Local (Aromatic)	Chinigura	64.00 ± 2.67	66.67 ± 2.11	60.00 ± 3.65	54.67 ± 2.49	61.34 ±2.61
	Kalijira	60.00 ± 2.11	62.67 ± 2.67	49.33 ± 1.63	45.33 ± 2.49	54.33 ±4.16
Mean ± SE		58.00 ± 2.05	**61.00** ± 2.03	53.53 ± 1.92	49.07 ± 1.93	-
F-value (Var.)		16.928	22.187	25.533	25.797	-
Significance		0.000	0.000	0.000	0.000	-
F-value (Rep.)		26.498	35.710	46.045	42.640	-
Significance		0.000	0.000	0.000	0.000	-
Variety $LSD_{0.05}$		3.141	2.717	2.396	22.402	-

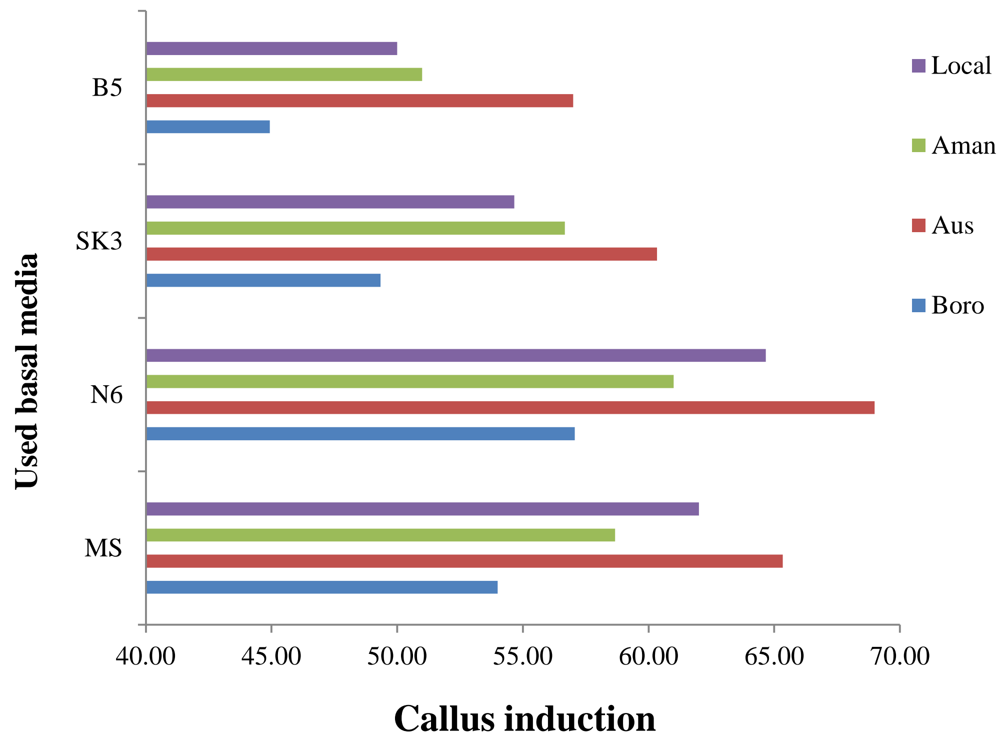


Fig. 05: Effect of basal media on callus induction to various groups of rice varieties in Bangladesh.

Table 06: Variances analysis of four basal media on callus induction

ANOVA of media	Source of Variation	Sum of Squares	df	Mean Square	F-value	Sig.
MS	Variety	7968.677	19	419.404	16.928	0.000
	Replication	2626.069	4	656.517	26.498	0.000
	Error	1882.963	76	24.776	-	-
	Total	12477.709	99	-	-	-
N6	Variety	7813.388	19	411.231	22.187	0.000
	Replication	2647.446	4	661.861	35.710	0.000
	Error	1408.626	76	18.535	-	-
	Total	11869.460	99	-	-	-
B5	Variety	7027.190	19	369.852	25.533	0.000
	Replication	2667.897	4	666.974	46.045	0.000
	Error	1100.883	76	14.485	-	-
	Total	10795.970	99	-	-	-
SK-3	Variety	7069.017	19	372.054	25.797	0.000
	Replication	2459.926	4	614.981	42.640	0.000
	Error	1096.119	76	14.423	-	-
	Total	10625.062	99	-	-	-

df = degrees of freedom, Sig. = Significance.

4.1.3.2 Effect of PGRs on callusing

To examined the effect of plant growth regulators on callus induction, six different hormonal combinations (T_1 - T_6) were added to N6 medium for the studied twenty rice varieties (**Table 7**). It was observed that all the tested genotypes performed well to induce callus. However, the variety BRRI dhan29 induced the highest number of callus (81.33%) when seeds were cultured in T_4 (3.0 mg/l 2,4-D + BAP 1.5 mg/l + Kin 1.0 mg/l). The nearer recorded values were 76.02% for BRRI dhan29 in T_6 (2,4-D 4.0 mg/l + BAP 2.5 mg/l + Kin 1.0 mg/l), 76.02% for BRRI dhan29; T_5 - 74.67% for BRRI dhan29; T_5 - (2,4-D 3.5 mg/l + BAP 2.0 mg/l + Kin 1.0 mg/l) 72.00% for BRRI dhan42 in T_4 (**Table 07**). On the other hand the lowest number of callus induction 32.00% was found for the variety BR3 in T_1 (2,4-D 1.0 mg/l + BAP 0.5 mg/l + Kin 0.5 mg/l).

The average value of six types of hormonal combinations (treatments) showed that the variety BRRI dhan29 performed the highest callus induction (68.19%). The other varieties performed with 64.00, 61.71, and 60.38% for BRRI dhan27, BRRI dhan28 and BRRI dhan58 respectively (**Fig. 06 & 08**). On the other hand the average values of twenty rice varieties expressed that the hormonal concentration T_4 influenced the varieties with the highest performance (58.20%) to produce callus and the lowest (37.67%) in T_1 (**Table 07**). Furthermore, by calculating the average values of hormonal combinations it was found that the varieties of Boro group gave the maximum callusing (54.14%). The group of local varieties produced the minimum callus induction (48.10%, **Fig. 07**). Considering the frequencies of callus induction, analysis of variance (ANOVA) showed the significant differences within the studied genotypes at $p \leq 0.001$ (**Table 09**).

Table 07: Effect of various PGRs on callus induction (CI) with N6 medium

Season	Variety	Treatments			
		Cont.	T_1	T_2	T_3
Boro Rice	BR3	54.67 ± 1.33	32.00 ± 2.49	38.67 ± 2.49	46.67 ± 3.65
	BR14	42.67 ± 2.67	37.33 ± 2.67	38.67 ± 3.27	40.00 ± 2.11
	BR19	52.00 ± 2.49	34.67 ± 1.33	44.00 ± 2.67	52.00 ± 2.49
	BRRI dhan28	53.33 ± 2.11	34.67 ± 3.27	42.67 ± 2.67	50.67 ± 3.40
	BRRI dhan29	69.33 ± 2.67	50.67 ± 1.63	57.33 ± 3.40	68.00 ± 3.27
	BRRI dhan47	52.00 ± 2.49	32.00 ± 2.49	37.33 ± 2.67	45.33 ± 2.49
	BRRI dhan50	54.67 ± 2.49	33.33 ± 2.11	42.67 ± 2.67	50.67 ± 1.63
	BRRI dhan55	48.00 ± 2.49	34.67 ± 2.49	40.00 ± 2.98	42.67 ± 3.40
	BRRI dhan58	49.33 ± 2.67	36.00 ± 3.40	38.67 ± 2.49	44.00 ± 2.67
	BRRI dhan59	46.67 ± 2.11	**32.00 ± 2.49**	37.33 ± 2.67	40.00 ± 2.11
Aus Rice	BRRI dhan24	66.67±3.65	48.00 ± 2.49	53.33 ± 2.11	62.67 ± 2.67
	BRRI dhan27	64.00±4.00	46.67 ± 2.11	56.00 ± 2.67	57.33 ± 2.67
	BRRI dhan42	65.33±4.42	45.33 ± 3.27	54.67 ± 3.89	60.00 ± 3.65
	BRRI dhan48	53.33±2.11	32.00 ± 2.49	41.33 ± 3.27	49.33 ± 2.67
Aman Rice	BR4	66.67±3.65	41.33 ± 3.27	54.67 ± 2.49	58.67 ± 2.49
	BR11	61.33±3.27	38.67 ± 2.49	52.00 ± 2.49	54.67 ± 3.27
	BRRI dhan30	41.33±1.33	33.33 ± 2.11	34.67 ± 3.27	36.00 ± 2.67
	BRRI dhan31	60.00±2.11	37.33 ± 2.67	46.67 ± 4.22	50.67 ± 4.52
Local (Aromatic)	Chinigura	60.00±2.11	37.33 ± 2.67	44.00 ± 1.63	50.67 ± 2.67
	Kalijira	57.33±2.67	36.00 ± 1.63	45.33 ± 2.49	53.33 ± 2.11
Mean ± SE		**55.93 ± 1.82**	**37.67 ± 1.29**	**45.00 ± 1.60**	**50.67 ± 1.82**
F-value (Var.)		22.94	10.76	15.65	18.87
Significance		0.000	0.000	0.000	0.000
F-value (Rep.)		33.71	22.90	31.58	28.98
Significance		0.000	0.000	0.000	0.000
Variety $LSD_{0.05}$		**2.396**	**2.475**	**2.559**	**2.647**

Contd.

Contd. (Table 07)

Season	Variety	Treatments		
		T_4	T_5	T_6
Boro Rice	BR3	49.33 ± 2.67	46.67 ± 3.65	46.67 ± 2.98
	BR14	45.33 ± 3.27	42.67 ± 3.40	41.33 ± 3.27
	BR19	54.67 ± 3.89	52.00 ± 2.49	50.67 ± 2.67
	BRRI dhan28	58.67 ± 3.89	52.00 ± 3.89	50.67 ± 4.00
	BRRI dhan29	**81.33 ± 3.27**	**74.67 ± 3.89**	**76.02 ± 2.67**
	BRRI dhan47	49.33 ± 1.63	46.67 ± 3.65	45.33 ± 3.27
	BRRI dhan50	57.33 ± 2.67	52.00 ± 1.33	50.67 ± 2.67
	BRRI dhan55	49.33 ± 1.63	44.00 ± 4.52	46.67 ± 2.98
	BRRI dhan58	50.67 ± 1.63	45.33 ± 3.89	46.67 ± 2.11
	BRRI dhan59	48.00 ± 1.33	42.67 ± 2.67	45.33 ± 2.49
Aus Rice	BRRI dhan24	76.00 ± 2.67	**70.67 ± 3.40**	70.67 ± 2.67
	BRRI dhan27	68.00 ± 3.27	65.33 ± 2.49	65.33 ± 3.89
	BRRI dhan42	**72.00 ± 3.27**	66.67 ± 3.65	68.00 ± 3.27
	BRRI dhan48	56.00 ± 1.63	50.67 ± 1.63	49.33 ± 1.63
Aman Rice	BR4	62.67 ± 2.67	64.00 ± 2.67	61.33 ± 3.27
	BR11	62.67 ± 1.63	61.33 ± 3.27	58.67 ± 2.49
	BRRI dhan30	44.00 ± 2.67	40.00 ± 2.11	40.00 ± 2.11
	BRRI dhan31	57.33 ± 2.67	58.67 ± 3.89	56.00 ± 2.67
Local (Aromatic)	Chinigura	61.33 ± 3.27	57.33 ± 2.67	56.00 ± 2.67
	Kalijira	60.00 ± 2.11	54.67 ± 1.33	53.33 ± 2.11
Mean ± SE		**58.20** ± **2.28**	**54.40** ± **2.26**	**53.93** ± **2.23**
F-value (Var.)		31.57	26.36	33.08
Significance		0.000	0.000	0.000
F-value (Rep.)		25.29	32.39	34.99
Significance		0.000	0.000	0.000
Variety $LSD_{0.05}$		**2.560**	**2.775**	**2.449**

Table 08: Average values of the treatments Cont. and T_1-T_6 of callus induction

Season	Variety	Statistical parameters		
		Average	SD	CV%
Boro Rice	BR3	44.95 ± 2.80	7.41	16.49
	BR14	41.14 ± 1.03	2.71	6.60
	BR19	48.95 ± 2.98	7.88	16.09
	BRRI dhan28	**61.71 ± 3.46**	9.16	14.85
	BRRI dhan29	**68.19 ± 4.09**	10.82	15.86
	BRRI dhan47	43.62 ± 1.92	5.09	11.67
	BRRI dhan50	44.38 ± 2.03	5.37	12.11
	BRRI dhan55	41.71 ± 2.15	5.69	13.65
	BRRI dhan58	**60.38 ± 2.83**	7.49	12.41
	BRRI dhan59	44.00 ± 2.63	6.97	15.84
Aus Rice	BRRI dhan24	38.48 ± 1.47	3.88	10.09
	BRRI dhan27	**64.00 ± 3.82**	10.10	15.78
	BRRI dhan42	58.48 ± 3.21	8.48	14.51
	BRRI dhan48	55.62 ± 3.18	8.42	15.14
Aman Rice	BR4	52.38 ± 3.08	8.14	15.53
	BR11	52.38 ± 3.36	8.90	16.99
	BRRI dhan30	51.43 ± 3.09	8.18	15.90
	BRRI dhan31	48.57 ± 2.63	6.96	14.34
Local	Chinigura	48.76 ± 3.09	8.18	16.77
(Aromatic)	Kalijira	47.43 ± 3.09	8.18	17.24

CV= Co-efficient of variations.

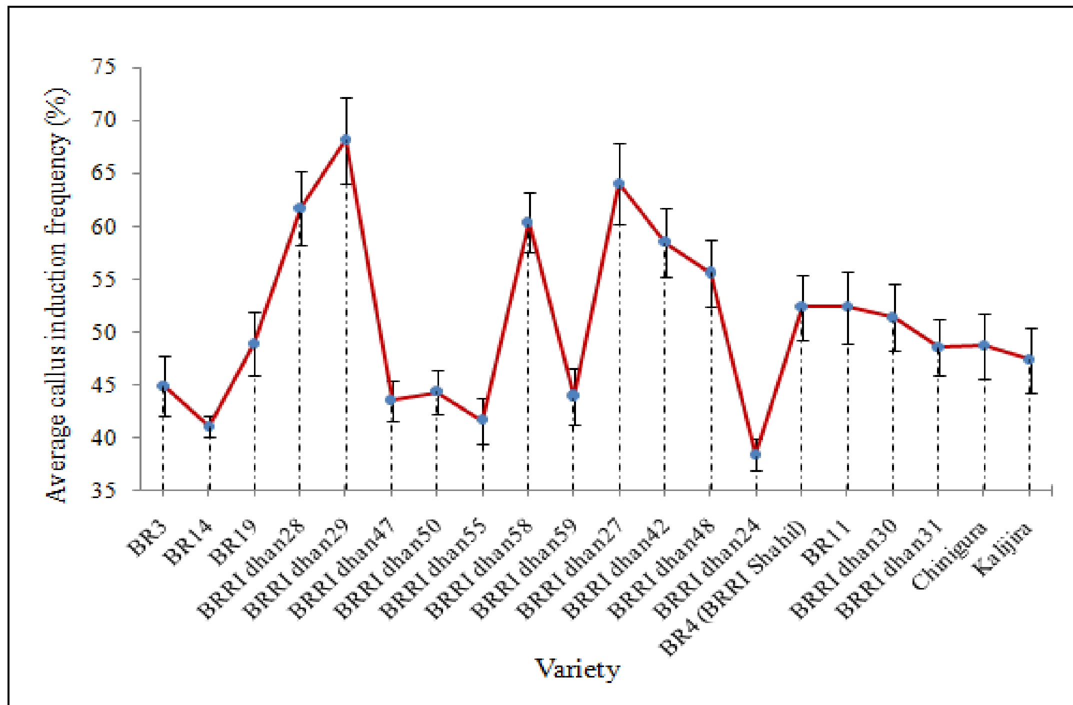


Fig. 06: Average callus induction frequency of studied varieties.

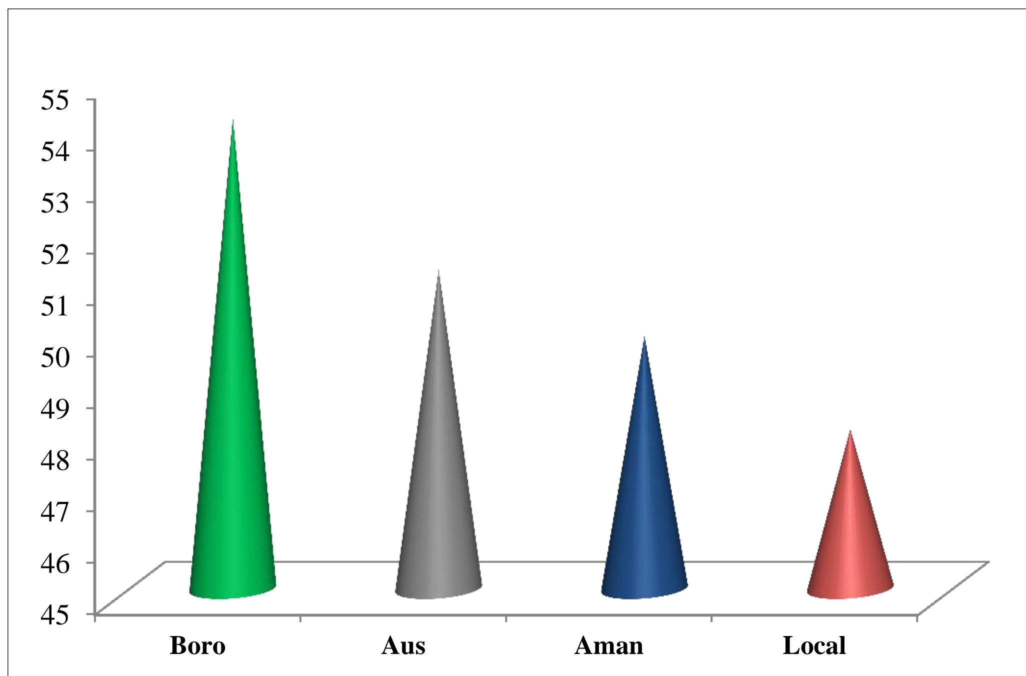


Fig. 07: Callus induction efficiency of different rice groups of studied varieties.

Table 09: Analysis of variances (ANOVAs) of different treatments tested for callus induction

ANOVA of treatment	Source of Variation	Sum of Squares	df	Mean Square	F-value	Sig.
Cont.	Variety	6283.617	19	330.717	22.935	0.000
	Replication	1944.084	4	486.021	33.705	0.000
	Error	1095.916	76	14.420	-	-
	Total	9323.618	99	-	-	-
T_1	Variety	3144.749	19	165.513	10.761	0.000
	Replication	1408.698	4	352.174	22.898	0.000
	Error	1168.912	76	15.380	-	-
	Total	5722.359	99	-	-	-
T_2	Variety	4886.700	19	257.195	15.646	0.000
	Replication	2076.211	4	519.053	31.575	0.000
	Error	1249.345	76	16.439	-	-
	Total	8212.256	99	-	-	-
T_3	Variety	6310.942	19	332.155	18.872	0.000
	Replication	2040.120	4	510.030	28.979	0.000
	Error	1337.596	76	17.600	-	-
	Corrected Total	9688.659	99	-	-	-
T_4	Variety	9871.387	19	519.547	31.571	0.000
	Replication	1664.707	4	416.177	25.289	0.000
	Error	1250.698	76	16.457	-	-
	Total	12786.792	99	-	-	-
T_5	Variety	9681.236	19	509.539	26.356	0.000
	Replication	2512.764	4	628.191	32.493	0.000
	Error	1469.307	76	19.333	-	-
	Total	13663.307	99	-	-	-
T_6	Variety	9466.793	19	498.252	33.078	0.000
	Replication	2108.110	4	527.028	34.989	0.000
	Error	1144.770	76	15.063	-	-
	Total	12719.673	99	-	-	-

df = Degrees of freedom, Sig. = Significance.

4.1.3.3 Effect of media on plant regeneration

A suitable hormonal combination BAP (1.0 mg/l) + Kin (1.0 mg/l) + NAA (0.5 mg/l) was added to the basal media of MS, N6, SK-3 and B5 to investigate their effect on regeneration for all the studied varieties. The results showed that N6 was investigated as the best medium when the callus of BRRI dhan29 (70.67%) was cultured for plant regeneration (**Table 10, Fig. 8A-F**). Furthermore, the variety BRRI dhan29 gave the maximum regeneration in all the media examined i.e. MS (64.00%), SK-3 (62.67%) and B5 (57.33%). However, the lowest value was recorded for BRRI dhan59 (25.33%) when the callus was cultured in B5 (**Table 10**). Analysis of variance (ANOVA) showed the significant difference among the genotypes at the probability of $p \leq 0.001$ (**Table 11**).

4.1.3.4 Effect of PGRs on plant regeneration

To prepare the regeneration media, four types of hormonal combinations along with Control were tested and phytohormones were added to MS basal medium. For plant regeneration, the calli of studied varieties were transferred to regeneration media; and the results were shown in **Table 12** and **Fig. 8 (A-F)**. In the results, it was found that the genotype BRRI dhan29 performed with the highest frequency of plant regeneration (72.00%) with BAP (2.0 mg/l) + Kin (1.0 mg/l) + NAA (0.5 mg/l). On the other hand, the lowest regeneration was recorded for BRRI dhan59 (25.33%) when the calli were transferred to GR4: BAP (3.0 mg/l) + Kin (2.0 mg/l) + NAA (0.5 mg/l). By analyzing the variances, it was shown that the tested varieties were differed significantly considering regeneration efficiency at $p \leq 0.001$ (**Table 11**).

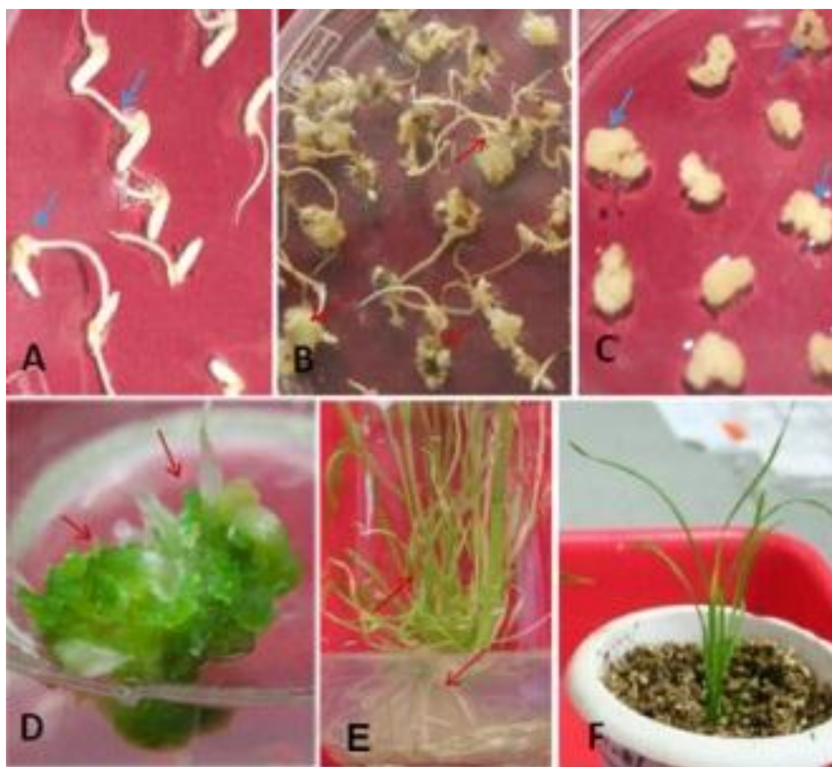


Fig. 8 (A-F): Inoculation of rice seeds and several steps for development of calli and its subsequent regeneration. A) Seven days of inoculated seeds started calli. B) Three weeks old calli, C) Three weeks old calli were transferred to regeneration medium, D) Seed derived calli is becoming greenish and started to regeneration, E) Green plantlets with good root and shoots and F) Plants were transferred in pots after acclimatization.

On an average of four tested hormonal concentration and combinations, BRRI dhan29 (62.40%) was the most efficient genotype to regenerate plant through *in vitro* culture (**Fig. 9**). On the other hand, the lowest frequency of regeneration was found for BRRI dhan24 (29.87%). However, average values of regeneration of the varieties showed that the hormonal concentration and combinations (GR2) like BAP (2.0 mg/l) + Kin (1.0 mg/l) + NAA (0.5 mg/l) influenced the calli to regenerate plants with the highest value of 51.07% (**Table 12**). The lowest frequency of regeneration (42.53%) was recorded (GR4) when BAP (3.0 mg/l) + Kin (2.0 mg/l) + NAA (0.5 mg/l) added in the medium (**Table 12**). Analysis of variance (ANOVA) showed the significant difference among the five different treatments on regeneration (**Table 13**).

Table 10: Effect of four media on regeneration (RG) for 20 rice varieties in Bangladesh

Season	Variety	Media				Mean ± SE
		MS	N6	SK-3	B5	
		RG (% ± SE)	RG (% ± SE)	RG (% ± SE)	RG (% ± SE)	
Boro Rice	BR3	58.67 ± 3.27	61.33 ± 3.89	58.67 ± 2.49	52.00 ± 3.27	57.67 ± 1.99
	BR14	48.00 ± 2.49	49.33 ± 2.67	42.67 ± 2.67	38.67 ± 2.49	44.67 ± 2.46
	BR19	57.33 ± 3.40	61.33 ± 3.27	53.33 ± 4.22	49.33 ± 3.40	55.33 ± 2.58
	BRRI dhan28	54.67 ± 2.49	58.67 ± 3.27	50.67 ± 2.67	46.67 ± 2.98	52.67 ± 2.58
	BRRI dhan29	**64.00 ± 3.40**	**70.67 ± 4.00**	**62.67 ± 4.00**	**57.33 ± 2.67**	63.67 ± 2.74
	BRRI dhan47	49.33 ± 1.63	48.00 ± 2.49	36.00 ± 2.67	36.00 ± 2.67	42.33 ± 3.67
	BRRI dhan50	58.67 ± 3.27	54.67 ± 2.49	49.33 ± 2.67	42.67 ± 2.67	51.34 ± 3.46
	BRRI dhan55	48.00 ± 2.49	54.67 ± 2.49	45.33 ± 2.49	42.67 ± 3.40	47.67 ± 2.58
	BRRI dhan58	57.33 ± 2.67	62.67 ± 3.40	54.67 ± 3.89	50.67 ± 2.67	56.34 ± 2.52
	BRRI dhan59	33.33 ± 2.11	36.00 ± 1.63	28.00 ± 2.49	**25.33 ± 2.49**	30.67 ± 2.43
Aus Rice	BRRI dhan24	42.67 ± 1.63	46.67 ± 2.11	37.33 ± 2.67	34.67 ± 2.49	40.34 ± 2.69
	BRRI dhan27	34.67 ± 2.49	37.33 ± 1.63	29.33 ± 2.67	26.67 ± 2.98	32.00 ± 2.43
	BRRI dhan42	48.00 ± 2.49	52.00 ± 3.27	45.33 ± 2.49	40.00 ± 2.98	46.33 ± 2.52
	BRRI dhan48	57.33 ± 3.40	50.67 ± 2.67	50.67 ± 1.63	48.00 ± 2.49	51.67 ± 1.99
Aman Rice	BR4	49.33 ± 3.40	53.33 ± 2.98	46.67 ± 2.98	44.00 ± 2.67	48.33 ± 1.99
	BR11	38.67 ± 2.49	40.00 ± 2.98	32.00 ± 2.49	30.67 ± 2.67	35.34 ± 2.34
	BRRI dhan30	54.67 ± 2.49	56.00 ± 1.63	49.33 ± 1.63	45.33 ± 2.49	51.33 ± 2.47
	BRRI dhan31	58.67 ± 3.89	61.33 ± 3.27	52.00 ± 1.33	46.67 ± 2.98	54.67 ± 3.31
Local (Aromatic)	Chinigura	56.00 ± 2.67	57.33 ± 1.63	49.33 ± 1.63	46.67 ± 2.98	52.33 ± 2.57
	Kalijira	46.67 ± 2.11	49.33 ± 2.67	41.33 ± 1.33	38.67 ± 2.49	44.00 ± 2.43
Mean ± SE		**50.80 ± 11.36**	**53.07 ± 11.87**	**42.13 ± 9.42**	**45.73 ± 10.23**	**-**
F-value (Var.)		41.488	35.159	44.199	62.479	-
Significance		0.000	0.000	0.000	0.000	-
F-value (Rep.)		70.083	52.358	52.279	121.332	-
Significance		0.000	0.000	0.000	0.000	-
$LSD_{0.05}$		1.859	2.101	1.999	1.498	-

Table 11: Analysis of variances (ANOVAs) of four basal media for regeneration

ANOVA of media	Source of Variation	Sum of Squares	df	Mean Square	F-value	Sig.
MS	Variety	6843.011	19	360.158	41.488	0.000
	Replication	2433.553	4	608.388	70.083	0.000
	Error	659.754	76	8.681	-	-
	Total	9936.318	99	-	-	-
N6	Variety	7406.418	19	389.811	35.159	0.000
	Replication	2321.982	4	580.496	52.358	0.000
	Error	842.614	76	11.087	-	-
	Total	10571.014	99	-	-	-
SK3	Variety	8428.724	19	443.617	44.199	0.000
	Replication	2098.853	4	524.713	52.279	0.000
	Error	762.793	76	10.037	-	-
	Total	11290.369	99	-	-	-
B5	Variety	6691.581	19	352.188	62.479	0.000
	Replication	2735.763	4	683.941	121.332	0.000
	Error	428.407	76	5.637	-	-
	Total	9855.750	99	-	-	-

df = Degrees of freedom, Sig. = Significance.

4.1.3.5 Root induction

To observe the effect of basal media on root induction in MS, ½MS, N6 and RRM (modified MS) were tested. For this purpose around 3-5 cm length of shoot of all the varieties were placed into the culture vessels or test tubes; and the results showed that BRRI dhan29 (90.67%) produced the highest frequency of rooting in RRM medium (**Table 14, Fig. 8A-F**). On the contrary, the lowest value was found for Kalijira (64.00%) when the shoots were cultured in N6 (**Table 14**). In addition to examine the rooting efficiency of the cultivars five concentration and combinations of PGRs were added to RRM and cultured for the shoots. Analysis of variance (ANOVA) showed the significant difference among the four different basal media for root induction (**Table 15**).

The highest result was recorded for BRRI dhan29 (100%) in T_4 where Kin (0.5) + NAA (0.5 mg/l) + sucrose (25 g/l) + phytagel (3.0 g/l) were used. **Table 16** showed the lowest value was found for Kalijira (72.00%) in Kin (0.5) + NAA (1.0 mg/l) + sucrose (30 g/l) + 3.0 g/l phytagel (Cont.). Analysis of variance showed the significance difference among the rice cultivars at $p \leq 0.001$ (**Table 15**).

In another study two basal media e.g. MS and N6 with various plant growth regulators were examined. It was observed that for callus induction (81.33%) and regeneration (72.00) N6 medium showed better responses than MS for BRRI dhan 29. However, for better regeneration MS medium showed highest results (84.00%) also in BRRI dhan 29. Here, Boro rice varieties showed quiet better results on callus induction, regeneration and for root induction than Aus, Aman and local two rice cultivars (**Table 17**).

Table 12: Effect of PGRs on plant regeneration for the twenty rice varieties

Variety	Treatment					Mean ± SE
	Cont. (% ± SE)	GR_1 (% ± SE)	GR_2 (% ± SE)	GR_3 (% ± SE)	GR_4 (% ± SE)	
BR3	56.00 ± 1.63	50.67 ± 1.63	58.67 ± 1.33	52.00 ± 2.49	48.00 ± 1.33	53.07±1.90
BR14	40.00 ± 2.11	36.00 ± 3.40	45.33 ± 3.27	37.33 ± 3.40	32.00 ± 3.27	38.13±2.21
BR19	56.00 ± 2.67	49.33 ± 2.67	56.00 ± 1.63	50.67 ± 1.63	46.67 ± 2.11	51.73±1.86
BRRI dhan28	56.00 ± 3.40	50.67 ± 3.40	60.00 ± 4.22	52.00 ± 3.89	48.00 ± 3.27	53.33±2.11
BRRI dhan29	65.33 ± 3.27	57.33 ± 2.67	**72.00** ± **4.42**	61.33 ± 3.89	56.00 ± 2.67	**62.40**±2.90
BRRI dhan47	54.67 ± 3.27	50.67 ± 3.40	54.67 ± 2.49	53.33 ± 3.65	48.00 ± 3.89	52.27±1.29
BRRI dhan50	50.67 ± 2.67	49.33 ± 2.67	54.67 ± 2.49	52.00 ± 1.33	48.00 ± 2.49	50.93±1.15
BRRI dhan55	42.67 ± 1.63	40.00 ± 2.11	44.00 ± 1.63	36.00 ± 1.63	33.33 ± 2.11	39.20±2.01
BRRI dhan58	45.33 ± 1.33	41.33 ± 1.33	50.67 ± 1.63	49.33 ± 1.63	46.67 ± 2.11	46.67±1.63
BRRI dhan59	49.33 ± 1.63	42.67 ± 1.63	53.33 ± 2.11	48.00 ± 1.33	45.33 ± 2.49	47.73±1.81
BRRI dhan24	32.00 ± 2.49	26.67 ± 3.65	36.00 ± 2.67	29.33 ± 3.40	**25.33** ± 3.89	**29.87**±1.91
BRRI dhan27	54.67 ± 1.33	52.00 ± 2.49	57.33 ± 1.63	52.00 ± 1.33	48.00 ± 1.33	52.80±1.55
BRRI dhan42	30.67 ± 2.67	28.00 ± 2.49	33.33 ± 3.65	37.33 ± 3.40	33.33 ± 2.11	32.53±1.55
BRRI dhan48	53.33 ± 2.11	50.67 ± 3.40	57.33 ± 1.63	49.33 ± 1.63	44.00 ± 2.67	50.93±2.21
BR4	44.00 ± 1.63	40.00 ± 2.11	48.00 ± 1.33	44.00 ± 1.63	40.00 ± 2.11	43.20±1.50
BR11	44.00 ± 2.67	44.00 ± 2.67	49.33 ± 1.63	48.00 ± 2.49	44.00 ± 3.40	45.87±1.16
BRRI dhan30	42.67 ± 1.63	40.00 ± 2.11	45.33 ± 2.49	41.33 ± 1.33	37.33 ± 2.67	41.33±1.33
BRRI dhan31	48.00 ± 1.33	45.33 ± 2.49	52.00 ± 2.49	49.33 ± 1.63	46.67 ± 2.98	48.27±1.15
Chinigura	37.33 ± 1.63	36.00 ± 1.63	37.33 ± 1.63	37.33 ± 1.63	36.00 ± 1.63	36.80±0.33
Kalijira	52.00 ± 2.49	48.00 ± 2.49	56.00 ± 1.63	48.00 ± 1.33	44.00 ± 2.67	49.60±2.04
Mean ± SE	**47.73 ± 1.96**	**43.93 ± 1.80**	**51.07 ± 2.05**	**46.39 ± 1.72**	**42.53 ± 1.66**	**-**
F-value (Var.)	35.489	20.390	29.672	20.709	21.610	-
Significance	0.000	0.000	0.000	0.000	0.000	-
F-value (Rep.)	28.915	23.646	24.407	22.315	36.332	-
Significance	0.000	0.000	0.000	0.000	0.000	-
$LSD_{0.05}$	**2.079**	**2.520**	**2.372**	**2.387**	**2.255**	-

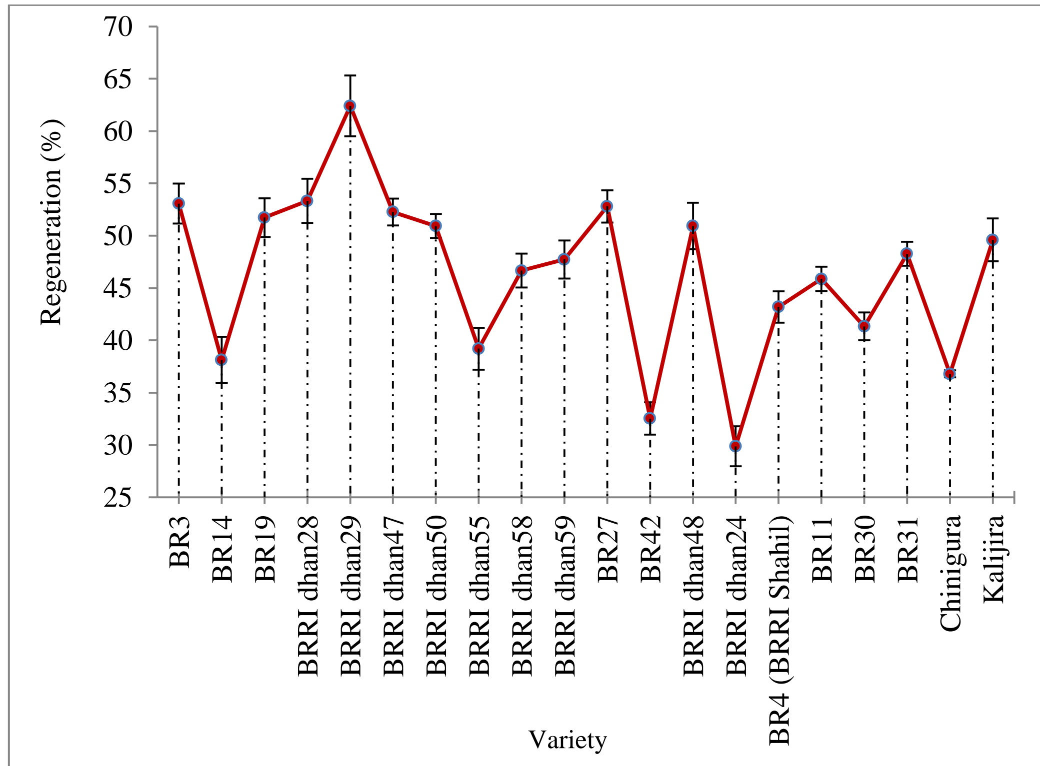


Fig. 09: Average values of four concentration and combinations of PGRs (Control and GR1-GR4) to plant regeneration for the studied varieties.

Table 13: Analysis of variances (ANOVAs) of five different treatments for plant regeneration

ANOVA of PGRs	Source of Variation	Sum of Squares	df	Mean Square	F-value	Sig.
Cont.	Variety	7317.003	19	385.105	35.489	0.000
	Replication	1255.065	4	313.766	28.915	0.000
	Error	824.696	76	10.851	-	-
	Total	9396.763	99	-	-	-
GR_1	Variety	6176.735	19	325.091	20.390	0.000
	Replication	1508.037	4	377.009	23.646	0.000
	Error	1211.724	76	15.944	-	-
	Total	8896.495	99	-	-	-
GR_2	Variety	7966.341	19	419.281	29.672	0.000
	Replication	1379.560	4	344.890	24.407	0.000
	Error	1073.933	76	14.131	-	-
	Total	10419.835	99	-	-	-
GR_3	Variety	5628.036	19	296.212	20.709	0.000
	Replication	1276.693	4	319.173	22.315	0.000
	Error	1087.049	76	14.303	-	-
	Total	7991.779	99	-	-	-
GR_4	Variety	5243.368	19	275.967	21.610	0.000
	Replication	1855.888	4	463.972	36.332	0.000
	Error	970.539	76	12.770	-	-
	Total	8069.795	99	-	-	-

df = degrees of freedom, Sig. = Significance.

Table 14: Effect of four basal media on rooting (RT) using 20 rice varieties in Bangladesh

Season	Rice cultivars	Media (mg/l)				Mean ± SE
		MS (% ± SE)	½ MS (% ± SE)	N6 (% ± SE)	RRM (% ± SE)	
Boro Rice	BR3	74.67 ± 3.27	81.33 ± 2.49	78.67 ± 3.89	84.00 ± 4.00	79.67 ± 1.99
	BR14	76.00 ± 3.40	78.67 ± 4.90	76.00 ± 5.81	82.67 ± 3.40	78.34 ± 1.58
	BR19	80.00 ± 4.22	84.00 ± 5.81	81.33 ± 6.11	86.67 ± 4.71	83.00 ± 1.48
	BRRI dhan28	84.00 ± 4.52	86.67 ± 5.58	80.00 ± 4.71	86.67 ± 6.99	84.34 ± 1.58
	BRRI dhan29	82.67 ± 4.99	86.67 ± 3.65	82.67 ± 3.40	**90.67** ± 4.52	85.67 ± 1.91
	BRRI dhan47	80.00 ± 2.98	82.67 ± 4.00	77.33 ± 5.42	85.33 ± 3.89	81.33 ± 1.72
	BRRI dhan50	77.33 ± 4.52	78.67 ± 4.90	72.00 ± 2.49	81.33 ± 4.90	77.33 ± 1.96
	BRRI dhan55	81.33 ± 3.27	84.00 ± 4.52	77.33 ± 4.00	85.33 ± 3.89	82.00 ± 1.76
	BRRI dhan58	76.00 ± 4.52	82.67 ± 4.00	74.67 ± 4.90	84.00 ± 3.40	79.34 ± 2.34
	BRRI dhan59	72.00 ± 2.49	76.00 ± 3.40	68.00 ± 4.90	78.67 ± 3.27	73.67 ± 2.33
Aus Rice	BRRI dhan24	74.67 ± 3.27	78.67 ± 2.49	70.67 ± 3.40	81.33 ± 2.49	76.34 ± 2.33
	BRRI dhan27	76.00 ± 4.52	81.33 ± 3.89	72.00 ± 2.49	84.00 ± 4.00	78.33 ± 2.69
	BRRI dhan42	81.33 ± 5.33	86.67 ± 5.58	81.33 ± 6.46	84.00 ± 4.52	83.33 ± 1.28
	BRRI dhan48	78.67 ± 4.90	81.33 ± 4.90	72.00 ± 5.33	78.67 ± 3.89	77.67 ± 1.99
Aman Rice	BR4	69.33 ± 4.00	70.67 ± 3.40	66.67 ± 3.65	78.67 ± 3.89	71.34 ± 2.58
	BR11	73.33 ± 4.71	80.00 ± 6.99	74.67 ± 5.73	82.67 ± 6.53	77.67 ± 2.20
	BRRI dhan30	70.67 ± 4.00	76.00 ± 3.40	66.67 ± 4.22	72.00 ± 2.49	71.34 ± 1.92
	BRRI dhan31	66.67 ± 4.22	73.33 ± 5.58	72.00 ± 5.33	78.67 ± 2.49	74.67 ± 1.77
Local (Aromatic)	Chinigura	66.67 ± 3.65	70.67 ± 4.99	65.33 ± 2.49	74.67 ± 3.89	70.22 ± 2.34
	Kalijira	66.67 ± 2.98	72.00 ± 2.49	**64.00** ± 4.00	76.00 ± 4.00	69.67 ± 2.69
Mean ± SE		76.37 ± 1.07	79.6 0 ± 1.15	73.67 ± 1.27	81.80 ± 1.02	77.76 ± 1.07
F-value (Var.)		9.229	7.413	6.960	6.499	-
Significance		0.000	0.000	0.000	0.000	-
F-value (Rep.)		82.198	94.793	72.060	91.169	-
Significance		0.000	0.000	0.000	0.000	-
$LSD_{0.05}$		0.379	0.400	0.456	0.381	-

Callus induction (CI): N6 and MS supplemented with 2, 4-D (3.0 mg/l) + BAP (1.5 mg/l) + Kin (1.0 mg/l) + CA (300 mg/l) + 7.0 g/l agar. Regeneration (RG): N6 and MS supplemented with BAP (2.0 mg/l) + Kin (1.0) + NAA (0.5 mg/l) + sucrose (30 g/l) + 7.0 g/l agar. Root induction (RI): N6 and MS + Kin (0.5) + NAA (0.5 mg/l) + sucrose (25 g/l) + 3.0 g/l phytagel.

Table 15: Analysis of variances (ANOVAs) of four different basal media for root induction

ANOVA of basal media	Source of Variation	Sum of Squares	df	Mean Square	F-value	Sig.
MS	Variety	63.242	19	3.329	9.229	0.000
	Rep.	118.575	4	29.644	82.198	0.000
	Error	27.408	76	0.361	-	-
	Total	211.390	99	-	-	-
½MS	Variety	56.639	19	2.981	7.413	0.000
	Rep.	152.472	4	38.118	94.793	0.000
	Error	30.561	76	0.402	-	-
	Total	239.640	99	-	-	-
N6	Variety	69.122	19	3.638	6.960	0.000
	Rep.	150.672	4	37.668	72.060	0.000
	Error	39.728	76	0.523	-	-
	Total	258.750	99	-	-	-
RRM	Variety	45.095	19	2.373	6.499	0.000
	Rep.	133.178	4	33.295	91.169	0.000
	Error	27.755	76	0.365	-	-
	Total	203.710	99	-	-	-

Table 16: Effect of various PGRs on rooting with RRM medium (modified MS)

Variety	Treatment					Mean ± SE
	Cont. (% ± SE)	T_1 (% ± SE)	T_2 (% ± SE)	T_3 (% ± SE)	T_4 (% ± SE)	
BR3	85.33 ± 3.89	81.33 ± 2.49	90.67 ± 4.71	86.67 ± 6.99	98.67 ± 1.33	88.53 ± 2.94
BR14	81.33 ± 2.49	80.00 ± 4.71	85.33 ± 3.89	78.67 ± 3.89	85.33 ± 3.40	82.13 ± 1.37
BR19	86.67 ± 4.71	80.00 ± 4.71	85.33 ± 3.89	81.33 ± 2.49	90.67 ± 4.71	84.80 ± 1.91
BRRI dhan28	84.00 ± 4.00	92.00 ± 2.49	86.67 ± 6.99	90.67 ± 4.71	97.33 ±1.63	90.13 ± 2.29
BRRI dhan29	92.00 ± 2.49	93.33 ± 2.98	97.33 ± 1.63	96.00 ± 1.63	**100.00** ± 0.00	95.73 ± 1.42
BRRI dhan47	84.00 ± 3.40	90.67 ± 4.71	85.33 ± 3.89	82.67 ± 6.53	90.67 ± 4.71	86.67 ± 1.69
BRRI dhan50	82.67 ± 6.53	81.33 ± 2.49	86.67 ± 4.71	84.00 ± 3.40	92.00 ± 2.49	85.33 ± 1.89
BRRI dhan55	84.00 ± 4.00	90.67 ± 4.71	85.33 ± 3.89	81.33 ±2.49	98.65 ± 4.00	88.00 ± 3.07
BRRI dhan58	85.33 ± 3.89	80.00 ± 4.71	81.33 ± 2.49	78.67 ± 3.27	84.00 ± 3.40	81.87 ± 1.24
BRRI dhan59	78.67 ± .27	80.00 ± 4.71	84.00 ± 4.52	80.00 ± 4.71	86.63 ± 6.99	81.87 ± 1.50
BRRI dhan24	82.67 ± 3.40	80.00 ± 4.71	85.33 ± 3.89	81.33 ± 2.49	86.67 ± 6.99	83.20 ± 1.24
BRRI dhan27	86.67 ± 6.99	82.67 ± 6.53	84.00 ± 4.52	82.67 ± 6.53	92.00 ± 2.49	85.60 ± 1.76
BRRI dhan42	84.00 ± 4.52	81.33 ± 2.49	86.67 ± 4.71	90.67 ± 4.71	98.62 ± 4.00	88.27 ± 3.02
BRRI dhan48	78.67 ± 3.89	81.33 ± 2.49	78.67 ± 3.89	80.00 ± 4.71	86.65 ± 6.99	81.07 ± 1.48
BR4	78.67 ± 3.89	80.00 ± 4.71	86.67±4.71	82.67 ± 6.53	98.63 ± 4.00	85.34 ± 3.60
BR11	74.67 ± 3.89	81.33 ± 2.49	78.67±3.89	78.67±3.89	98.67 ± 4.00	81.87 ± 4.47
BRRI Dhan30	81.33 ± 4.90	80.00 ± 4.71	81.33±2.49	78.67±3.27	90.66 ± 4.71	82.40 ± 2.13
BRRI dhan31	78.67 ± 2.49	84.00 ± 4.52	81.33±2.49	86.67±6.99	90.60 ± 4.71	84.27 ± 2.08
Chinigura	76.00 ± 4.00	81.33 ± 2.49	84.00±4.52	81.33±2.49	86.68 ± 6.99	81.87 ± 1.77
Kalijira	**72.00** ± 2.49	78.67 ± 3.89	78.67±3.89	78.67±3.89	80.00 ± 4.71	78.14 ± 0.90
Mean	**81.87 ± 1.05**	**83.00 ± 1.03**	**84.67 ± 0.97**	**83.07 ± 1.07**	**91.67 ± 1.34**	**-**
F-value (Var.)	6.512	7.031	6.45	8.431	9.462	-
Significance	0.000	0.000	0.000	0.000	0.000	-
F-value (Rep.)	62.148	84.393	69.073	71.153	86.532	-
Significance	0.000	0.000	0.000	0.000	0.000	-
$LSD_{0.05}$	0.357	0.325	0.432	0.362	0.403	-

Cont. = RRM + Kin (0.5) + NAA (1.0 mg/l) + sucrose (30 g/l) + 3.0 g/l phytagel, T_1 = RRM + Kin (0.5) + NAA (1.0 mg/l) + sucrose (20 g/l) + 3.0 g/l gelrite, T_2 = RRM + Kin (0.5) + NAA (1.0 mg/l) + sucrose (30 g/l) + 3.0 g/l gelrite, T_3 = RRM + Kin (0.5) + NAA (0.5 mg/l) + sucrose (20 g/l) + 3.0 g/l phytagel, T_4 = RRM + Kin (0.5) + NAA (0.5 mg/l) + sucrose (25 g/l) + 3.0 g/l phytagel.

Table 17: Comparison of studied rice cultivars on CI, regeneration and root induction using MS and N6 media

Season	Rice cultivars	Media					
		MS			N6		
		CI (% ± SE)	RG (% ± SE)	RI (% ± SE)	CI (% ± SE)	RG (% ± SE)	RI (% ± SE)
Boro Rice	BR3	48.67 ± 1.63	54.00 ± 2.49	82.67 ± 4.99	49.33 ± 2.67	58.67 ± 1.33	81.33 ± 6.11
	BR14	41.00 ± 2.98	38.67 ± 2.49	74.67 ± 4.52	45.33 ± 3.27	45.33 ± 3.27	72.00 ± 4.81
	BR19	51.67 ± 2.49	54.33 ± 2.67	76.00 ± 3.27	54.67 ± 3.89	56.00 ± 1.63	77.33 ± 5.42
	BRRI dhan28	53.33 ± 4.90	54.67 ± 2.49	80.00 ± 4.22	58.67 ± 3.89	60.00 ± 4.22	80.00 ± 4.71
	BRRI dhan29	75.67 ± 2.98	64.00 ± 3.27	84.00 ± 3.40	**81.33 ± 3.27**	**72.00 ± 4.42**	82.67 ± 3.40
	BRRI dhan47	53.67 ± 3.27	58.33 ± 3.40	80.00 ± 2.98	49.33 ± 1.63	54.67 ± 2.49	78.67 ± 4.71
	BRRI dhan50	53.33 ± 5.33	48.00 ± 2.49	81.33 ± 3.27	57.33 ± 2.67	54.67 ± 2.49	72.00 ± 4.49
	BRRI dhan55	48.33 ± 4.52	46.67 ± 1.63	77.33 ± 4.52	49.33 ± 1.63	44.00 ± 1.63	74.67 ± 3.40
	BRRI dhan58	47.00 ± 2.49	42.00 ± 2.11	78.67 ± 2.49	50.67 ± 1.63	50.67 ± 1.63	77.33 ± 4.00
	BRRI dhan59	43.33 ± 2.49	57.33 ± 3.40	76.00 ± 4.52	48.00 ± 1.33	53.33 ± 2.11	72.00 ± 3.65
Aus Rice	BRRI dhan24	69.00 ± 5.33	34.67 ± 2.49	70.67 ± 4.52	76.00 ± 2.67	36.00 ± 2.67	72.00 ± 5.33
	BRRI dhan27	61.67 ± 3.27	48.67 ± 2.49	72.00 ± 4.90	68.00 ± 3.27	57.33 ± 1.63	68.00 ± 4.90
	BRRI dhan42	67.00 ± 2.98	33.33 ± 2.11	81.33 ± 5.33	72.00 ± 3.27	33.33 ± 3.65	81.33 ± 6.46
	BRRI dhan48	52.67 ± 3.89	47.33 ± 1.63	69.33 ± 4.00	56.00 ± 1.63	57.33 ± 1.63	64.67 ± 5.73
Aman Rice	BR4	54.33 ± 2.49	45.00+3.89	76.00±4.00	62.67+2.67	48.00+1.33	70.67±4.90
	BR11	52.00 ± 2.67	49.33+3.40	73.33±4.71	62.67+1.63	49.33+1.63	74.67±2.49
	BRRI dhan30	40.33 ± 2.49	58.67+3.27	74.67±3.27	44.00+2.67	45.33+2.49	64.00±4.22
	BRRI dhan31	52.00 ± 2.98	57.67+2.49	66.67±2.98	57.33+2.67	52.00+2.49	73.67± 4.00
Local (Aromatic)	Chinigura	60.00 ± 2.05	51.33 ± 2.67	69.67 ± 3.65	61.33 ± 3.27	37.33 ± 1.63	65.33 ± 2.49
	Kalijira	58.00 ± 2.67	56.00 ± 3.40	66.33 ± 4.22	60.00 ± 2.11	56.00 ± 1.63	64.67 ± 1.27
	Mean	54.15 ± 2.03	50 ± 1.85	75.53 ± 1.17	58.20 ± 2.28	51.07 ± 2.05	73.35 ± 1.33

4.1.4 Discussion

4.1.4.1 Effect of media on callus induction

Aananthi et al. (2010) tested two basal media MS and N6 for callus induction using five indica rice (*Oryza sativa* L.) cultivars *viz.* ASD 16, White Ponni, Pusa Basmati 1, Pusa Sugandh 4 and Pusa Sugandh 5. They recorded the highest callus induction for White Ponni (88.75%) in MS + 2.0 mg/l 2,4-D and Pusa Basmati 1 (84.97%) in MS + 2.5 mg/l 2,4-D. The authors described that MS performed better than N6. The results of present study, argued with the previous reports and claimed that the basal medium N6 performed the best callus induction than MS, SK-3 and B5 when a suitable hormonal concentration 2,4-D (2.0 mg/l) + BAP (0) + Kin (0) was added to callus induction medium. In this case, the variety BRRI dhan29 gave the maximum callus (78.67%) in N6; while the highest values were 74.67, 69.33 and 64.00% in MS, SK-3 and B5 for the same variety BRRI dhan29 respectively. Therefore, the present investigations noticed that all the tested varieties produced better callusing in N6 medium than MS, SK-3 and B5 except BRRI dhan50. The variety BRRI dhan50 gave maximum callusing in MS (61.33%) and minimum in B5 (50.67%).

4.1.4.2 Effect of PGRs to callus induction

In the present study, six (T_1-T_6) different concentrations and combinations of plant growth regulators were added in N6 medium and examined the effect on callus induction for studied twenty rice varieties. The results showed that the frequencies of callus induction was ranged as 32.00 - 81.33% and the highest efficiency was found for BRRI dhan29 in T_4 = 2,4-D (3.0 mg/l) + BAP (1.5 mg/l) + Kin (1.0 mg/l). The lowest number of callus induction was found for the varieties of BR3, BRRI dhan47, BRRI dhan48 and BRRI dhan59 in T_1 = 2,4-D (1.0 mg/l) + BAP (0.5 mg/l) + Kin (0.5 mg/l).

Tiwari et al. (2012) reported that for maximum CI, the optimum hormonal combination was 1.5 mg/l 2,4-D + 0.1 mg/l NAA + 0.1 mg/l BAP with MS medium. They have recorded 85% and 90% callus induction for indica rice varieties of Pusa Basmati1 and Kalanamak respectively. Present investigation was nearly similar to the previous reports on

callus induction efficiency while the rice varieties and the hormonal concentration and combinations were different. Upadhyaya et al. (2015) used different concentrations of 2,4-D (1.0, 1.5, 2.0, 2.5 mg/l) for callus induction from mature embryos of three japonica rice cultivars *viz.* Sita, Rupali and Masuri. They recorded 63.36% to 92.23% callus induction and mentioned that all the varieties produced calli at the highest rate in 2.0 mg/l 2,4-D concentration.

Sah et al. (2014) reported that embryogenic callus induction in *Oryza sativa* var. Kitaake a japonica rice cultivar. They used MS medium supplemented with 3.0 mg/l 2,4-D + 0.25 mg/l BAP for callus induction. Mannan et al. (2013) induced calli from Bangladeshi aromatic rice var. Kalijira and Chinigura using MS medium supplemented with different concentrations of 2,4-D (1.0, 1.5, 2.0, 2.5 mg/l). They recorded highest 97.22 and 94.44% callus induction in 2.0 mg/l 2,4-D for Kalijira and Chinigura respectively. They also expressed that on the combined effect of 2.0 mg/l 2,4-D with Kin (0.25, 0.5, 1.0, 1.5 mg/l) to induce calli; and recorded the highest values (97.22%) for the varieties Kalijira and for Chinigura (94.44%) when 2.0 mg/l 2,4-D was used singly; while they reported that callus initiation days were decreased at combined condition of 2.0 mg/l 2,4-D + 0.5 mg/l Kin. However, in this study, it was observed that the combined effect of 2,4-D, BAP and Kin was most suitable PGRs to induce the highest callus induction for BRRI dhan29 (81.33%) in T_4 = 2,4-D (3.0 mg/l) + BAP (1.5 mg/l) + Kin (1.0 mg/l). Previously it was reported that 2.5 mg/l 2,4-D individually influenced better to induce efficient calli than combined with Kin for BRRI dhan29 and recorded highest 78% callus induction.

Zuraida et al. (2010) reported that callus induction frequency depends on genotype; and most indica rice cultivars had poor callusing potentiality. Abe and Futsuhara (1986) mentioned that potentiality of callus induction depends on a number of factors, like genotype, physiological and biochemical status of the explants, composition and concentration of different ingredients of culture medium while among these factors, genotypic difference is the most important one. It was reported that all the indica sub-species have no equal potentiality for *in vitro* responses (Seraj et al. 1997, Khanna and Raina 1998). Genotypic effect also reported by several authors in indica rice (Khatun et al.

2010), maize (Morshed et al. 2014), wheat (Saha et al. 2015), sugarcane (Roy et al. 2011) and *Rorippa indica* (Xu et al. 2016). Makerly et al. (2012) recorded highest percentage of callus induction in MS supplemented with NAA for Malaysian indica rice cultivars MR232 (41%) and MR220 (37%); and also reported that the varieties responded lower in 2,4-D.

Upadhyaya et al. (2015) used 2.0 mg/l 2,4-D with MS and induced calli in 10 h light and 14 h of dark cycle. Three varieties of indica rice, Sita, Rupali and Masuri resulted optimum frequency of callus initiation at 2.0 mg/l 2,4-D concentration whereas at a high or low concentration the cultivars showed similar tendency of decrease in callus initiation. Therefore, they stated that the use of 2,4-D with 2.0 mg/l was adequate for production of high amount of callus in rice. In the present investigation the results were differed with their reports, and mentioned that studied varieties responded with high efficiency of callus induction in 3.0 mg/l 2,4-D concentration along with 1.5 mg/l BAP and 1.0 mg/l Kin.

4.1.4.3 Effect of basal media on regeneration efficiency in rice

To regenerate plants a suitable hormonal combination of BAP (1.0 mg/l) + Kin (1.0 mg/l) + NAA (0.5 mg/l) was added as constant to the basal media e.g. MS, N6, SK-3 and B5 to test their effect on regeneration; and the results showed that N6 (70.67%) was the best medium. Hoque et al. (2007) reported on plant regeneration for Bangladeshi indica rice varieties *viz*. BR14, BRRI dhan28, BRRI dhan38, BRRI dhan39 and BRRI dhan40 and mentioned that highest regeneration (44.30%) for BRRI dhan38 by using MS basal medium supplemented with 1.0 mg/l Kin + 1.0 NAA mg/l. They also stated that Bangladeshi indica rice cultivar as BRRI dhan38 and BRRI dhan39 performed with lower efficiency than the Japonica rice cultivar in Taipei-309. In the present investigation, N6 gave best performance than the previous reports. It could be happened due to different hormonal combinations and the genotypes also. However, the embryogenic and non-embryogenic callus formation as well as plant regeneration is reported to be genetically determined as described for different cereals including rice (Khanna and Raina 1998, Hoque and Mansfield 2004, Khalequzzaman et al. 2005, Khatun et al. 2010) and in wheat

(Özgen et al. 1996) and barley (Powell and Dunwell 1987, Haque and Islam 2014). Abe and Futsuhara (1986) reported that many Japonica rice cultivars respond better than the indica varieties. Upadhyaya et al. (2015) used 2,4-D (2.0 mg/l) with MS to induce calli in 10 h light and 14 h of dark cycle. They added that in addition 2.0 mg/l concentration was also found to be more suitable for plant regeneration i.e. shoot initiation or plantlet formation. After proliferation of calli with somatic embryogenic features were identified and sub-cultured in the same media and concentration of growth regulator for regeneration i.e. shoot, root or plantlet formation.

4.1.4.4 Effect of PGRs on regeneration

Zinnah et al. (2013) measured the highest frequency of regeneration (80%) for BRRI dhan38 and 60% for Chini Kanai in MS medium that supplemented with 2.0 mg/l Kin + 1.0 mg/l NAA + 2 mg/l BA. Haque et al. (2013) added 1.0 mg/l Kin and 1.0 mg/l NAA along with four concentrations of BA (1.0, 2.0, 3.0 and 4.0 mg/l) that supplemented with MS medium for plant regeneration from BRRI dhan53; and recorded highest regeneration (75%) with 3.0 mg/l BA. Reddy et al. (2013) used 0.5 mg/l NAA + 2.0 mg/l Kin + 0.50 mg/l BA with MS medium for plant regeneration from 'Gorah' an indigenous rice line in South Bengal, India. Mannan et al. (2013) measured 83.33 and 91.67% regeneration in 0.5 mg/l BAP + 0.1 mg/l IBA for the rice varieties of Kalijira and Chinigura respectively. Alam et al. (2012) examined four rice varieties and mentioned that among four varieties the highest regeneration was found for BRRI dhan29 (84.33%) which required only 14.80 days for green bud formation and the lowest in BINA dhan7 (39.67%) required maximum 15.47 days for green bud formation. They also found that BRRI dhan29 produced highest number of shoots (4.67) per callus while BINA dhan7 showed minimum number of shoots (2.87) per callus. Where BRRI dhan47 was the best performer considering efficiency to induce calli in MS supplemented with 2.0 mg/l 2 4-D + 0.8 mg/l BAP (Alam et al. 2012). By using MS + 2.0 mg/l BAP + 0.5 mg/l NAA + 1.0 mg/l Kin, maximum 31.75, 38.10 and 30.16% regeneration was recorded for BR10, BRRI dhan32 and BRRI dhan47 respectively (Siddique et al. 2014).

However, in the present study the range of plant regeneration efficiency was 25.33 - 72.00% for twenty tested rice varieties in five types of hormonal combinations and combinations of GR_1-GR_4 along with control. In the results the highest plant regeneration was recorded for BRRI dhan29 in GR_2 = BAP (2.0 mg/l) + Kin (1.0 mg/l) + NAA (0.5 mg/l) and the lowest for BRRI dhan55 in GR_4 = BAP (3.0 mg/l) + Kin (2.0 mg/l) + NAA (0.5 mg/l). By analyzing the variances to regenerate plant *in vitro* it was indicated that the studied varieties were significantly differed at $p \leq 0.001$. Alam et al. (2012) examined the regeneration efficiency of four rice varieties and mentioned that BRRI dhan29 (84.33%) was the highest performer among the varieties. Present reports quiet similar to their investigations where the genotype BRRI dhan29 produced 72.00% green plants from *in vitro* callus culture. Furthermore, it was also observed that out of twenty rice varieties BRRI dhana29 produced maximum regenerations in most of the case for hormonal concentration and combinations examined under this study. Therefore, it could be claimed that BRRI dhan29 was the most suitable variety to induce callus and subsequent plant regeneration efficiently. It was observed that out of five hormonal concentration and combinations all the genotypes gave their highest performance in GR_2. Hence, the present study suggested that the hormonal combination GR_2 = BAP (2.0 mg/l) + Kin (1.0 mg/l) + NAA (0.5 mg/l) was the most effective treatment to regenerate plants *in vitro*.

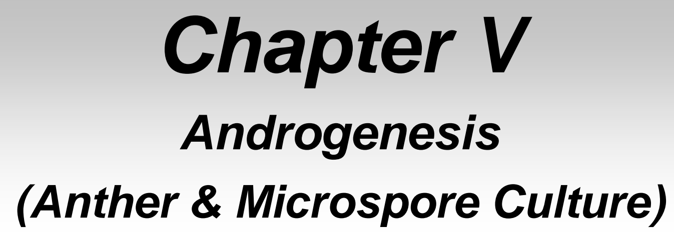
4.1.4.5 Induction of root

Four basal media such as, MS, ½MS, N6 and RRM were tested to find out the most effective medium for rooting of all the experimented rice varieties. In this study, the medium RRM influence the varieties to produce maximum rooting frequencies range of 64.00 to 90.67% where BRRI dhan29 performed best in RRM and Kalijira was worst in N6. In the previous reports it was expressed that half strength MS medium produced 26.10% well developed root in half strength of MS medium from LX278 an elite line of japonica × indica cross (Evangelista 2009). Alam et al. (2012) reported the highest number of roots for BRRI dhan29 (3.66) and lowest for BINA dhan7 (3.11) by using the half strength of MS medium. In the same medium three weeks old *in vitro* regenerated plants

produced vigorous roots for Bangladeshi indica rice BRRI dhan14, BRRI dhan28, BRRI dhan29, BRRI dhan38, BRRI dhan39, BRRI dhan40 and Joponica rice Taipei 309 (Hoque et al. 2007). The findings of this studies agued the previous reports and claimed that the medium RRM best than other media e.g. MS, ½MS and N6 were tested in this study. Besides, the results of testing the effect of the PGRs on rooting was found that the range of the frequencies of root inductions were 72 to 100%. In this case, the highest value was recorded for BRRI dhan29 (100%) in T_4 = Kin (0.5) + NAA (0.5 mg/l) + sucrose (25 g/l) + phytagel (3.0 g/l) and the lowest was for Kalijira (72.00%) in Kin (0.5) + NAA (1.0 mg/l) + sucrose (30 g/l) + 3.0 g/l phytagel (Cont.). However, the comparison of the effect of tested media expressed the betterment of N6 than MS on CI, regeneration and root induction for studied varieties.

4.1.5 Conclusion

The present study provides a simple protocol to produce plants *in vitro* with high frequency of callus induction and its subsequent regeneration for Bangladeshi indica rice genotypes. For callus induction six types of concentration and combinations of plant growth regulators were tested to produce callus which influenced the explants for all the twenty rice varieties examined. Among them T_4 (3.0 mg/l 2,4-D + BAP 1.5 mg/l + Kin 1.0 mg/l) was the most effective combination for consideration of the capability to induce callus. The variety BRRI dhan29 (81.33%) showed supremacy on the others genotypes for both callusing and regenerating plants. Four different hormonal combinations and concentration were used to regenerate plants and the same variety BRRI dhan29 (72.00%) considered as the supreme member regarding its remarkable ability to regenerate plants through *in vitro* methods. Therefore, for further experiments could be conducted to increase the regeneration ability of BRRI dhan29, BRRI dhan24, BRRI dhan42 would be very much helpful for advance research in the field of biotechnology and genetic engineering for crop improvement in Bangladesh.


Chapter V
Androgenesis
(Anther & Microspore Culture)

5.0 Androgenetic studies for rice improvement

5.1 Anther Culture

The *in vitro* production of doubled haploids (DHs) through anther and isolated microspore culture is a powerful tool to generate pure lines much faster techniques than conventional breeding methods (Cao et al. 1995). In case of conventional inbreeding procedures it takes 6-8 generations or more to achieve complete homozygsity, whereas doubled haploidy comes within one generation (Reinert and Bajaj 1977). For successful development of DH plants, till various approaches have been employed with varying degrees of success (Chopkar et al. 2016). However, genotype dependency still plays an important role for getting success through *in vitro* culture in any crop plants. The genotype of anther donor plant plays an important role in callus induction and regeneration in cereal and other crops (Islam 2001, Bhojwani and Dantu 2010, Naik et al. 2017). For success in androgenesis- growth conditions of donor plants, light intensity, temperature, nutrition, pre-treatments factors are very important in rice and other cereal crops. Anther culture allows a rapid production of appropriate genotypes for breeding purposes in an effort to identify promising homozygous lines within shortening of time (Schmid et al. 1996, Redha et al. 1998, Burun and Emiroglu 2003, Lazaridou et al. 2005). In cereals, production of haploids through anther culture has major obstacle with low regeneration rate and albino plants. But application of different stress pre-treatment may improve induction and regeneration efficiency (Wędzony et al. 2000, Shariatpanahi et al. 2006a, Islam and Tuteja 2012). Anther and microspore culture methods have the potential to produce more than a thousand haploid plants per cultured anther (Chen 1983). In the case of anther culture, plants may originate from anther somatic tissue (2n) other than microspores (n). Therefore, microspore culture is the preferred method for haploid production in many cereal crops (Jähne and Lörz 1995). There are so many steps are need to standardize and culture factors for getting success in DHs production.

Anther culture is very quick as compared to microspore culture and it also influences development of microspores acting as a conditioning factor (Ruwani et al. 2018). 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. There are some reports that glutamine alone or in combination with serine and myo-inositol could replace the anther wall factor for isolated microspore culture (Bajaj 1983). 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 (Parra-Vega et al. 2015). Sometimes the development of microspores inside the anther may be interrupted due to growth inhibiting substances leaking out of the anther wall in contact with nutrient medium (Patel 2016).

The culture medium is one of the most important factors for the induction of androgenesis and the subsequent development of new structure and plants (Kunz et al. 2000). Getting success in plant tissue culture need to find out suitable genotypes and to optimize media and its components is very important things. In general harvesting flower buds/spikes just before first mitosis are most responsive in almost crop species. Microspore conditions at the early to mid uni-nucleated stage are better for androgenetic responses especially in cereal crops. Under this study for success in androgenesis-genotypes, media, various physical and chemical pre-treatment factors are employed and evaluated their effects on anther and isolated microspore culture in rice.

5.1.1 Effect of genotypes in anther culture responses of rice

5.1.1.1 Introduction

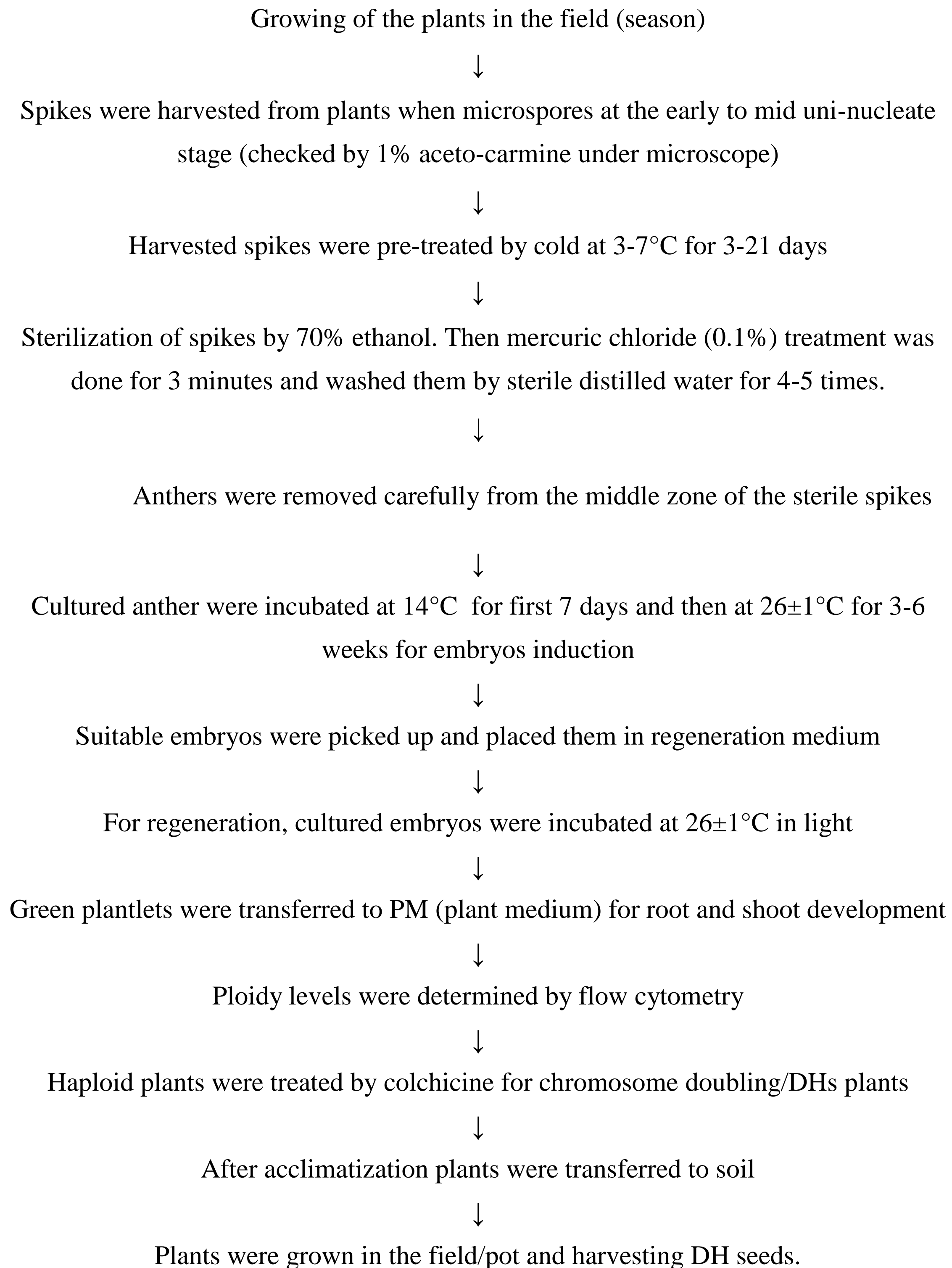
Many crop plants are quite recalcitrant in their responses though *in vitro* culture (George et al. 2008). For androgenetic studies induction in microspores can be limited by various factors and is highly genotype-dependent (Dunwell et al. 1985, Serrat et al. 2014). Success in microspore culture is predominantly dependent on the genotype of the anther donor material. Genetic factors are also important in determining the age of anther and microspores in rice (Rout et al. 2016). Several workers reported that the growing conditions of the donor plants might have a profound influence on embryo induction and regeneration (Ali and Jones 2000, Islam et al. 2001, Cha-um et al. 2009). In general, japonica rice genotypes responded better than indica has been reported by Zapata et al. (1983), Manimekalai and Rangaswamy (1983 and 1987), Quimio and Zapata (1990). Several researchers like Niizeki and Oono (1968), Zapata et al. (1983), Mikami and Kinoshita (1988), Boyadzhiev and Kong (1989), Quimio and Zapata (1990) worked on genotype differences for culture ability in rice. In cereal crops, still the major problem is albinisms for androgenetic studies. Many factors have been found to affect the degree of albinisms, such as the genotype and physiological state of the donor plants (Wojnarowiez et al. 2004, Jacquard et al. 2006, Torp and Andersen 2009). Genotypic variation to the extent of green and albino plant production in rice also reported by Datta et al. (1990). Japonica rice cultivars seem to be good for callus induction as compared to indica rice varieties are reported by Quimio and Zapata (1990). The main objective of this study was to find out a suitable rice genotype for their androgenic responses for further advance biotechnological research on rice improvement in Bangladesh.

5.1.1.2 Materials and Methods

5.1.1.2.1 Plant materials and media

Under this study twenty three (23) rice varieties (**Table 03**) were tested for their androgenic responses especially for anther culture (**Table 18**). Plants are grown in the research field of the Institute of Biological Sciences, University of Rajshahi during the growing seasons (2014-2016). As explants sources spikes were also collected from the Regional Rice Research Center, Shyampur, Rajshahi.

5.1.1.2.2 Methods (major steps for rice anther culture)



5.1.1.3 Results and Discussion

5.1.1.3.1 Results

Twenty three rice varieties were tested for their androgenetic responses and each genotype were cultured in SK3 (semi-solid) callus induction medium. Here almost all varieties showed more or less embryos induction and subsequent regeneration (**Table 18**). Among the responding genotypes BRRI dhan29 (22.36%), BRRI dhan30 (27.85%) and IR-43 (47.07%) showed better results on embryoids induction in SK3 medium (**Fig. 10A**). Better regeneration were also recorded in IR-43 (16.19%), BRRI dhan30 (12.54%) and BRRI dhan29 (8.49%) **Fig. 10 (C-D**). All of the genotypes produced more or less (0.94-24.59%) albino plants (**Fig. 10E**). In case of average mean value (ELS, RP and GRP), IR-43 showed better performance than other rice (cultivars 24.59%, **Table 18**). The percentages rate of green plants was recorded in IR-43 (10.50%). The preset study showed variation in embryoids induction and regeneration among the studied genotypes (**Fig. 10A-F**). Here mean values of each genotype were recorded on the basis of EI, RP, GR and AP is mentioned in **Table 18**. Analysis of variance (ANOVA) showed significant differences in the frequency of EI, RP, GRP and ARP at p <001 (**Table 18 & 19**).

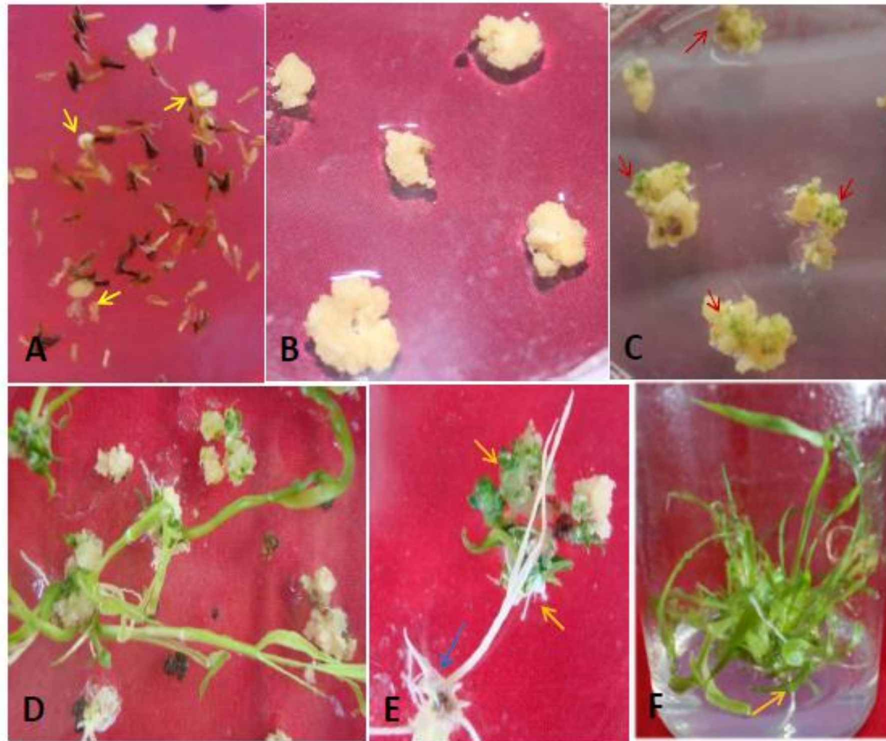


Fig. 10 (A-F): Anther culture and its responses in rice. A) Anther derived embryoids, B) Embroyids were sub-cultured in regeneration medium, C) Anther-derived embryoids showed green spots that becommg regeneration, D) Anther derived embrvoids showing good plantlets, E) Albino and green regenerated plants grown from anther derived embryoids, F) Plants with shoot and roots.

Table 18: Different rice varieties were tested for their responses to anther culture

Genotypes	Per 100 anthers				Mean for EI, RP & GP
	Embryo induction (EI)	Regenerated plantlets (RP)	Green plants (GP)	Albino plants (AP)	
BR-3	1.83±0.18ij	1.09±0.13jklm	0.72±0.27hi	0.50±0.26ghi	1.21±0.10
BR-4	11.16±1.18de	3.82±0.24f	1.94±0.20f	1.52±0.29cd	5.64±0.40
BR-11	9.56±0.45e	3.50±0.38fg	1.58±0.21fg	1.19±0.14de	4.88±0.33
BR-14	1.90±0.15hij	1.04±0.11klm	0.45±0.08hi	0.43±0.05ghij	1.13±0.11
BR-19	1.97±0.40hij	1.47±0.29ijklm	0.48±0.11hi	0.43±0.07ghij	1.31±0.27
BRRI dhan27	3.10±0.31ghij	1.60±0.05hijkl	0.61±0.08hi	0.31±0.03hij	1.77±0.08
BRRI dhan28	4.32±0.41fghi	2.28±0.24hi	1.07±0.08gh	0.77±0.02efg	2.56±0.08
BRRI dhan29	**22.36±0.91c**	8.49±0.50d	6.35±0.34d	1.88±0.06bc	12.40±0.57
BRRI dhan30	**27.85±1.43b**	12.54±0.82b	7.31±0.48c	2.06±0.34b	**15.90±0.32**
BRRI dhan31	5.41±0.31fg	1.67±0.13hijkl	0.76±0.07hi	0.61±0.05fgh	2.61±0.17
BRRI dhan42	4.63±0.64fgh	1.79±0.17hijk	1.05±0.06gh	0.66±0.11fgh	2.49±0.27
BRRI dhan47	2.92±0.30ghij	0.96±0.15klm	0.47±0.07hi	0.31±0.05hij	1.45±0.15
BRRI dhan48	1.72±0.35ij	0.75±0.14klm	0.33±0.04hi	0.25±0.03hij	0.94±0.17
BRRI dhan50	1.13±0.19j	0.30±0.06m	0.00±0.00i	0.00±0.00j	0.48±0.08
BRRI dhan55	6.37±0.38f	2.62±0.20gh	1.66±0.09gh	0.98±0.14ef	3.55±0.18
BRRI dhan56	1.13±0.12j	0.50±0.03lm	0.24±0.03i	0.15±0.03ij	0.62±0.05
BRRI dhan58	5.62±0.66fg	2.21±0.21hij	1.11±0.16gh	0.83±0.12efg	2.98±0.25
BRRI dhan59	2.58±0.51hij	1.04±0.23klm	0.58±0.11hi	0.27±0.04hij	1.40±0.18
IR-43	**47.07±2.49a**	16.19±0.69a	**10.50±0.40a**	2.90±0.06a	24.59±0.62
IR-54	27.06±0.98b	10.94±0.93c	9.43±0.62b	2.23±0.15b	15.81±0.75
Chinigura	2.92±0.23ghij	0.70±0.15klm	0.26±0.07i	0.12±0.06ij	1.29±0.11
Kalijira	13.16±1.47d	4.87±0.21e	2.76±0.15e	1.11±0.12e	6.93±0.58
Jaya	2.13±0.32hij	0.86±0.10klm	0.57±0.10hi	0.23±0.04hij	1.19±0.14
Mean (SD)	**9.04** (11.43)	3.53 (4.28)	2.18 (3.04)	0.86 (0.79)	-
F-value (Var.)	192.366	150.634	175.003	33.622	-
Significance	0.000	0.000	0.000	0.000	-
F-value (Rep.)	1.235	2.126	0.063	0.426	-
Significance	0.301	0.131	0.939	0.656	
Variety $LSD_{0.05}$	0.856	0.361	0.238	0.138	

Data were recorded on the basis of number of inoculated anthers. Medium = SK3, Twenty three rice varieties were tested for their responses anther culture.

Table 19: ANOVA for response to anther culture (Data source Table 18)

Source of Variation	Dependent Variable	Sum of Squares	df	Mean Square	F-value	Sig.
Variety	EI	8785.834	22	399.356	192.366	0.000
	RP	1226.485	22	55.749	150.634	0.000
	GP	619.269	22	28.149	175.003	0.000
	AP	39.861	22	1.812	33.622	0.000
Replication	EI	5.130	2	2.565	1.235	0.301
	RP	1.574	2	0.787	2.126	0.131
	GP	0.020	2	0.010	0.063	0.939
	AP	0.046	2	0.023	0.426	0.656
Error	EI	91.345	44	2.076	-	-
	RP	16.284	44	0.370	-	-
	GP	7.077	44	0.161	-	-
	AP	2.371	44	0.054	-	-
Total	EI	14520.738	69	-	-	-
	RP	2104.640	69	-	-	-
	GP	955.504	69	-	-	-
	AP	93.156	69	-	-	-
Corrected Total	EI	8882.309	68	-	-	-
	RP	1244.344	68	-	-	-
	GP	626.366	68	-	-	-
	AP	42.278	68	-	-	-

EI = Embryo induction, RP = Regenerated plants, GP = Green plants and AP = Albino plants.

5.1.1.3.2 Discussion

Genotype is an important factor for success of *in vitro* culture for an efficient callus induction and regeneration through anther culture (Islam et al. 2001). Niizeki and Oono (1968) was the first reporter who successfully worked in rice anther culture. Then some new cultivars have been developed using anther culture methods in rice (Brown and Thorpe 1995, Zapata et al. 2004). Mishra and Rao (2016) found that *in vitro* response of anthers for both *japonica* and *indica* rice varieties are very much genotype specific and related some other factors (Maw and Kenji 2017). The success in anther culture depends on first genotype and then duration of cold pretreatment, culture medium and growth regulators (PGRs) was reported by Ponitka and Ślusarkiewicz-Jarzina (2007), Niroula and Bimb (2009) and Kahrizi et al. (2011). It appears that optimum culture medium and pretreatment conditions vary from genotype to genotype. In cereals, production of haploid plants through anther culture has major obstacle with low regeneration rate and albino plant production. Application of different stress pre-treatment factors using suitable genotypes may improve induction and regeneration efficiency in cereal crops for anther and microspore culture of rice (Datta 2005). Ball et al. (1992) mentioned that the anther culture process is still under strong genetic control. In a study Guha-Mukherjee (1999) found the effect on rice anther culture where only two cultivars showed callusing. Lentini et al. (1995) examined the effect of maltose and silver nitrate on callus induction and found only one genotypes exhibited callusing out of 35 indica rice cultivars. Niroula and Bimb (2009) found only five genotypes responded on embryos induction out of eighteen genotypes in case of anther culture of rice. Under this study we evaluated 23 rice cultivars for their responses on anther culture and those cultivars sources was BRRI, IRRI and also two local cultivars were used. Here we found almost genotypes showed more or less callusing in SK3 medium. May be the differences comes genotypes sources, media composition, PGRs and culture condition that was not same with other findings.

5.1.2 Effect of culture media for anther culture responses in rice

5.1.2.1 Introduction

The composition of culture media is one of the most important factors in the success of androgenesis in cereal crops (Chu 1978). In case of androgenesis the nutrient medium not only provides nutrition to the microspores but also directs the pathway of embryo development. The available literature does not allow recommendation of any anther culture medium for general application purpose in cereal crops. The most widely used basal media is MS, N6, B5, Potato-2 (Chuang et al. 1978), LS, SK3 (modified SK-1, Raina 1989), FGH (Hunter 1987), MO19 (Raina and Zapata 1997), AMS3 (Islam 2000), etc.

Niizeki and Oono (1968) used Blaydes (1966) medium in their first report of successful rice anther culture. Use of modified MS medium containing reduced nitrogen and 10 per cent (w/v) ficoll might favor microspore embryogenesis and green plant regeneration in rice (Datta et al. 1990). Oono (1975) examined several other media and recommended MS medium is the most suitable medium for rice anther culture. Chu et al. (1975) reported that for rice anther culture N6 medium is widely used for Japonica rice cultivars. They also pointed out that the growth and differentiation of callus derived from microspores influenced by major salts, especially by amonium salt and found lower concentration of NH+ ion is beneficial for androgenetic studies. Boyadzhiev and Kong (1989) reported that N6 is better for callus induction and MS medium for obtaining better regenerations in rice. Under this study for callus induction N6, FHG and AMS3, for regeneration MO19, and for plant development PM medium was employed. The main objective of this study was to optimize suitable media, PGRs and other culture conditions for advance biotechnological research for anther culture of rice.

5.1.2.2 Material and Methods

5.1.2.2.1 Plant materials and media

To evaluate the effect of media three good androgenic rice varieties *viz*. BRRI dhan29, BRRI dhan30 and IR-43 were considered for this study. Out of several media for callus induction and regeneratin four media (N6, AMS3, FHG and SK3) are employed (**Table 4 & 20**) to testify their effect on callus induction and regeneration.

5.1.2.2.2 Methods

Anther culture methods and other procedures are described in **Section 5.1.1.2.**

5.1.2.3 Results and Discussion

5.1.2.3.1 Results

Under this study four different types of induction media *viz*. FHG, AMS3, N6 and SK3 were employed for embryoids development (**Table 04 & 20**). In a previous study 23 rice varieties were tested for their androgenetic responses (**Table 18**) and out of them only three (BRRI dhan29, BRRI dhan30 and IR-43) good responding genotypes were considered for this study. Among the four inductions medium (FHG, AMS3, N6, and SK3) highest embryos showed in IR-43 (**Table 20**). In case of average mean value studied genotypes showed better response on callusing (36.62%) in SK3 medium. Second heights embryoids were recorded in N6 medium (32.43%) that was 2-3 times higher that AMS3 (22.16%) and FGH (16.38%). For green plant regeneration SK3 (12.39%) and N6 (12.19%) showed quiet similar results than others (AMS3-7.70% and FHG-7.32%). For albino plant development AMS3 (2.16%) and SK3 (2.67%) showed better results for reducing albinism than others (**Table 21**). ANOVA showed significant differences on ELS, GRP and ALP in different culture media for anther culture (AC) responses at $p < 0.01$ (**Table 21 & 22**).

Table 20: Media constituents (induction, regeneration and rooting) for rice anther culture

Media constituents	Medium (mg/l)			
	FHG	AMS_3	M-019*	PM*
Macronutrients				
$(NH_4)_2SO_4$	-	250	264	-
$MgSO_4.7H_2O$	370	200	370	71.5
$Ca(NO_3)_2.4H_2O$	-	-	-	500
KCl	-	40	-	65.0
$CaC1_2.2H_2O$	440	100	440	-
KNO_3	1900	950	3101	950
NH_4NO_3	165	-	-	825
$NaH_2PO_4.H_2O$	-	-	-	
KH_2PO_4	170	285	540	350
Micronutrients				
$MnSO_4.4H_2O$	16.9	8.0	22.3	4.9
$ZnSO_4.7H_2O$	8.6	3.0	8.6	2.7
$CuSO_4.5H_2O$	0.025	-	0.025	-
$CoC1_2.6H_2O$	0.025	-	-	-
K1	0.83	0.5	0.83	0.75
H_3BO_3	6.2	3.0	6.2	1.6
$Na_2M_OO_4.2H_2O$	0.25	-	0.25	-
Iron				
$FeSO_4.7H_2O$	40.0	27.8	27.85	13.9
Na_2 EDTA	40.0	41.0	37.25	18.6
Vitamins				
Pyridoxine HCl	-	0.5	2.5	5.0
Nicotinic Acid	-	0.5	2.5	5.0
Thiamine HCl	0.4	1.0	2.5	1.0
Myo-Inositol	100	100	100	100
Hormones				
2, 4-D	2.0	2.0	0.5	-
Kinetin	0.5	0.5	0.5	-
IAA	-	1.0	2.5	1.0
Carbon sources				
Sucrose	-	-	40 g/	20 g/l
Maltose	62 g/l	90 g/l	-	
Amino acids				
L-glutamine	730	500	-	-
L-proline	-	200	-	-
L-asparagine	-	50	-	-
Casein hydrolysate	100	-	-	-
Glycine	2.0	2.0	2.0	2.0
Gelling agents				
Agar	8.0 g/l	-	8.0 g/l	8.0 g/l
Phytagel	-	-	3.0 g/l	3.0 g/l

M-019 (Raina and Irfan 1998). pH of all media was adjused at 5.8. N6 & SK3 media constituents are briefly described in Table 4 (Chapter III). M-019* and PM* are used for some experimental purpose of this study.

Table 21: Culture media and its productivity on anther culture (AC) response in rice

Media	Variety	Per 100 anthers			Mean for ELS & GRP
		ELS	GRP	ALP	
N6	BRRI dhan29	19.99±0.77f	9.89±0.06d	2.39±0.36de	14.94±0.42
	BRRI dhan30	28.33±2.03de	11.50±0.76cd	3.72±0.15abcd	19.92± 1.40
	IR43	**48.97**±1.55b	15.19±0.42a	4.93±0.98a	32.08 ±0.99
	Mean (individual aspect)	**32.43±4.37**	**12.19±0.82**	**3.68±0.48**	**-**
AMS3	BRRI dhan29	13.87±0.70gh	5.71±0.60e	1.65±0.44e	9.79 ±0.65
	BRRI dhan30	16.30±0.65fg	6.06±0.24e	2.30±0.29de	11.18± 0.45
	IR43	**36.30**±1.42c	11.34±0.39cd	2.53±0.41de	23.82± 0.91
	Mean (individual aspect)	**22.16±3.59**	**7.70±0.94**	**2.16±0.23**	**-**
FHG	BRRI dhan29	10.37±0.69h	5.71±0.24e	3.27±0.50bcd	8.04± 0.47
	BRRI dhan30	14.27±0.82gh	6.10±0.38e	4.07±0.23abc	10.19± 0.60
	IR-43	24.50±1.04e	10.15±0.64cd	4.48±0.38ab	17.33± 0.84
	Mean (individual aspect)	**16.38±2.15**	**7.32±0.74**	**3.94±0.26**	**-**
SK3	BRRI dhan29	25.80±0.90de	11.33±1.45cd	2.41±0.50de	18.57± 1.18
	BRRI dhan30	29.60±0.71d	12.17±0.44bc	2.82±0.17cde	20.89± 0.58
	IR-43	**54.46**±3.15a	13.66±0.71ab	2.80±0.18cde	34.06± 1.93
	Mean (individual aspect)	**36.62±3.84**	**12.39±1.56**	**2.67±0.46**	**-**
F-value (Var.)		90.754	25.344	4.884	-
Significance		0.000	0.000	0.001	-
F-value (Rep.)		0.098	0.208	0.479	-
Significance		0.907	0.814	0.626	-

N_6 = Chu et al. (1975), SK3 = Modified SK-1 medium (Raina 1989), AMS3 = Modified MS (Islam 2000), FHG = (Hunter 1987).

Table 22: ANOVA for culture media and its productivity on anther culture (AC) response in rice (Data source Table 21)

Source of Variation	Dependent Variable	Sum of Squares	df	Mean Square	F-value	Sig.
Variety	ELS	6342.227	11	576.566	90.754	0.000
	GRP	354.820	11	32.256	25.344	0.000
	ALP	32.462	11	2.951	4.884	0.001
Replication	ELS	1.244	2	0.622	0.098	0.907
	GRP	0.529	2	0.265	0.208	0.814
	ALP	0.578	2	0.289	0.479	0.626
Error	ELS	139.768	22	6.353	-	-
	GRP	28.000	22	1.273	-	-
	ALP	13.292	22	0.604	-	-
Total	ELS	32524.592	36	-	-	-
	GRP	3912.105	36	-	-	-
	ALP	395.275	36	-	-	-
Corrected Total	ELS	6483.239	35	-	-	-
	GRP	383.349	35	-	-	-
	ALP	46.332	35	-	-	-

5.1.2.3.2 Discussion

Media composition is one of the most important factors for the success of androgenesis in rice and other cereal crops. Four different types of induction media *viz*. FHG, AMS3, N6 and SK3 were used and found their effect on callus induction. Niizeki and Oono (1968) used Blaydes medium with some modification in their first report of successful in rice anther culture. They used the basic nutrient medium same as described by Blaydes (1966) except growth regulators. This was supplemented with various growth substances such as indol-3-acetic acid, kinetin, 2,4-dichlorophenoxyacetic acid, adenine sulfate and yeast extract singly or in combination. Oono (1975) examined different media for callus induction and he recommended MS as the most suitable medium for rice anther culture. Niizeki and Oono (1968) reported that callus formations occurred in anthers of 6 rice varieties (Eiko, Fujisaka 5, Kusabue, Minehikari, Norm 20 and Toride 2). The appropriate medium for callus formation from the anther was found to be the basic medium supplemented with 1~2 mg/1 IAA, 1~2 mg/1 kinetin and 1~2 mg/l 2,4-D. Under this study MS medium was not used but AMS3 used as induction medium that modification with MS. Chu et al. (1975) reported a suitable medium (N6) for rice anther culture. However, it is widely used for wheat anther culture. It was observed that N6 medium gave very better responses for embryoids induction than AMS3 and FHG. The present results agreed well with the finding of Chu et al. (1975). Ogawa et al. (1995) mentioned that amino acids induced a higher degree of plantlet regeneration and green plant production than the medium containing alanine or no amino acid. Raina (1997) reported that in addition of maltose to the induction medium enhanced plantlet regeneration rate in rice.

Boyadzhiev and Kong (1989) reported that N6 was better for callus induction while MS gave better regeneration in rice. In most of the cases they preferred semi-solid medium for anther culture. In the present study most of the cases liquid induction medium showed better performance on embryoids production than semi-solid medium. There is a

little discrepancy of our results with the findings of Boyadzhiev and Kong (1989). In another study Ku et al. (1978) mentioned that in liquid induction medium, amino acids play an important role for production of embryos that agreed well also with our present findings. They reported that several amino acids, vitamins and inositol stimulate androgenetic responses in maize. Here we also used amino acids as L-proline and L-glutamine and other media components like vitamins, myo-inositol that gave better responses on anther culture of rice. Cho and Zapata (1988) reported that L-proline and L-glutamine promoted callus formation in microspore cultures of a japonica cultivar.

Ogawa et al. (1995) found glutamine is a stimulatory chemical for pollen callusing in microspore cultures of an indica cultivar but alanine was far better than glutamine for plant regeneration and green plant production. Chu (1975) had demonstrated that the level of ammonium nitrogen in the culture medium is critical for androgenesis in rice. On this basis he developed the N6 medium which most widely used for rice anther culture (Raina 1989 and 1997). Chu (1975) reported that the N6 medium has proved significantly better than others for japonica rice cultivars. Reddy et al. (1985) considered 8 indica rice cultivars for their study and found He2 medium was better than N6 medium. Raina and Zapata (1997) reported that based on their detailed study on the medium requirement of the indica cultivar IR-43 evolved a new medium called M-019 (Raina and Irfan 1998). Under this study M-019 was used as regeneration medium and that showed better regeneration also in case of IR-43 cultivars. As Bangladeshi rice cultivars BRRI dhan29 and BRRI dhan30 showed quiet better responses on callus induction and regeneration where as medium SK3 was considered. For regeneration PM (modified MS) showed better responses in regeneration in the studied genotypes. In some cases differences may be happen due to the donor plants of spikes (genotype), various induction media, semi-solid and liquid medium, pre-treatments factors and other culture conditions.

5.1.3 Anther transferring time to improve androgenetic responses and reducing albinism

5.1.3.1 Introduction

Production of doubled haploids through anther and isolated microspore culture has major obstacle with low regeneration rate and albino plants in cereal crops. For anther culture in cereal crops albino plants represented by the formation of high frequency regenerants devoid of chlorophyll in monocots that plants cannot survive in nature and have no agronomic value (Muñoz-Amatriaín el al. 2009, Wędzony et al. 2009). Clément et al. (2005) reported that about albinism pointed out that the role of the programmed cell death as the process responsible for chloroplast DNA damage during microspore embryogenesis which resulted albino plant production. The high frequencies of albinos among regenerated plants continue to be a major drawback preventing the widespread use of anther culture for crop improvement.

Application of different methods such as early transfer of anthers and various stresses pre-treatment may enhance callus induction and regeneration in maize, wheat and rice (Barloy and Beckert 1993, Islam 2001, Khatun 2010). Ball et al. (1992) mentioned that the anther culture process is still under strong genetic control, but improved analyses of the functions of media components may reduce differential responses and improve efficiency of culture methods. Henry and De Buyser (1981) transferred anthers to regeneration medium after two weeks of culture initiation from induction to regeneration medium and found that green plantlets were increased in wheat. In maize, the time of embryo transfer to the regeneration medium showed significantly higher green plants regeneration (Barloy and Beckert 1993, Saisingtong et al. 1998, Khatun et al. 2012). Büter (1997) reported that the plant regeneration frequencies were increased in maize by the optimal transfer time of anthers to the regeneration medium. The main objective of this study was to observe the regeneration efficiency of inoculated anthers by early transferring methods along with or without embryo like structures (ELS) to regeneration medium with different time points.

5.1.3.2 Materials and Methods

5.1.3.2.1 Plant materials and media

For all treatment (T) three days cold (4°C) pre-treated spikes were used for this experiment. The SK3 (liquid and semi-solid) medium and BRRI dhan30 rice variety was considered for its better performances in anther culture responses in a previous finding under this study.

5.1.3.2.2 Methods

Rice anther culture was carried out on transferring time (days) for T_1 = 5 d, T_2 = 10 d, T_3= 15 d, T_4 = 20 d and T_5 = 25 days. For Cont. 1 (0 d) = anthers were inoculated in liquid induction medium, and for Cont. 2 (0 d) in semi-solid induction medium. For each time point inoculated anthers with or without embryo like structures (ELS) were transferred from liquid induction to semi-solid regeneration medium. Then cultures were incubated at 26-27°C for embryoids formation. For both Control and treatments (T), ELS were transferred to PM = plant medium and placed them in culture room at 26°C in light for regeneration.

5.1.3.3 Result and Discussion

5.1.3.3.1 Results

Under this study inoculated anthers for all treatments (T) were transferred from induction medium (SK3) to semi-solid regeneration medium (PM) with or without embryo like structures (ELS) in different days. The SK3 medium was supplemented with Yeast extract (1 g/l) along with other media constituents (**Table 23**). As per the anther inoculation to liquid or semi-solid medium and the effect of incubation period two Controls were considers under this study. It was observed that T1 (11.69%) and T2 (13.67%) produced green plant regeneration which is higher that Cont. 1 (10.45%) and Cont. 2 (11.71%). On the other hand regeneration potentials were decreased when inoculated anthers were transferred to regeneration medium after 15 days (**Table 23**). For green plant regeneration T2 showed better results (13.67%) than both Controls and other

treatments (T). Albino plant production reduced (2.38%) in T2 than both Controls and treatments that was 2-3 times higher. In respect to regeneration Cont. 2 was not worthies where green and albino plant production was almost identical (**Table 23**). ANOVA showed the highly significant differences in embryoids induction, regeneration and reducing albinisms at 0.01% level of significance (LS) that was an important target for this study (**Table 23 & 24**).

Table 23: Improvement of plant regeneration efficiency through anther transferring time for reducing albinism in rice

Anther transferring time (days)	Per 100 anthers			Mean for ELS & GRP
	ELS	GRP	ALP	
Cont. 1 (Liq.)	29.08±0.58a	10.45±0.33b	5.12±0.49b	19.77±0.45
Cont. 2 (Semi-solid)	24.60±2.36bc	11.71±0.65b	3.74±0.13c	18.15±1.25
T_1	22.72±1.41c	11.69±0.31b	3.91±0.21c	17.20±0.85
T_2	26.62±0.88ab	13.67±0.25a	2.38±0.03d	20.15±0.53
T_3	18.17±0.60d	8.27±0.82d	5.21±0.28b	13.22±0.12
T_4	14.89±0.49de	6.37±0.20e	6.12±0.07a	10.63±0.15
T5	12.53±0.82e	4.81±0.14f	6.67±0.17a	8.67±0.48
Mean (SD)	21.2295 (6.11705)	9.5667 (3.09948)	4.7367 (1.45317)	-
F-value (Treat.)	28.462	45.414	37.987	-
Significance	0.000	0.000	0.000	-
F-value (Rep.)	1.399	0.314	1.127	-
Significance	0.284	0.736	0.356	-
Treat. $LSD_{0.05}$	2.339	0.956	0.486	-

Cont. 1 = Anthers were inoculation in liquid medium and Cont. 2 in semi-solid induction medium. T_1 = 5 d, T_2 = 10 d, T_3= 15 d, T_4 = 20 d and T_5 = 25 d.

Table 24: ANOVA for improvement of plant regeneration efficiency through anther transferring time for reducing albinism (Data source Table 23)

Source of Variation	Dependent Variable	Sum of Squares	df	Mean Square	F-value	Sig.
Treatment	ELS	688.689	6	114.781	28.462	0.000
	GRP	183.626	6	30.604	45.414	0.000
	ALP	39.748	6	6.625	37.987	0.000
Replication	ELS	11.283	2	5.642	1.399	0.284
	GRP	0.423	2	0.212	0.314	0.736
	ALP	0.393	2	0.197	1.127	0.356
Error	ELS	48.393	12	4.033	-	-
	GRP	8.087	12	0.674	-	-
	ALP	2.093	12	0.174	-	-
Total	ELS	10212.912	21	-	-	-
	GRP	2114.079	21	-	-	-
	ALP	513.390	21	-	-	-
Corrected Total	ELS	748.366	20	-	-	-
	GRP	192.136	20	-	-	-
	ALP	42.234	20	-	-	-

5.1.3.3.2 Discussion

Albino plant production in cereal anther and microspore culture is a great problem and till it has no agronomic value. High frequency of albino plant formation in anther and microspore cultures is a general phenomenon in most cereals like wheat (Andersen et al. 1987), barley (Knudsen et al. 1989), rice (Guiderdoni et al. 1992), rye (Immonen 1999), etc. There is not enough report and suitable solutions for reducing albinism in cereal crops. But with suitable genotypes, improving culture conditions and applying physical and chemical factors may be possible to improve androgenic responses and reducing albinism. Early transfer of anthers from induction medium to regeneration medium may enhanced the gametic embryogenesis and reducing albinism are reported in maize (Barloy and Beckert 1993, Saisingtong 1998), wheat (Schmid et al. 1996, Islam 2000) and rice (Khatun et al. 2012). Early transfer of anthers along with or without embryo like structures is an efficient tool for improving the quality of regeneration (Büter 1997). Hence, this experiment was conducted to improve embryo quality by increasing more green plantlets and limiting albinos in wheat anther culture (Barloy and Beckert 1993, Islam 2000).

In the present study two controls were considered where inoculated anthers were transferred to regeneration medium for different time periods (within 5-25 days). Results indicated that transfer of anthers with or without ELS from induction to regeneration medium posed a significant impact on improving regeneration potentiality and reducing albinisms. Inoculated anthers from liquid induction to regeneration medium within 10-15 days showed significantly higher results on regenerating and less number of albino plants in this study. In a previous report early transfer of anthers (21 days of inoculation) of maize anthers with or without ELS to regeneration medium gave promising results on induction and regeneration (Barloy and Beckert 1993, Saisingtong 1998). The present results agreed well with the previous findings done by Barloy and Beckert (1993) and Saisingtong (1998). Though the anther transferring time was little different in the present study. Under the present investigation T3-T5 could not able to show any promising results because the anthers were transferred lately to the regeneration medium. May be

during the incubation period to induction medium a lot of microspores were released in liquid or semi-solid induction medium and those was not possible to collect due to early stages of embryogenesis. Early transfer of maize (before three weeks) also showed negative effect both on induction and regeneration (Barloy and Beckert 1993). Büter (1997) reported that, plant regeneration frequencies might be manipulable by transferring of anthers to the regeneration medium. Nägeli et al. (1999) reported that earlier transfer of embryos to the regeneration medium showed significantly higher regeneration frequencies in maize microspore culture. All the reported findings are consistent with the results of the present findings. Barloy and Beckert (1993) mentioned that quality of embryos might influence regeneration and reported that embryos quality might also be modified by medium composition and timing of anther transfer. The present investigation transfer of rice anthers after two weeks (15 days) of culture proved to be the best time for increasing green plant regeneration as well as reducing albinism.

5.2 Microspore culture

Isolated microspores have remarkable features to be utilized in plant biotechnology and are very promising targets for genetic manipulation because they are unicellular, highly synchronized and ensures that all plantlets are derived from the haploid microspores (Touraev et al. 1997, Kunz et al. 2000, Clément et al. 2005, Anitasari et al. 2018). This system is an important and useful tool in plant breeding program for the production of homozygous line rapidly. In combination with genetic transformation technology fertile homozygous plants can be produced rapidly (Guo and Pulli 2000, Islam and Tuteja 2013a). 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 an important technique (Coumans and Zhang 1995, Ferrie and Caswell 2011). It has been reported that most of the cases, the production of embryos per anther obtained in isolated microspore culture is generally higher than anther culture (Jähne and Lörz 1995, Nägeli 1999). In certain plant species such as *Hordium vulgare* the isolated microspore culture procedure has produced five fold green plants compared to anther culture (Hoekstra et al. 1993, Shim et al. 2009). For androgenetic research still a great lacking of embryo induction and high percentage of albinism among regenerated plants (Islam 2010c). Albino plants are very often produced during the regeneration of microspore-derived plants in cereals (Olmedilla 2010). The frequency of albino plants may vary from 5-100% in rice anther culture Talebi et al. (2007).

There are some reports on isolated microspore culture techniques in cereal and other crop plants, e.g. barley (Köhler and Wenzel 1985, Wei et al. 1986, Cistué et al. 1995, Esteves and Belzile 2019), durum wheat (Cistué et al. 2009), rice (Datta et al. 1990, Raina and Irfan 1998, Islam et al. 2013b), rye (Wenzel et al. 1975, Datta and Wenzel 1987), maize (Pescitelli et al. 1990, Nägeli 1999, Obert et al. 2004), wheat (Kunz et al. 2000, Islam et al. 2001, Liu et al. 2002, Kasha et al. 2003, Shirdelmoghanloo et al. 2009, Slama-Ayed et al. 2010, Santra et al. 2012, Ekhveh et al. 2013), Sugarcane (Anitasari et al. 2018), *Brassica napus* (Pechan and Keller 1989, Weber et al. 2005, Abdollahi et al. 2011, Li et al. 2018), cauliflower (Bhaita et al. 2017), carrot (Górecka et al. 2010), chickpea (Grewal et al. 2009), *Corchorus* (Ali and Jones 2000), horse chestnut (Dragosavac et al. 2010), *Medicago* (Ochatt et al. 2009), pepper (Bárány et al. 2001 & 2005, Kim et al. 2008 & 2013), *Raphanus sativus* (Chung et al. 2018).

5.2.1 Various microspore isolation procedures

5.2.1.1 Introduction

The isolated microspore culture offered the following advantages compared to anther culture- gametic origin, *in vitro* selection, mutagenic studies, response, transformation, observation and statistics evaluation. Till many researchers have done several experiments on influencing of androgenesis in cereal crops. But there is not enough report on rice about the suitable microspore isolation procedure. Under this circumstance experiments were carried out on isolated microspore culture procedures to develop an efficient embryoids induction and regeneration systems using some elite rice cultivars.

5.2.1.2 Material and Methods

5.2.1.2.1 Plant materials and media

For microspore isolation procedure six rice varieties *viz*. BR-4, BR-11, BRRI dhan29, IR-43, IR-54 and Kaljira were considered for their quite better responses in anther culture under this study. To optimize different microspore isoalted procedure IR-34 variety was considered as plant material that showed good androgenetic responses in a previous study (Khatun et al. 2012). Seeds were grown in the experimental field at the Institute of Biological Sciences during the rice cultivation period. Spikes were harvested and subjected to cold pre-treatment at 4ºC-5ºC for 3-21 days in dark. Spikes were also collected from the Regional Rice Research Center, Shyampur, Rajshahi. Anthers containing microspores at the early to mid uninucleated stage were used from the central part of the spikes.

Three different types of media *viz.* PC (pre-culture), WM (washing) and induction (AMC) medium were used for this study (Kunz et al. 2000) are shown in **Table 25**. The WM and AMC were modified by AM (Schmid 1990). The pH of all media was adjusted at 5.8 before autoclaving.

Table 25: Chemical composition of pre-culture (PC), washing (WM) and induction (AMC) media for rice micrsopore culture

Media constituents	Pre-culture medium (mg/l)	Washing medium (mg/l)	Induction medium (mg/l)
Macro-nutrients			
KNO_3	-	500	1000
$(NH_4)_2SO_4$	-	50	100
$Ca(NO_3)_2.4H_2O$	-	50	100
KH_2PO_4	-	100	200
$MgSO_4.7H_2O$	-	62.5	125
KCl	-	17.5	35
Micro-nutrients			
$MnSO_4.4H_2O$ (H_2O)	-	4.4 (3.3)	4.4 (3.3)
$ZnSO_4.7H_2O$	-	1.5	1.5
H_3BO_3	-	1.6	1.6
KI	-	0.8	0.8
Iron			
$FeCl_3$	-	27.0	27.0
Na_2EDTA	-	37.3	37.3
Vitamins			
Thiamin HCl	-	1.0	1.0
Growth regulators			
2,4-D	-	1.5	1.5
Kinetin	-	0.5	0.5
Carbon			
Sucrose	-	60 g/l	-
Maltose (monohydrate)	-	-	90 g/l
D-Manitol	72.87 g/l	-	-
Amino acids			
L-glutamine	-	1000	1000
L-serine	-	100	100
L-ascorbic acid	50	-	-
L-proline	125	-	-
Gelling agents			
Phytagel	-	-	3.0 g/l
Agar	-	-	7.0 g/l

pH of all media was adjusted at 5.8.

5.2.1.2.2 Methods

Microspore conditions were observed by 1% aceto-carmine staining under microscope prior to culture. Different steps of microspore isolation and subsequent cuture procedure of doubled haploid (DH) plants production are describe in **Fig. 11.**

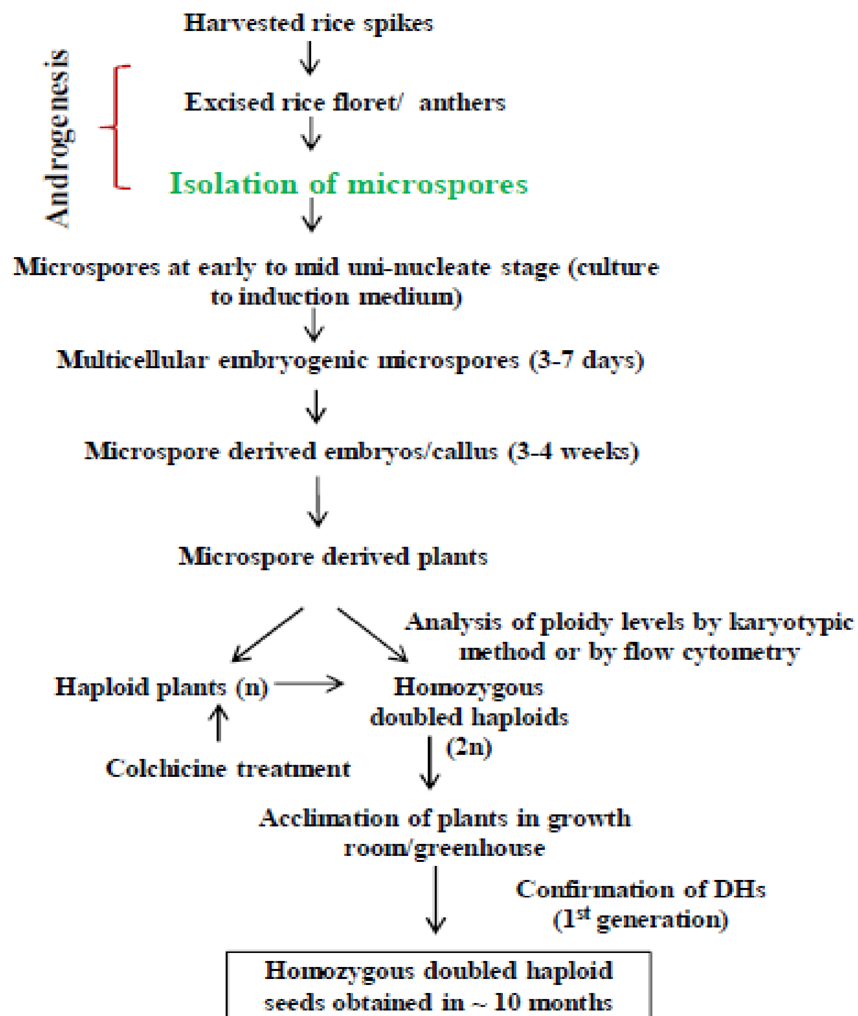


Fig. 11: A flow chart showing on different steps of microspore culture for doubled haploid production.

5.2.1.2.2.1 Microspore isolation procedures

5.2.1.2.2.1.1 P_1 = Squeezed of rice floret by glass rod

Cold pre-treated spikes were surface sterilized with 70% ethanol and anthers were removed with a fine tweezers (forceps) and kept in a sterile petri dishes with 4 ml washing medium (WM). Here microspores were released from rice floret (along with anthers lemma and palea were closed) by squeezing with a sterile glass rod. The suspension was diluted with 15 ml WM and filtered through a sieve with a 100 µm mesh and centrifuged for 3-4 minutes with 750-1000 rpm. Then the sediment was carefully re-suspended in 3 ml induction medium and transferred to a sterile petri dish (35 × 10 mm). Inoculated petri dishes were sealed with parafilm and incubated at 26±1ºC in dark for embryo induction. After 3-4 weeks the targeted embryos (around 1-3 mm), were removed weekly and transferred to the regeneration medium. Then regenerated plantlets were transferred to PM = plant medium (Schmid 1990) for root and shoot development.

5.2.1.2.2.1.2 P_2 = Squeezed of excised anther by glass rod

Here microspores were released in the medium from excised anther by a sterile glass rod. Other steps for microspore isolation procedure, incubation, culture procedures, transferred of embryoids to the regeneration medium are described briefly in P_1 (**Section 5.2.1.2.2.1.1**).

5.2.1.2.2.1.3 P_3 = Squeezed of rice floret by homogenizer

In this case sterilized rice floret was placed in a 50 ml tube with 20-25 ml washing medium (WM). Then squeezed them by a homogenizer (Polytron, PT-1200, Kinematica AG, Switzerland) with medium speed for 1 min. The anthers and debris were removed by passing a 100 µm stainless steel mesh sieve and the microspores were collected and the solution was washed by WM and further centrifuged with 750 - 1000 rpm for 3 minutes. The debris was removed through a 100 µm stainless steel mesh sieve and the microspores were collected in a 50 ml falcon tube. The subsequent procedure was followed as described previously in P_1 (**Section 5.2.1.2.2.1.1**).

5.2.1.2.2.1.4 P_4 = Microspore isolation from anthers by homogenizer

Here microspores were released in the medium from excised anther by squeezing with homogenizer (polytron). The subsequent other procedures are the same mentioned in P_1 (**Section 5.2.1.2.2.1.1**).

5.2.1.2.2.1.5 P_5 = Squeezed of rice floret by blender

Rice floret from spikes were placed in a sterilized microblender chamber containing 20-30 ml liquid WM. The microblender chamber covered with autoclaved aluminum foil during blending and anthers were blended with low speed 30-45 seconds. Then the debris was removed through a 100 µm stainless steel mesh sieve and microspores were collected following the procedures are described in P_1 (**Section 5.2.1.2.2.1.1**).

5.2.1.2.2.1.6 P_6 = Microspore isolation from anthers by blender

In this case microspores were released in the medium from excised anther by squeezing with a blender. The microblender chamber covered with aluminum foil during blending and anthers were blended with low speed 10-15 seconds. The subsequent procedures are followed as P_1 (**Section 5.2.1.2.2.1.1**) and P_3 (**Section 5.2.1.2.2.1.4**).

5.2.1.3 Data recording and statistical analysis

Data were recorded on the basis of embryo yield and its regeneration potentials from three major microspore isoaltion procedures. For Control, excised anthers were directly cultured in induction medium (AMC, Kunz et al. 2000). For microspore isolation procedure data were recorded on the basis embryogenesis (number of regenerated embryoids, embryoids per 10^5 microspores from 100 anthers) and for regeneration e.g. total regenerated plantlets (TRP), green regenerated plants (GRP) and albino plants (ARP) per 100 embryoids. Three replications were considered and 5×10^5 microspores were isolated from 500 anthers for all cases. For control, 500 anthers were inoculated in induction medium (AMC).

5.2.1.4 Results and Discussion

5.2.1.4.1 Results

Under this study six rice varieties *viz*. BR-4, BR-11, BRRI dhan29 (BRRI rice), IR-43 and IR-54 (IRRI rice) and Kaljira (local variety) were considered for isolated microspore culture (**Table 26**). Out of six varieties IR-43 (71.71%), and BRRI dhan29 (61.29%), BR-11 (53.29%) showed best performance for showing uni-nucleate microspores with exine and intine are visible (**Fig. 12A**) and multicelular pollen which appears to be the callusing type (**Fig. 12B,C**). On the other hand for green plant regeneration BR4 (32.66%) and IR-43 (30.15%) showed best results than other cultivars. For green plant regeneration BR4 produced less embryos (51.87%) induction than IR-43 (71.71%), but for green plant regeneration (**Fig. 12D**) it shows highest results that is very important for advance resaerch of rapid development of DHs production. Less number of albino plants (9.58%) were also recorded in BR4 in comparison with EM production frequency under all the studied rice varieties (**Table 26**). All of the responding varieties showed more or less green and albino plants also (**Fig. 12E**). Variances were analyzed for the efficiency on embryoids induction and regeneration of six BRRI, IRRI and local cultivers were evaluted that are mentined in **Table 26 & 27**.

Under this study embryoids induction and its subsequent regeneration procedures were evaluated and for that six microspore isolation procedures e.g. P_1 = squeezed of rice floret by sterile glass rod, P_2 = squeezed of excised anthers by sterile glass rod, P_3 = squeezed of rice floret by homogenizer (polytron), P_4 = squeezed of excised anther by homogenizer, P_5 = squeezed of rice floret by blender, P_6 = squeezed of excised anther by blender were employed. The percentages of embryoids induction from different isolation procedures are shown in **Table 28**. It was observed that out of three major steps, microspore isolation procedure by glass rod (P_1) was much easier and simplest where 65-123% of embryoids from 100 anthers were obtained. In this case second heighst

regeneration (91%) in P_2 and less number of albinos (8.00%) were recorded by glass rod isolation procedure (P_1). Highest percentage of embryoids (177%) were recorded in P_4 where anthers were squeezed by homogenizer (**Table 28).** ANOVA for embryoids induction and regeneration by different microspore isolation procedure are shown in **Table 29**.

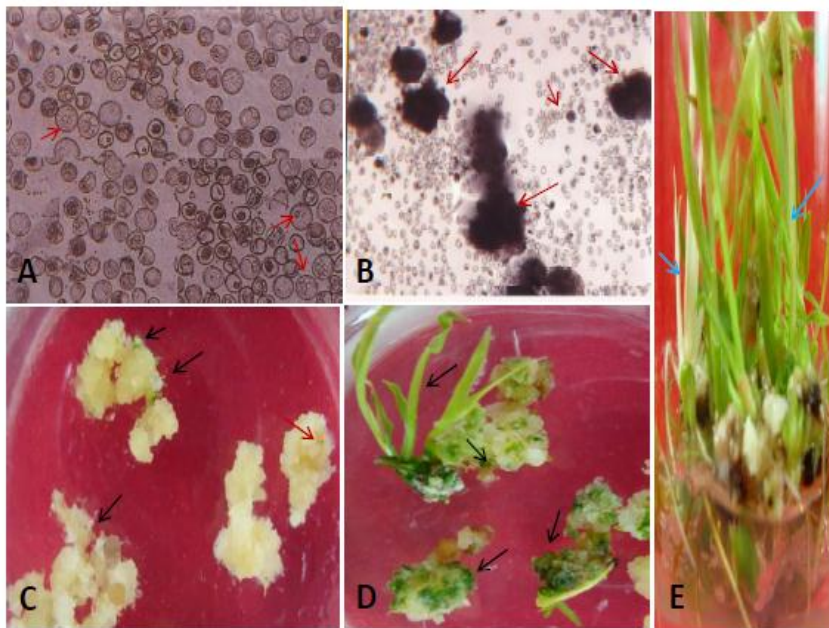


Fig. 12 (A-E): Stages of isolated microspore culture and its subsequent regeneration in rice. A) Microspore isolation after 3-5 days of culture initiation, B) Embryoids formation after 2-3 weeks of culture initiation, C) Microspore-derived embryoids were transferred to regeneration medium, D) Embryoids showing green plantlets in regeneration medium and E) Green and albino plants along with good shoot and roots.

Table 26: Efficiency of embryo induction and regeneration from isolated microspore culture of six rice cultivars

Variety	Embryos per 10^5 microspore			Mean for EM & GRP
	Embryos (EM)	Green plants (GRP)	Albino plants (ALP)	
BR4	51.87±3.13b	32.66±1.44a	9.58±0.74c	42.27±2.14
BR11	53.29±4.60b	27.68±1.07b	10.40±0.53c	40.49±2.84
BRRI dhan29	61.01±0.86b	25.26±1.28b	13.71±0.94b	43.13±0.24
IR-43	71.71±3.97a	30.15±2.60ab	16.55±1.54a	50.93±3.22
IR-54	40.66±1.92c	26.91±1.31b	13.18±0.61b	33.79±1.05
Kaljira	13.83±1.56d	6.99±0.54d	4.91±0.34d	10.41±1.00
Mean (SD)	48.7289 (19.24937)	24.9417 (8.88694)	11.3883 (3.99880)	-
F-value (Var.)	38.444	37.114	22.482	-
Significance	0.000	0.000	0.000	-
F-value (Rep.)	0.168	1.014	1.311	-
Significance	0.847	0.397	0.312	-
Treat. $LSD_{0.05}$	7.173	3.353	1.897	-

∗10^5 microspores = 100 anthers.

Table 27: ANOVA for efficiency of embryos induction and plants regeneration of six rice genotypes (Data source Table 26)

Source of variation	Dependent variable	Sum of squares	df	Mean square	F-value	Sig.
Treatment	ELS	5977.695	5	1195.539	38.444	0.000
	GRP	1260.893	5	252.179	37.114	0.000
	ALP	244.395	5	48.879	22.482	0.000
Replication	ELS	10.477	2	5.238	0.168	0.847
	GRP	13.782	2	6.891	1.014	0.397
	ALP	5.700	2	2.850	1.311	0.312
Error	ELS	310.981	10	31.098	-	-
	GRP	67.946	10	6.795	-	-
	ALP	21.742	10	2.174	-	-
Total	ELS	49040.236	18	-	-	-
	GRP	12540.182	18	-	-	-
	ALP	2606.331	18	-	-	-
Corrected Total	ELS	6299.153	17	-	-	-
	GRP	1342.621	17	-	-	-
	ALP	271.837	17	-	-	-

Table 28: Induction of embryoids and regeneration efficiency by different microspore isolation procedure in rice

Procedure	ELS per 10^5 microspores/ 100 anthers	Regenerated embryos (%)	Regeneration (per 100 embryos)		
			TRP	GRP	ARP
Cont.	28.00±0.58g	16.67±0.17g	22.67±0.17g	16.00±0.58	7.00±0.58
P1	65.00±0.59f	44.00±0.23f	55.00±0.58f	47.00±0.23	8.00±0.58
P2	123.00±0.17c	110.00±0.58b	102.00±0.23b	91.00±0.58b	11.00±0.17
P3	103.00±0.58d	78.00±0.58e	65.00±0.17d	53.00±0.58	12.00±0.23
P4	177.00±0.17a	136.00±0.23a	124.00±0.59a	108.00±0.23a	16.00±0.58
P5	99.00±0.59e	83.00±0.58d	58.00±0.13e	43.00±0.58	15.00±0.23
P6	137.00±0.23b	97.00±0.58c	94.00±0.59c	69.00±0.58c	25.00±0.23
Mean (SD)	104.5714 (45.99627)	80.6667 (38.03825)	74.3810 (32.45031)	61.0000 (29.46184)	13.4286 (5.82728)
F-value (Treat.)	6168.625	5259.273	3777.897	2529.625	97.000
Significance	0.000	0.000	0.000	0.000	0.000
F-value (Rep.)	0.125	0.636	0.551	0.125	0.125
Significance	0.884	0.546	0.590	0.884	0.884
Treat. $LSD_{0.05}$	1.245	1.115	1.123	1.245	1.245

Control = Excised anthers (100) directly cultured in liquid AMC (Kunz et al. 2000), P = Procedure, P_1 = squeeze of rice floret by sterile glass rod, P_2 = squeeze of excised anthers by sterile glass rod, P_3 = squeeze of rice floret by homogenizer, P_4 = squeeze of excised anther by homogenizer, P_5 = squeeze of rice floret by blender, P_6 = squeeze of excised anther by blender, 10^5 microspores = 100 anthers, TRP= Total regenerated plants, GRP = Green regenerated plants and ARP = Albino regenerated plants.

Table 29: ANOVA for embryoids induction and regeneration by different microspore isolation procedure in rice (Data source Table 28)

Source of variation	Dependent variable	Sum of squares	df	Mean square	F-value	Sig.
Treat.	ELS	42299.143	6	7049.857	6168.625	0.000
	RE	28926.000	6	4821.000	5259.273	0.000
	TRP	21048.286	6	3508.048	3777.897	0.000
	GRP	17346.000	6	2891.000	2529.625	0.000
	ARP	665.143	6	110.857	97.000	0.000
Rep.	ELS	0.286	2	0.143	0.125	0.884
	RE	1.167	2	0.583	0.636	0.546
	TRP	1.024	2	0.512	0.551	0.590
	GRP	0.286	2	0.143	0.125	0.884
	ARP	0.286	2	0.143	0.125	0.884
Error	ELS	13.714	12	1.143	-	-
	RE	11.000	12	0.917	-	-
	TRP	11.143	12	0.929	-	-
	GRP	13.714	12	1.143	-	-
	ARP	13.714	12	1.143	-	-
Total	ELS	271952.000	21	-	-	-
	RE	165587.500	21	-	-	-
	TRP	137243.500	21	-	-	-
	GRP	95501.000	21	-	-	-
	ARP	4466.000	21	-	-	-
Corrected Total	ELS	42313.143	20	-	-	-
	RE	28938.167	20	-	-	-
	TRP	21060.452	20	-	-	-
	GRP	17360.000	20	-	-	-
	ARP	679.143	20	-	-	-

ELS = Embryo like structures, RE = Regenerated embryo, TRP = Total regenerated plants, GRP = Green regenerated plants and ARP = Albino regenerated plants.

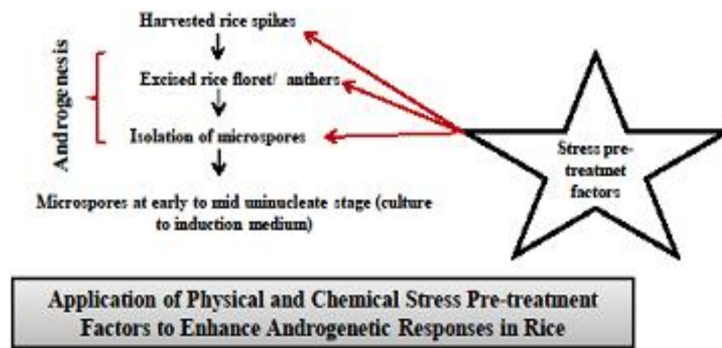
5.2.1.4.2 Discussion

Out of six microspore isolation procedure the best results were recorded in P_4 where anther were squeezed by homogenizer and in this case excised anthers were used as explants. The efficiency of isolated microspore culture technique of rice have been studied by major three methods and successfully standarized suitable isoaltion techniques. It was observed that better embryo yields of microspores isolation by hmomogenizer is better than glass rod and blender isoaltion procedures. Major disadvantages of both systems are produced lesser green and albino plants obtained little higher that other two procedures of galss rod and polytron isoaltion system. In *Brassica napus* the technique of pestle maceration by which the anthers are pressed through a sieve with a certain mesh size by using a teflon rod reported by Lichter (1982). This technique also successfully adapted for wheat (Tuvesson and Öhlund 1993), barley (Hoekstra et al. 1992) and maize (Pescitelli et al. 1990). In maize and barley the microblending technique was found to be superior for isolation of microspores compared to the maceration method with respect to yield of microspores (Olsen 1991). Similar observation was done by Gustafson et al. (1995). They compared four isolation methods of microspore and obtained a highest initial microspore viability (75%) with blending isolation technique. The system for homogenizer (polytron) and blending isolation techniques in this study gave highest embryoids per 100 anthers. However, for isolation technique of microspores by homogenizer and blender also gave quite similar results with the system Olsen (1991). By using a microblender a large number of microspores were free from floret and anther tissues and cellular debris raised. For microspore isolation, a series of sieve were used with different pore sizes in order to separate microspores of different stages (e.g. non-vacuolated early microspores and vacuolated microspores or bicellular pollen grains) before centrifugation, or to eliminate the subcellular components (Bedinger and Edgerton 1990). Regeneration from microspores obtained by microblending was described for wheat (Mejza et al. 1993), barley (Olsen 1991) and maize (Pescitelli et al. 1990). Gustafson et al. (1995) compared four microspore isolation methods e.g. blending, stirring, maceration and floating and

obtained highest initial microspore viability (75%) with blending isolation technique. They reported that blender isolation without mannitol conditioning and an initial density 2×10^5 microspore ml^{-1} was best for continued microspore viability. Those techniques are as follows: isolation of microspores by sterile glass rod, by homogenizer from anthers and by blender from anthers. Albino plants are still a great problem in many cereal crops for androgenetic studies and still it has no agronomical value. However, under this study microspore isolation by sterile glass rod and homogenizer showed quite better performances for reducing albino plants comapred with control and other microspore isolation procedures. May be some physiological changes happen during slow microspore isolation time and it may be concluded that for high embryo yield and reducing albinisims microspore isolation by homozeginer and glass rods are better more faster techniques.

5.3 Enhancement of abiotic stress tolerance in plants by physical and chemical stress pre-treatment factors through androgenesis (AC & MC) in rice

Several stress factors can trigger the reprogramming of microspores in case of androgenetic studies and that have been co-related to change the ultrastuctural changes of cells to embryos and finally successful production of haploid (n) and dihaploid (2n) plants in cereal and other crops. Improving anther and isolated microspore culture have focused on "stress" treatments to induce androgenesis by redirecting the preprogrammed gametophytic to the sporophytic development pathway in cereal crops are reported by Touraev et al. (1996 a and b, 1997), Hu and Kasha (1999), Zhou and Konzak (1997), Simmonds (1989), Reynolds (1997) and Yousuf et al. (2012). There are some reports on various stress pretreatment factors and their application in case of androgenetic studies e.g. physical (cold, heat shock, starvation, drought, osmotic pressure, gamma irradiation, oxidative stress, reduced atmospheric pressure, etc) and chemical (colchicine, heavy metal, ABA, CGA, AEC, Azetidine, 2-NHA, etc) either single or in combination uses that showed very positive effect of callus induction, regeneration and reducing albinisms. Hoveida et al. (2017) evaluated that the efficiency of different chemical and physical stresses on anther cultures of borage (*Borago officinalis* L.) and found interesting results on callus induction, regeneration and doubling efficiency. They applied 0.2% n-butanol in addition with 200 mg/l colchicine to flower buds for 4 days and found the highest percentages of callogenesis compared to control and other colchicines treatments. Under this study some physical and chemical stress pre-treatment factors were applied to find out their effect on androgenetic responses and development of haploid and doubled haploid plants in rice.



5.3.1 Physical stress pre-tretement factors

5.3.1.1 Effect of cold stress pre-treatment factors in rice anther culture

5.3.1.1.1 Introduction

Cold pretreatment of anthers/spikes/flower buds either pre- or post-culture at 3 to 5°C for 2-4 days influenced symmetric rather than asymmetric division of the microspore nuclei (Yie et al. 1997, Obert and Barnabás 2004). They suggested that cold or heat acts as a shock treatment causing a 90° shift in division plane of microspore causing a symmetrical division that enhancing anther culture response in maize. Zheng and Ouyang (1980) found that after low temperature pretreatment to suitable explants, symmetrically dividing microspores were found and that influences DHs production in wheat. It has been reported that cold-shock pretreatment of young spikes was very effective for anther culture of rice (Zhou and Cheng 1982). Zhou et al. (1983) found that the cold shock pre-treatment requirements were varied among the *Indica* and *Japanica* rice genotypes. Cold pre-treatment at 3°C the tillers soaked in water significantly that may help to improve the androgenetic responses in *Triticum* (Pan et al. 1975, Picard and De Buyser 1975). For maize anther and microspore culture, tassels are usually pretreated by cold, placing the tassels at around 7°C in the dark for 7-21 days showed very positive effects on callus induction was reported by Gaillard et al. (1991) and Barnabás (2003). Cold pretreatment at 7°C for 10 days in the dark conditions and then immature anthers

containing microspores at the late uninuclear developmental stage were placed on a modified liquid YP medium and found very positive results on callus induction in maize was reported by Genovesi and Collins (1982). Ouyang (1986) reported that cold treatment at mid or late uninucleate stage enhanced development of microspores towards the pre-mitosis stage within 2-3 days. They also pointed out that when cold treatment duration exceeded a certain limit, the induction frequency decreased markedly. They also observed that cold treatment not only significantly increased anther response but also enhanced green plant regeneration in wheat. Sen et al. (2011) reported that cold pretreatment influenced on callus induction and plant regeneration in rice anther culture. The present research has been undertaken to determine the effect of suitable duration of cold pretreatment for rice anther culture for further advance research in plant biotechnology.

5.3.1.1.2 Material and Methods

5.3.1.1.2.1 Plant materials and media

As rice variety, BRRI dhan29 and as medium SK3 was considered for this study. Under this study for Control, anthers were seperated from the sterilized spikes directly and cultured in the induction medium.

5.3.1.1.2.2 Methods

Harvested spikes were pre-treated by cold at 4°C chamber for T_1 = 1 d, T_2 = 3 d, T_3 = 5 d, T_4 = 7 d, T_5 = 9 d, T_6 = 11 d, T_7 = 13 d and T_8 = 15 days. For all treatments cultures were incubated at 14°C for first 7 days and then incubated at 25±1°C chamber in dark for embryos induction. Harvesting spikes and its preparation for stock solution and media preparation, sterilization, inoculation, incubation, embryoids transferred to regeneration medium and other procedures are explained in **Section 3.3.1.2.5**.

5.3.1.1.3 Results and Discussion

5.3.1.1.3.1 Results

Under this study harvested spikes were pre-treated by cold at 4°C chamber for different days (1-15 days) before inoculation. For Control harvested spikes were inoculated to induction medium after sterilization at the same day. For all cases inoculated spikes were incubated first 7 days at 14°C chamber and the for embryos induction cultures were incubated at 25±1°C for 6-8 weeks. For this study spikes were collected from BRRI dhan29 and excised anthers were inoculated in liquid SK3 medium. It was observed that T2 showed highest embryoids induction (28.85%) than Control (17.83%). The effects of cold pre-treatment on embryoids induction were increased (20.51% - 28.85%) where Control showed 17.83%. For total regenerated plants (TRP) 3 days cold pre-treated spikes (anther sources) also showed highest regeneration (15.78%) in comparison with Cont. (8.81%) and other pretreatments (**Table 30**).

Due to increasing the days of cold pre-treatment the TRP gradually decreased. For green plant regeneration T_2 showed nearly doubled production of plants (12.93%) than Control (6.12%). It was observed that due to long time cold pre-treatment green plant regeneration (GRP) rate were gradually decreased than other pre-treatments (T) and Control. For albino plant production 3 days cold pre-treated showed lower (2.91%) regeneration. In comparison with callus induction rate T_6-T_8 showed increasingly higher albino plants than control and treatments (**Table 30**). On the basis of ELS, TRP and GRP, for overall mean value showed significantly higher percentages of results in T_2 (19.19%), T_3 (16.56%) and T_4 (17.56%) in comparison with Control (10.92%). Analysis of variance showed significant differences in the frequency of embryoids induction and green plant regeneration depending on the cold pre-treatment duration (**Table 30 & 31**).

Table 30: Effect of cold pre-treatment (4°C) in anther culture of rice

Cold pre-treatment (days)	Per 100 anthers				Mean for ELS, TRP & GRP
	ELS	TRP	GRP	ALP	
Cont.	17.83±0.44d	8.81±0.46d	6.12±0.24e	2.23±0.47d	10.92±0.23
T_1	20.51±0.58cd	9.56±0.36d	7.58±0.36de	2.48±0.41d	12.55±0.20
T_2	28.85±1.67a	15.78±1.33a	12.93±0.87a	2.91±0.34cd	19.19±0.86
T_3	26.56±1.17ab	12.52±0.29bc	10.60±0.40bc	2.95±0.10cd	16.56±0.22
T_4	27.59±1.99ab	14.22±0.72ab	10.85±0.41b	4.47±0.60ab	17.56±0.94
T_5	24.37±1.32bc	11.22±1.05cd	9.73±0.44bc	3.82±0.30bc	15.11±0.91
T_6	23.78±1.39bc	10.55±1.08cd	9.53±0.52bc	4.78±0.21ab	14.62±0.95
T_7	23.30±1.93bc	10.41±0.48cd	9.00±0.90cd	5.47±0.36a	14.24±1.10
T_8	20.85±0.46cd	9.66±0.88d	6.37±0.21e	4.55±0.23ab	12.30±0.45
Mean (SD)	23.7381 (3.95514)	11.4163 (2.52435)	9.1904 (2.26035)	3.7396 (1.21682)	-
F-value (Treat.)	7.070	7.422	16.891	10.185	-
Significance	0.000	0.000	0.000	0.000	-
F-value (Rep.)	0.900	0.217	0.967	1.292	-
Significance	0.426	0.808	0.401	.302	-
Treat. $LSD_{0.05}$	2.337	1.477	0.931	0.620	-

Cont.: Without cold pre-treatment. For T_1 = 1, T_2 = 3, T_3 = 5, T_4 = 7, T_5 = 9, T_6 = 11, and T_7 = 13 and T_8 = 15 harvested spikes were pre-treated by cold at 4°C.

Table 31: ANOVA for of cold pre-treatment (4°C) in anther culture (Data source Table 30)

Source of Variation	Dependent Variable	Sum of Squares	df	Mean Square	F-value	Sig.
Treatment	ELS	309.363	8	38.670	7.070	0.000
	TRP	129.767	8	16.221	7.422	0.000
	GRP	117.274	8	14.659	16.891	0.000
	ALP	31.347	8	3.918	10.185	0.000
Replication	ELS	9.849	2	4.925	0.900	0.426
	TRP	0.947	2	0.474	0.217	0.808
	GRP	1.679	2	0.840	0.967	0.401
	ALP	0.994	2	0.497	1.292	0.302
Error	ELS	87.510	16	5.469	-	-
	TRP	34.967	16	2.185	-	-
	GRP	13.886	16	0.868	-	-
	ALP	6.155	16	0.385	-	-
Total	ELS	15621.214	27	-	-	-
	TRP	3684.641	27	-	-	-
	GRP	2413.338	27	-	-	-
	ALP	416.087	27	-	-	-
Corrected Total	ELS	406.722	26	-	-	-
	TRP	165.681	26	-	-	-
	GRP	132.839	26	-	-	-
	ALP	38.497	26	-	-	-

5.3.1.1.3.2 Discussion

Under this study cold pre-treatment for different days at 4°C to harvested spikes were done before inoculation that showed significant results on androgenic responses. Pre-treatment such as cold to flower buds or excised anthers were shown to be very effective in cereal and other crops. It has been reported that low temperature showed symmetrically dividing microspores to be the main sources of androgenic haploids (Zheng and Ouyang 1980). Bajaj (1983) reported that cold pre-treatment used to increase the frequency of androgenic responses for anther and isolated microspore culture. He mentioned that cold treatment appears to inhibit spindle formation and some cases may cause an abnormal first mitosis by triggering the pollen nucleus to form two similar-looking nuclei. Lazar et al. (1985) reported that variation of intensity and duration of cold pretreatment increased the embryo yield and regeneration. Powell (1987) reported that the benefits of cold pre-treatment to harvested spikes could be attributed to slow degradation of the tapetum and locular matrix in barley. Croughan and Chu (1991) reported that cold pretreatment is very effective in enhancing callus formation and regeneration. A pretreatment of spikes at low temperature has been a very positive effect on callus induction as well as regeneration in rice was reported by Genovesi and Maggill (1979). They mentioned that cold treatment did not induce androgenesis, but it might enhance the viability of cultured pollen, and causes repressing of the gametophytic differentiation, which resulted in higher frequency of androgenenic responses in rice. Picard and De Buyser (1975) found that cold pre-treatment at 3ºC of the tillers soaked in water time significantly improves the androgenic success mainly during the less favorable winter period. In some cases, especially after low temperature pretreatment, symmetrically dividing microspores were found to be the main sources of androgenic haploids in rice (Reddy et al. 1985). Marsolais and Kasha (1983) also reported that cold pretreatment is not always necessary to obtain the higher percentages of plants derived from anthers or isolated microspores. However, it is a little discrepancy from the present study; it might be attributed to the genotype, media and PGRs concentration and combination differences.

Chaleff and Stolarz (1981) reported that about the effect of cold treatment in rice and they found more androgenic response occurred when the anthers were pretreated at 7°C for 3 days. Pan et al. (1975) showed that variation in intensity and duration in cold pretreatment lead to significant differences in callus formation frequency. Under this study 3 days cold pretreatment showed highest embryoids induction in comparison with control. These results agreed well with the previous report of Chaleff and Stolarz (1981) and Pan et al. (1975). But in this case cold pre-treatment was done at 4°C for 3 days that was quite different than previous study. Zapata et al. (1982) reported that rice anthers pretreated by cold shock at 8°C for 8 days displayed best result on callus induction and regeneration. Under this study regenerated plants were increased with cold pre-treatment at different days. But 3 days cold pre-treated spikes showed more positive responses than others. Pande (1997) observed that cold pre-treatment was found to be essential for anther culture responses of Indica cv. IR-43. He also mentioned that with longer pretreatment duration than 11 days showed more albino plant production. About albinism Torp and Andersen (2009) reported that for reducing the frequency of albino plant production avoid prolonged cold pretreatment duration. Under this study longer duration (15 days) of cold pre-treatment also showed increasing the number of albino plants than short duration (1-3 days). These results agreed well with the findings of Torp and Andersen (2009).

5.3.2 Effect of drought stress pre-treatment (physical) factors to improve anther culture responses in rice

5.3.2.1 Introduction (Drought)

Drought is an environmental stress factors that causes agricultural losses in the worldwide (Mahajan and Tuteja 2005). Minimizing these looses is a major concern for plant scientist to improve plants through various biotechnological approaches. There are three components of drought resistance *viz*. dehydration avoidance, dehydration tolerance and dehydration escapes. Dehydration avoidance is the ability of the plant to maintain its hydration state whereas dehydration tolerance refers to a plants ability to function after dehydration (Blum 1988). Plant response to drought stress is complex as it involves a number of physio and biochemical processes at the cellular level and different interacting component traits with different responses at the whole plant level reported by Witcombe et al. (2008) and Kadam (2012).

Ben-Gal et al. (2009) mentioned that drought usually leads to oxidative stress due to stomatal closure which causes the over-reduction of photosynthetic electrons. Drought tolerance is a difficult trait to define as it encompasses a wide range of characteristics involving multiple genetic, physiological, cellular and biochemical strategies in the plant. Mitra (2001) reported that drought involves the absence of rainfall for a period of time, long enough to cause moisture-depletion in soil and water deficit with a decrease of water potential in plant tissues. Verslues et al. (2006) mentioned that drought is simulated by adding osmotica, such as mannitol, sorbitol or polyethylene glycol (PEG) which lower the water potential of the medium (Feng et al. 2011). They used PEG, sucrose, mannitol or sorbitol as osmotic stress agents in the tissue culture medium for *in vitro* selection. Hassan et al. (2004) reported that PEG is stimulate water stress in plants and it has high molecular weight is a non-penetrating inert osmoticum lowering the water potential of nutrient solutions without being taken up or being phytotoxic. Polyethylene glycol (PEG) is used for *in vitro* selection of drought tolerant cell/callus of wheat where for too high osmotic stress kills the tolerant cells, while sub-optimal level of osmoticum

results in survival of non-tolerant cell lines (El-Hennawy et al. 2018). Chlorophyll a and b, total chlorophyll, proline, total phenolic contents, total peroxidase and catalase activities increased under stress conditions, suggesting the tolerance of callus to drought stress (Pant et al. 2014). Karmakar et al. (2012) found that stress causes oxidative damage to plants either directly or indirectly by triggering an increased level of production of reactive oxygen species (ROS) which include superoxide radical. However, a major challenge in the use of functional genomics to enhance the development of drought tolerance is to define the system and focus on key traits of interest.

In recent years, tissue culture based *in vitro* selection has emerged as a feasible and cost-effective tool for developing stress-tolerant plants. Islam (2010a) reported that drought stress (1-5 hrs) showed significantly better embryoids induction for anther culture in wheat. Plants tolerant to biotic and abiotic stresses can be acquired by applying the selecting agents such as NaCl (for salt tolerance), PEG or mannitol (for drought tolerance) in addition to the culture medium. Touraev et al. (1997) reported that application of stress pre-treatments factors in barley microspore culture, no green plants were found. The application of biotechnology in combination with conventional breeding methods may help to improve plants resistance to various abiotic stresses (Redha and Islam 2010). Stress pre-treatment factors to different explants such as spikes/flower bud, excised anthers, or isolated microspores may influences androgenetic responses and helpful to develop plants tolerance to abiotic stresses (Islam and Tuteja 2012). However, the success of work it depends to different explants types, levels and duration of pre-treatments, the regeneration efficiencies vary as well culture media also. Under this study drought stress pre-treatments along or in combination such as heat, sugar starvation by PEG and mannitol were applied, and also evaluated the culture media for anther and isolated microspore culture in rice. The main objective of this study was to determine the effect of drought stress pre-treatment factors to improve androgenetic responses in rice.

5.3.2.2 Materials and Methods

5.3.2.2.1 Plant materials

As variety BRRI dhan29 and medium SK3 was considered to conduct this experiment.

5.3.2.2.2 Methods

Under this study for Control (without drought treatment, 0 h) excised anthers were directly cultured in induction medium. For treatments (T), excised anthers were pre-treated by drought in laminar air flow cabinet (r.t) for T_1 = 2 h, T_2 = 4 h, T_3= 6 h, T_4 = 8 h, T_5 = 10 h and T_6 = 12 h. For all treatments cultures were incubated at 14°C for first 7 days and then incubated at 26±1°C chamber in dark for embryos induction. Harvesting spikes and its preparation, sterilization procedure, stock solution and media preparation, inoculation, incubation, embryoids transferred to regeneration medium and other procedures are explained previously (**Section 3.3.1.2.5**).

5.3.2.3 Results and Discussion

5.3.2.3.1 Results

To evaluate the effect of drought stress pre-treatment 3 days cold pre-treated spikes of BRRI dhan29 were used. Anthers were excised from the sterilized spikes in laminar air flow cabinet and subjected them as drought stress pre-treatment with various durations. It was observed that application of drought stress to excised anthers T_2 (4 h) showed highest embryo yield (37.71%) than Control (19.91%) and other treatments of this study (**Table 32**). T_3 (6 h) showed slightly lower embryo yield (37.08%) but for plant regeneration it gave highest green plants (16.26%) than all over the treatments (T) and Control considering under this study (**Table 32**).

In case of albinism less percentage (2.87%) of plants was recorded with short duration of drought pre-treatment (2 h) in T_1. It was observed that increasing drought stress pre-treatments it showed higher percentages of albino plants (T_3-T_6). In T_5 (10 h) and T_6 (12 h) drought stress pre-treatment showed less green plants (8.54% and 5.81%) where

albino plants were increased (9.16% and 8.29%) that plants have no agronomic value (**Table 32**). On the basis of ELS and GRP the overall mean value was higher in T_3 (26.67%) and lower in T_6 (11.53%). Analysis of variance (ANOVA) showed the highly significant differences in the frequency of embryo yield and green plant regeneration. Whereas increasing albino plant production with prolonged duration of drought stress pre-treatment to inoculated anthers (**Table 32 & 33**).

Table 32: Anther induction and regeneration efficiency under different durations of drought stresses in rice

Drought stress (hrs)	Per 100 anthers			Mean for ELS & GRP
	ELS	GRP	ALP	
Cont.	19.91±1.84c	8.06±0.46c	3.06±0.19c	13.99±1.11
T_1	24.61±0.93b	9.38±0.16c	2.87±0.19c	16.99±0.42
T_2	37.71±0.71a	13.91±1.18b	3.97±0.15c	25.81±0.90
T_3	37.08±1.83a	16.26±0.55a	5.76±0.40b	26.67±0.74
T_4	33.81±1.24a	12.95±0.63b	6.52±1.17b	23.38±0.94
T_5	28.24±1.50b	8.54±0.61c	9.16±0.58a	18.39±0.93
T_6	17.25±1.18c	5.81±0.74d	8.29±0.36a	11.53±0.30
Mean (SD)	28.3738 (8.01867)	10.7005 (3.67632)	5.6610 (2.49391)	-
F-value (Treat.)	43.262	36.688	26.535	-
Significance	0.000	0.000	0.000	-
F-value (Rep.)	2.579	2.575	2.872	-
Significance	0.117	0.117	0.096	-
Treat. $LSD_{0.05}$	2.511	1.243	0.977	-

Cont. = without drought (0 d). Excised anthers were pre-treated at r.t (laminar air flow cabinet) for drought for T_1 = 2 h, T_2 = 4 h, T_3= 6 h, T_4 = 8 h, T_5 = 10 h and T_6 = 12 h.

Table 33: ANOVA for anther induction and regeneration efficiency under different durations of drought stress pre-treatment factors (Data source Table 32)

Source of Variation	Dependent Variable	Sum of Squares	df	Mean Square	F-value	Sig.
Treatment	ELS	1206.246	6	201.041	43.262	0.000
	GRP	250.770	6	41.795	36.688	0.000
	ALP	111.919	6	18.653	26.535	0.000
Replication	ELS	23.970	2	11.985	2.579	0.117
	GRP	5.866	2	2.933	2.575	0.117
	ALP	4.037	2	2.019	2.872	0.096
Error	ELS	55.765	12	4.647	-	-
	GRP	13.670	12	1.139	-	-
	ALP	8.436	12	0.703	-	-
Total	ELS	18192.516	21	-	-	-
	GRP	2674.810	21	-	-	-
	ALP	797.366	21	-	-	-
Corrected Total	ELS	1285.981	20	-	-	-
	GRP	270.306	20	-	-	-
	ALP	124.392	20	-	-	-

5.3.2.3.2 Discussion

Various stress pre-treatments such as cold, heat shock, drought, osmotic shock and starvation during the development of microspores inside in the anthers is known to promote or is essential for the induction of androgenesis in several crop plants. There are some reports on successful application of abiotic stress pre-treatments for crop improvement (Maraschin et al. 2006, Shariatpanahi et al. 2006b, Seguí-Simarro and Nuez 2007, Ochatt et al. 2009, Żur et al. 2009). Zoriniants et al. (2005) reported that a variety of pretreatments have been used such as applying as stress to the developing microspores at a critical stage, causing a block or delay in their development (Clément et al. 2005, Obert et al. 2009). The mechanisms underlying the stress-induced switch from gametophytic to sporophytic pathway on cellular, biochemical, and molecular processes taking place during transition to the embryogenic state have been reported by Shariatpanahi et al. (2006a). Under this study drought stress might be considered as parallel to the thermal shock treatment.

In this case an open exposure of starvation subjected to excised anthers of BRRI dhan29 at room temperature was referred as drought stress pre-treatments. The effect of different durations (hrs) of drought stress pre-treatment to excised anthers were evaluated their responses on the basis of embryo yield and green plant regeneration. Interesting results was obtained of simple exposure of drought stress pre-treatment at room temperature of excised anthers brought about a remarkable progress in improving anther culture responses in rice. The drought stress pre-treatments varied from 2-12 hrs. In this case T_2 (3 h) proved to be more suitable for embryo yield and green plant regeneration in rice. Drought exposure for a prolonged period gradually decreased the embryo yield as well as regeneration in rice anther culture. It is very interesting to note that drought stress for longer duration caused to increase the albino plant production in comparison to short duration of drought stress pre-treatments to excised anthers. Application of drought to excised anthers prior to culture in liquid induction medium was very effective for embryoids induction as well as green plant regeneration was optimized with 3 h stress pre-treatments in rice.

5.3.3 Combined effect of drought and heat stress pre-treatment to rice anther culture

5.3.3.1 Introduction

Heat stress is a complex function of intensity, duration and rate of increase in high temperature that causes agricultural looses in many countries in the world. Extreme temperatures can cause premature death of plants. But some cases optimum heat stress may influences to enhance embryoids yield in cereal crops for androgenetic responses (Ahmed et al. 1992, Ahmed and Hall 1993). However, optimization the range and duration of heat for each crop species is very important. There are some reports on heat shock that shown to cause changes in microtubule and cytoskeleton in cultured *Brassica* microspores (Hause et al. 1993, Cordewener et al. 1994, Simmonds and Keller 1999). The stimulatory effect of thermal shocks for anther culture has been successfully adopted in *Datura* by Nitsch and Norreel (1973), tomato (Debergh and Nitsch 1973) and tobacco (Bajaj and Reinert 1975) and *Anemone coronaria* (Dei et al. 2018). Heat shock has been used as a trigger to induce microspore embryogenesis in rapeseed was reported by Custers et al. (1994) and also for wheat (Touraev et al. 1996b), tobacco (Touraev et al. 1996a), eggplant (Miyoshi 1996), timothy (Guo and Pulli 2000), oats (Kiviharju and Pehu 1998), flax (Chen et al. 1998) and *Hepatica nobilis* (Nomizu et al. 2004). Heat pretreatment of inoculated anthers or microspores at 30-35ºC was found effective for callus induction and regeneration in cereals crops are reported by Reddy et al. (1985) and Li et al. (1997). Evidence of the beneficial effects of thermal shocks has been reported in oats and Proso millet (Wu et al. 2012). Islam (2010a) reported that drought stress (1-5 hrs) significantly better for anther culture in wheat. High temperature shock at 35°C for 12 h elevated 11 times higher embryo yield in comparison to control in potato (Shen and Veilleux 1995). Mannitol pretreatment of isolated tobacco microspores in combination with heat shock of freshly isolated microspores have proven to be successful methods for induction of microspore regeneration in *Brassica napus* (Custers et al. 1994). Under this study application of heat shock along or in combination with drought starvation and mannitol pre-treatment on rice anther an isolated microspore culture were evaluated.

5.3.3.2 Materials and Methods

5.3.3.2.1 Plant materials and media

As variety BRRI dhan29 and medium SK3 was considered to conduct this experiment.

5.3.3.2.2 Methods

For all treatments (T) spikes were pre-treated by cold at 4°C for 3 days. Cont. = directly cultured in liquid induction medium (without drought and or heat pre-treatment). Excised anthers were pre-treated by drought for 3 hrs in laminar air flow cabinet (r.t) along with various temperature (°C) for T_1 = 1 h (27°C), T_2 = 2 h (29°C), T_3= 3 h (31°C), T_4 = 4 h (33°C) and T_5 = 5 h (35°C). Harvesting spikes and its preparation, sterilization procedure, stock solution and media, inoculation, incubation at 14°C for 1st 14 days and then at 26°C until embryos induction, transferred embryoids to regeneration medium and other procedures are explained in **Section: 5.1.1.2.1**.

5.3.3.2.3 Results and Discussion

5.3.3.2.3.1 Results

To evaluate the combined effect of heat and drought stress pre-treatments cold pre-treated spikes were selected from BRRI dhan29. Here combined stress pre-treatments (drought- 3 hrs and heat pre-treatment with different hours) were done before adding medium to the inoculated anthers. It was observed that highest embryo yield (38.99%) was recorded when excised anthers were pre-treated at 27°C for 1 h. Green plant regeneration was higher (17.77%) which was also recorded in T_1. Very close results on green plants were also recorded in T_2 (16.21%). Due to higher temperature to inoculated anthers embryo yield was decreased 3-4 fold in T_5 than T_1 and T_2. Here T_5 showed 6 times lesser green plants (3.59%) than T_1 (17.77%). It was observed that increasing the temperature pre-treatment at 33-35°C and 4-5 h duration showed very less number of green plants and increasing albino plant production compared to Control and other treatments (T) of this study (**Table 34**). In overall mean performance T_1 (27°C, 1 h) showed the highest results (28.38%) on ELS and GRP than Cont. (20.44%); and lowest for T_5 (7.59%). ANOVA showed significantly higher embryo yield and regeneration with 1 hr drought + less incubation period (T_1) by heat pre-treatment subjected to inoculated anthers.

Table 34: Anther induction and regeneration efficiency under different durations (drought 3 h) + various heat stress pre-treatment factors in rice.

Drought stress (Heat/ hrs)	Per 100 anthers			Mean for ELS & GRP
	ELS	GRP	ALP	
Cont. (26°C, 0 h)	27.50±0.46c	13.38±0.25b	2.90±0.21d	20.44±0.35
T_1 (27°C, 1 h)	38.99±1.51a	17.77±0.34a	3.23±0.55d	28.38±0.86
T_2 (29°C, 2 h)	34.87±1.05b	16.21±0.71a	4.95±0.16c	25.54±0.88
T_3 (31°C, 3 h)	29.66±0.79c	11.82±0.85b	8.91±0.31a	20.74±0.67
T_4 (33°C, 4 h)	21.09±1.50d	9.02±0.85c	7.46±0.61ab	15.05±1.16
T_5 (35°C, 5 h)	11.58±0.88e	3.59±0.23d	6.38±0.58bc	7.59±0.40
Mean (SD)	27.2811 (9.39053)	11.9650 (4.91589)	5.6389 (2.32872)	-
F-value (Treat.)	70.679	63.676	25.124	-
Significance	0.000	0.000	0.000	-
F-value (Rep.)	0.274	0.231	0.223	-
Significance	0.766	0.798	0.804	-
Treat. $LSD_{0.05}$	2.611	1.438	1.059	-

Cont. = directly cultured in liquid induction medium (no drought pre-treatment). T_1 = 1 h (27°C), T_2 = 2 h (29°C), T_3= 3 h (31°C), T_4 = 4 h (33°C) and T_5 = 5 h (35°C).

Table 35: Anther induction and regeneration efficiency under different durations (drought 3 h) + various heat stress pre-treatment factors (Data source Table 34)

Source of Variation	Dependent Variable	Sum of Squares	df	Mean Square	F-value	Sig.
Treatment	ELS	1455.645	5	291.129	70.679	0.000
	GRP	397.752	5	79.550	63.676	0.000
	ALP	85.112	5	17.022	25.124	0.000
Replication	ELS	2.261	2	1.130	0.274	0.766
	GRP	0.576	2	0.288	0.231	0.798
	ALP	0.302	2	0.151	0.223	0.804
Error	ELS	41.191	10	4.119	-	-
	GRP	12.493	10	1.249	-	-
	ALP	6.775	10	0.678	-	-
Total	ELS	14895.759	18	-	-	-
	GRP	2987.723	18	-	-	-
	ALP	664.537	18	-	-	-
Corrected Total	ELS	1499.096	17	-	-	-
	GRP	410.821	17	-	-	-
	ALP	92.190	17	-	-	-

5.3.3.3.2 Discussion

Several physiological factors such as high temperature, drought and/starvation either single or in combination was used prior to culture of excised anthers that influences androgenic responses in rice. Combined effect of drought and starvation for shorter period (3-4 days) stimulated androgenic responses and increased microspore survival rate which may cause to reduce the frequency of albino plant production in cereal and other crops are reported by Shariatpanahi et al. (2006a). Efficient androgenesis is usually induced by the successful application of different stress pretreatment (Touraev et al. 1996a). Since so many stress factors can trigger the reprogramming of microspores and that have been co-related to change the ultrastuctural changes of cells to embryos and finally haploid plants (Shariatpanahi et al. 2006a). Stress acts as a trigger to induce microspore embryogenesis and represents a key point where the reprogramming of microspores takes place. Hosp et al. (2007a) reported that application of various stress pre-pretreatments to higher plants showed that microspores are able to reprogram their regular gametophytic development towards the sporophytic pathway to form haploid embryos and plants. Ho et al. (1978) obtained high embryo yield and plantlets in wheat anther culture following heat stress pretreatment at 28°C - 32°C. Cao et al. (1994) provided three different temperature treatments, such as 25°C (continuous) and 33°C for 1 and 3 days to *Brassica* anther culture and obtained the highest embryo yield per bud at 33°C for one day heat pre-treatment. Touraev et al. (2009) reported that a combined effect of starvation and heat stress of heat shock at 33°C or 37°C along with starvation influence the efficiency of induction in tobacco microspore culture (Touraev et al. 1996b). Under this study combined effect with drought (3 hrs) + shorter duration of heat stress enhanced embryo yield as well as green plants in rice. The highest embryo yield (38.99%) was recorded when excised anthers were pre-treated at 27°C for 1 hr. However, high temperature applied by Touraev et al. (1996b) and found the positive responses on embryo yield is not similar with the present findings because they have done work on tobacco plants. May be the experimental pattern, media and PGRs was different than the present study.

Keller and Armstrong (1979) found embryo yield was increased by subjecting short-duration of high temperature shock (i.e., 45°C for one hour followed by 40°C for 3 hours) prior to anthers planting in *Brassica*. Similarly, Ockendon and Sutherland (1987) found that *Brussels sprouts* (*B. oleracea* var. *gemmifera)* yielded up to 357 embryo yield per 100 anthers using a thermal shock treatment of 16 hours at 35°C at the starting of culture. Touraev et al. (1996b) applied heat shock treatment along with starvation stress on wheat and observed very efficient induction and regeneration. Bueno et al. (1996) also obtained haploid embryos and regenerated plantlets in *Quercus* sp. by combining a starvation treatment in anther culture with a mid heat shock at 33°C for five days. Under this study heat pre-treatment was applied to excised anthers for different hours that application duration and donor plant was not same.

5.3.4 Effect of chemical stress pre-treatment factors

5.3.4.1 Enhancement of anther culture responses in rice by chemical stress pre-treatment factors

5.3.4.1.1 Introduction

From gametophytic to sporophytic development to switch microspores some chemicals (colchicine, CGA, AEC, Azetidine, ethanol, feminizing agents, ABA, etc) had a positive effect on androgenesis from the treated microspores (Dermen 1940, Schmid and Keller 1986, Shariatpanahi et al. 2006a). For symmetrical divisions of microspores and increasing the doubling efficiency colchicines showed positive effects of DHs production in wheat (Barnabás 1991) and maize (Michard and Beckert 1997). For androgenetic studies application of various chemicals such as colchicine, heavy metal, ABA, CGA, AEC, Azetidine, 2-NHA, etc either single or in combination showed very positive effect in cereal crops. Schmid and Keller (1986) reported that chemical substances, like gametocide (CGA) influence androgenic response very strongly in wheat (Schmid 1988). They also used other chemical substances like Azetidine (3, 4-dinito-N4, N4-dipropylsulfanilimide) and AEC (analog of amino acid) for enhancing callus induction and doubling efficiency by external uses in plants as well as in medium (Schmid 1988). Miao et al. (1988) applied lysine and proline e.g. S-2-aminoethyl-L-cysteine (AEC) and azetidine-2-carboxylic acid (A2C) to improve maize callus culture.

Chromosome doubling has become an important tool in breeding programmes as it offers the ability of introducing novel traits into existing plants. Antimitotic agents are compounds obtained from nature that mimic biological agents (Liang 1991, Abraha et al. 2008, Goyal and Khan 2009). These agents arrest cells in mitosis by interfering with microtubule function at the metaphase plate thus inhibiting cell division. As a result the chromosomes are duplicated but not divided. The plants that develop from explants treated with antimitotic agents are called doubled haploids or polyploids (Goyal and Khan 2009). Doubled haploid (DHs) plants are highly valued by both consumers and breeders as these plants usually show larger flower, leaves and fruit, thus making them

more marketable. Treatments with antimitotic agents such as colchicine, trifluralin, oryzalin, pronamide have been commonly employed to induce chromosome doubling of several plant species in various *in vitro* systems, e.g anther culture, microspore culture, ovule culture, flower bud culture, embryo and cell suspension cultures (Abraha et al. 2008). There are some reports using various herbicide treatments on the growth and regenerative capacity of callus along with the ploidy and seed set of regenerated plants was reported by Wan et al. (1991). As herbicides/components such as polyethylene glycol (PEG), pronamide, amiprophosmethyl (APM) and brassinosteroids (BR) are good candidates and have been used to generate doubled haploid (DH) plants and to improve the microspore embryogenesis in various *Brassica* species (Ferrie and Keller 2008, Geiger and Gordillo 2009, Hantzschel and Weber 2010). Some available chemicals as antimitotic agents are employed under this study are mentioned herein:

5.3.4.1.1.1 Colchicine

Colchicine ($C_{22}H_{25}N$) is a chemical derived from a species of saffron named *Colchicum autumnale*. Colchicine acts at the end of the mitotic prophase, either by inhibiting the development of the mitotic spindle or by leading it to an abortive spindle by precipitation of the proteins that make up its fibres (Jackson 1975). Colchicine is the most important chemical agent for chromosome doubling which is used very frequently for androgenetic studies in cereal crops. It disrupts mitosis by binding to tubulin, the protein sub unit of microtubles, thus inhibiting the formation of microtubules and the polar migration of chromosomes, which results in a cell with a doubled chromosome number (Wan et al. 1991). Several researchers used colchicine in the induction medium with different plant species such as wheat (Barnabás et al. 1991, Navarro-Alvarez et al. 1994, Islam 2010b), maize (Michard and Beckert 1997, Saisingtong 1998, Ren et al. 2018), rice (Alemanno and Guiderdoni 1994) and *Brassica napus* (Chen et al. 1994). Hassai and Liang (1991) mentioned that chromosome doubling of plants produced from microspores, using colchicine by *in vivo* and *in vitro*. Redha et al. (1998) used colchicine in the induction medium for 3 days and observed embryo production with higher percentage of doubled haploid (DHs) plants. Islam (2010b) found that colchicine application on anther culture

medium showed significant increase in embryo formation and green plant regeneration in wheat. The direct treatment of colchicine to isolated microspore culture decreased 3-4 fold embryoids induction but increasing fertile/DHs plants (Islam 2010b). Several microtubule depolymerising herbicides also showed to be efficient for *in vitro* chromosome doubling of microspores. It has been reported that colchicine added directly to the microspore cultures improved embryogenesis and increased diploidization rate up to 80-85% (Weber et al. 2005, Islam 2010b). Other antimitotic agents such as amiprophosmethyl (AMP), oryzalin, and pronamided have also showed potential for *in vitro* chromosome doubling in other plant species.

5.3.4.1.1.2 Oryzalin

Oryzalin is an antimitotic agent and dinitroaniline herbicide, low toxicity with a high affinity to plant tubulin. It is a well known herbicide and an agent for polyploidization in many crop species (Chauvin et al. 2003). Several authors have found that the orizaline can be less phytotoxic than the colchicine and also more efficient (Wang et al. 1991). Cox (2001) mentioned that oryzalin microtubule disrupting herbicide derived from the toluidine chemical family from Dow AgroScience and is sold under many trade names including Dirimal, Ryzelan, and Surflan. It is a surface-applied herbicide that inhibits the growth of germinating weed seeds by blocking cell division in the meristems (Extension Toxicology Network 1996). The mode of action for oryzalin is that it disrupts mitosis by inhibiting the formation of microtubules. Since the discovery of its potential for polyploid formation, oryzalin has been used to induce polyploids in many crop and ornamental species (Chalak and Legave 1997). It increases the number of tetraploids and increases the number of chimera (Tosca et al. 1995). Oryzalin binds strongly to plant tubulins to form tubulin-oryzalin complex. Oryzalin treatment was found to have a lower survival rate compared with the other two methods, although the doubling rate was better than colchicines (Ren et al. 2018). In some cases these compounds showed much higher affinity to plant microtubules than colchicines reported by Voughan and Voughn (1987), Morejohon and Fosket (1984).

5.3.4.1.1.3 Pronamide

Pronamide is an herbicide that could be a candidate which disrupts the spindle microtubule formation in a different manner to colchicine and trifluralin (Falconer and Seagull 1987, Vaughan and Vaughn 1987, Wan et al. 1991). In a previous study, it was suggested that pronamide either destabilizes the microtubules or limits the supply of proteins needed for microtubule development (Vaughan and Vaughn 1987). Under this study as antimitotic agent e.g. colchicines, azetidine and pronamide was applied to improve chromosome doubling efficiency and as some other chemicals namely sucrose, PEG, mannitol for anther culture in rice.

5.3.4.1.2 Materials and Methods

5.3.4.1.2.1 Plant materials and media

As variety BRRI dhan29 and medium SK3 was considered to conduct this experiment.

5.3.4.1.2.2 Methods

Under this study three antimitotic agents *viz*. azetidine, colchicine and pronamide are used in addition to the induction medium. Three different concentrations (100, 150 and 250 mg/l) were evaluated and incubated at 26ºC for three days. For control inoculated anthers were incubated without any antimitotic agent free medium. After the pre-treatment duration anthers were rinsed 2-3 times with the same fresh (antimitotic agents free) medium.

5.3.4.1.2.3 Data recording and statistical analysis

Data were recorded on the basis of embryogenesis, regeneration and diploidization of microspores particularly on the following traits, i) Embryo like structures = ELS/100 anthers, ii) green regenerated plants (GPR) per 100 embryos (GPR/100 ELS), iii) albino regenerated plants (ARP) per 100 embryos (ARP/100 ELS), iv) doubling index (DI) = doubled haploids plants / total green regenerated plants × 100, and mean for ELS and GRP. For each case 3 replications were taken and in a column the mean values followed by same letter (s) are not significantly different at $p<0.05$ according to DMRT. Ploidy status of regenerates plants were analyzed by flow cytometry (staining of nuclei isolated from green young leaves with DAPI solution).

5.3.4.1.3 Results and Discusssion

5.3.4.1.3.1 Results

Under this study for chromosome doubling three antimitotic agents (colchicine, oryzalin and pronamide) were considered to evaluate their doubling efficiency in rice anther culture. For each antimitotic agent three doses (100, 150 and 250 mg/l) were individually applied in addition to the induction medium and incubated them in liquid medium for 3 days (**Table 36**). It was observed that out of three dosages 150 mg/l azetidine showed higher embryo yield (30.51%) than others. For colchicine 150 mg/l also showed higher embryoids (35.24%) than Control and other dosages. In case of pronamide 100 mg/l showed better responses on embryo induction (30.27%). For overall embryo yield colchicine showed better and for regeneration it also shows highest regeneration (16.54%) than azetidine and pronamide (**Table 36**). For albino plant production higher percentages of plants (5.95%) were recorded in case of colchicine than azetidine (5.05%) and pronamide (4.81%). Under this study it was observed that all three antimitotic agents showed more or less doubling efficiency. But colchicine was the most effective chemicals for chromosome doubling (6.99%) than azetidine (5.72%) and pronamide (4.34%) for overall dosages (**Table 36**). Considering three dosages 150 mg/l colchicine showed most suitable dosages for doubling efficiency (7.95%). Less number of doubling efficiency was recorded in pronamide (150 mg/l) that was nearly half in comparison with colchicine. In overall mean performance 150 mg/l colchicine showed significantly higher performance (25.89%) on ELS and GRP. Analysis of variance showed that the antimitotic agents were differient significantly for ELS, ALP and DI; whereas, non-significant differences were found for GRP at LS of 0.215 (**Table 36 & 37**).

Table 36: Effect of various antimitotic agents on anther culture responses for doubling efficiency in rice

Antimitotic agents	Dosages (mg/l)	ELS (%)	GRP (%)	ALP (%)	Doubling Index (DI)	Mean for ELS & GRP
Azetidine	100	30.08±1.00abc	14.00±0.36b	3.97±0.19d	5.26±0.86cd	22.04±0.68
	150	30.51±1.22abc	15.87±0.25ab	4.78±0.28cd	6.30±0.54bc	23.19±0.74
	250	28.55±1.29bc	15.27±0.62ab	6.39±0.60ab	5.60±0.34bcd	21.91±0.96
	Mean	**29.71±0.66**	**15.05±0.35**	**5.05±0.41**	**5.72±0.35**	**-**
Colchicine	100	34.00±3.61ab	14.79±1.02ab	4.05±0.12d	6.04±0.51bcd	24.40±2.32
	150	35.24±2.33a	16.54±0.64a	6.39±0.71ab	**7.95**±0.23a	**25.89**±1.49
	250	27.55±0.39c	15.07±0.97ab	7.41±0.30a	**6.99**±0.23ab	21.31±0.68
	Mean	**32.26±1.72**	**15.47±0.52**	**5.95±0.55**	**6.99±0.33**	**-**
Pronamide	100	30.27±0.51abc	16.37±0.82ab	3.71±0.21d	3.25±0.26e	23.32±0.67
	150	29.48±0.33bc	16.22±0.77ab	4.93±0.30cd	4.70±0.48d	22.85±0.55
	250	21.84±1.18d	14.45±0.38ab	5.80±0.34bc	5.07±0.10cd	18.15±0.78
	Mean	**27.20±1.40**	**15.68±0.46**	**4.81±0.33**	**4.34±0.32**	**-**
F-value (Treat.)		5.167	1.556	11.559	9.629	-
Significance		0.003	0.215	0.000	0.000	-
F-value (Rep.)		0.571	0.361	1.296	1.498	-
Significance		0.576	0.702	0.301	0.253	-

Inoculated anther were pre-treated by antimitotic agents for 3 days in liquid induction medium (AMC) and then transferred to semi-solid plant medium (PM).

Table 37: ANOVA for effect of various antimitotic agents on anther culture responses for doubling efficiency in rice (Data source Table 36)

Source of Variation	Dependent Variable	Sum of Squares	df	Mean Square	F-value	Sig.
Treatment	ELS	354.535	8	44.317	5.167	0.003
	GRP	19.528	8	2.441	1.556	0.215
	ALP	39.898	8	4.987	11.559	0.000
	Double Index	44.394	8	5.549	9.629	0.000
Replication	ELS	9.794	2	4.897	0.571	0.576
	GRP	1.134	2	0.567	0.361	0.702
	ALP	1.118	2	0.559	1.296	0.301
	Double Index	1.727	2	0.863	1.498	0.253
Error	ELS	137.240	16	8.577	-	-
	GRP	25.093	16	1.568	-	-
	ALP	6.903	16	0.431	-	-
	Double Index	9.220	16	0.576	-	-
Total	ELS	24357.219	27	-	-	-
	GRP	6448.460	27	-	-	-
	ALP	797.787	27	-	-	-
	Double Index	927.562	27	-	-	-
Corrected Total	ELS	501.568	26	-	-	-
	GRP	45.756	26	-	-	-
	ALP	47.919	26	-	-	-
	Double Index	55.341	26	-	-	-

5.3.4.1.3.2 Discussion

For better androgenic responses gametophytic to sporophytic development to switch microspores, a signal is required. Physical and chemicals stress pre-treatment factors showed a very positive effect on androgenic responses in cereal crops are reported by Schmid and Keller (1986), Zheng et al. (2001) and Shariatpanahi et al. (2006b). For symmetrical division of microspores and increasing the doubling efficiency colchicine in addition to medium enhanced DHs production in wheat (Barnabás 1991), maize (Michard and Beckert 1997) and rice (Alemanno and Guiderdoni 1994). Different chemical substances such as colchicine, CGA (gametocide), AEC, azetidine, supermidine, ethrel treatment showed a very positive effect on callus induction and DHs production (Schmid and Keller 1986, Bajaj and Rajam 1996). Under this study three doses (100, 150 and 250 mg/l) of each antimitotic agents were applied in addition to the induction medium and incubated them in liquid medium for 3 days. For chromosome doubling colchicine, pronamide, oryzalin and amiprophosmethyl (AMP) were used in different crops plants as chemical stress pre-treatments factor (Soriano et al. 2007). Chemical treatments either single or in combined may influenced androgenic responses and increasing the doubling efficiency in wheat (Islam 2000). Out of the mentioned chemicals colchicine is the most important agent for chromosome doubling which is used very frequently in several crop plants and its improvement. It was observed three dosages of this study 150 mg/l azetidine showed highest embryo yield (30.51%) than others. For colchicine 150 mg/l also showed higher embryoids (35.24%) than Control and other dosages. Redha (1998) used colchicine in the induction medium for 3 days and observed embryo production with higher percentage of doubled haploid (DH) plants. Direct application of colchicine to the induction medium of anther culture using different concentrations and durations caused an increase in the frequency of fertile plants up to 76% in cereals crops (Redha et al. 1998, Obert and Barnabás 2004). Under this study the overall mean performance with 150 mg/l colchicine also showed significantly higher embryo yield and regeneration (25.89%) and fertile plants was 7.95%. The fertile plant was very less in comparison with the study Obert and Barnabás (2004). May be the

differences happen due to cause of genotypes, culture media, culture condition and various PGRs. Hansen and Andersen (1998) applied colchicine treatment to microspore culture, which caused an increase in the frequency of fertile plants up to 53%. Hassawi and Liang (1991) used three antimitotic agent *viz.* colchicines, trifluralin and oryzalin for different durations (48 and 72 h) in wheat. They found colchicine was the most effective compound for chromosome doubling. Barnabás et al. (1991) mentioned that the frequency of symmetrical divisions of microspores was significantly increased after *in vitro* colchicine treatment. The best concentration of colchicine in the medium was about 250 mg/l, and the optimum duration for the calli in the colchicine-containing medium was 1 day (Ouyang 1986). Zhuang and Jia (1980) increased the diploid plants by soaking the haploid callus for 72 h in colchicine solution (0.01 - 0.04%) before transferring them to regeneration medium. Efficient recovery of doubled haploid plants can be achieved by treating haploid maize callus culture with colchicine or antimicrotubule herbicides (Wan et al. 1991). Saisingtong et al. (1996) examined the effect of colchicine in maize anther culture and obtained 50% of doubled haploids (DH) when the treatment of optimal colchicine concentration was 250 mg/l for 7 days; on that case they found only 8-20% of spontaneous chromosome doubling in control.

Soriano et al. (2007) applied different concentration (0, 150 and 300 mg/l) of colchicine and found chromosome doubling efficiency increase significantly with 300 mg/l concentration. Using colchicine (250 mg/l) for 72 hrs + cold pre-treatment at 4ºC durations was very effective for the production of doubled haploids (Motallebi-Azar and Panahadeh 2010). Islam (2010c) reported that when 150 mg/l colchicine was applied directly to the isolated microspore culture medium for three days the number of embryos decreased significantly, while the chromosome doubling efficiency was increased. Alemanno and Guiderdoni (1994) reported that the optimal colchicine treatment showed 65% of DH plants in rice anther culture, but control showed only 31% doubled haploid plants. Hu and Liang (1979) reported that in rice anther culture, DH plants induced when anthers were treated by colchicine. Zhuang and Jia (1980) observed that the increased diploid plant by soaking the haploid callus for 72 h in colchicine solution (0.01 - 0.04%)

before transferring them to regeneration medium. Alemanno and Guiderdoni (1994) reported that the effect of colchicine (250 - 500 mg/l for 1-2 days) to rice anthers improved embryo induction and increased the doubling frequency. In optimal condition they obtained 31% - 65.5% doubled haploid (DH) plants. Hu and Liang (1979) observed that 50-250 mg/l colchicine treatment of rice anthers for 4 days enhanced embryo induction and green plant regeneration.

Under this study using colchicine also showed the most effective chemicals for chromosome doubling (6.99%) than azetidine (5.72%) and pronamide (4.34%) for overall dosages. Considering three dosages 150 mg/l colchicine showed most suitable dosage for doubling efficiency (7.95%). Hu and Liang (1979) also found the most effective antimitotic agents as colchicine. It is agreed well with the previous findings. In a report Navarro-Alvarez et al. (1994) mentioned that with lower concentration of colchicine is preferable than higher concentration because it had less effect on the critical step of embryoids formation. In the present investigation, it was observed that higher concentration (250 mg/l) of colchicine showed less percentage of doubling efficiency (15.07%) increasing albino plants (7.41%) than Control and other pre-treatments. The present results showed quiet similar effect with the findings of Navarro-Alvarez et al. (1994). Here the variety, medium and culture conditions were different than the previous study. The present evidence is also agreed well with the findings of Barnabás et al. (1991) and Hansen and Andersen (1998). Out of three antimitotic agents less number of doubling efficiency was recorded in pronamide (150 mg/l) that was nearly half in comparison with colchicine. As per the report of Vaughan and Vaughn (1987) mentioned that pronamide is less toxic than azetidine. Here we also found higher embryo yield in azetidine than pronamide and that may happen for the effect of high toxicity. Wang et al. (1991) reported that oryzaline can be less phytotoxic but less effective than colchicine. The results on embryo yield for azetidine (250 mg/l) but for chromosome doubling efficiency colchicines showed best results to developed fertile plants (DHs) that are very important for crop improvement.

5.3.5 Effect of osmoticum agents on anther and isolated microspore culture responses in rice

5.3.5.1 Introduction

Mannitol is the most suitable osmoticum for subsequent embryo and plant development especially in cereal crops. Chemically, mannitol is an alcohol and a sugar, or a polyol; it is similar to xylitol or sorbitol. However, mannitol has a tendency to lose a hydrogen ion in aqueous solutions, which causes the solution to become acidic. For this reason, it is not uncommon to add a substance to adjust its pH, such as sodium bicarbonate (https://www.drugbank.ca/drugs). Application of different pre-treatments may increase induction and regeneration efficiency in crop plants. Osmotic pre-treatment and starvation increased the number of viable isolated microspores and these stress pre-treatments factors changed the gametophytic pathway to sporophytic in wheat (Shirdelmoghanloo et al. 2009). They also found that after cold pre-treatment, the microspores of anthers were synchronized in 0.3 M mannitol solution for 3 days at 32ºC. Wei et al. (1986) who first time reported ab initio microspore culture of barley, followed a protocol involving isolation of microspores in 0.3 M mannitol and treating them in this solution for 3 days. According to them, mannitol pre-treatment for 3 days was essential and for 7 days optimum to induce androgenesis in this system.

Wojnarowiez et al. (2004) tested sugars for anther culture in barley to elucidate their influence on both the overall embryo yield and the structure of plastids in relation to albinism. They obtained best results using mannitol (364 mOsm/kg) and found 139.7 percentages of green plants per 100 plated anthers. Pre-treatment stresses with mannitol showed significant progress for microspore development in durum wheat (Slama-Ayed et al. 2010), rice (Raina and Irfan 1998) and maize (Pescitelli et al. 1990). Osmotic shock also affect for microspore development in barley (Shim et al. 2009). In barley, a mannitol pretreatment improved response (Ziauddin et al. 1992), whereas the inclusion of culture media macronutrients in the mannitol pretreatment were needed to obtain the microspore

division and sprophytic development in wheat (Hu et al. 1995), and rice (Ogawa et al. 1994). Cold pretreatment with or without mannitol or chemical + heat showed very positive effects on embryo production and green plant regeneration in isolated microspore of wheat (Shirdelmoghanloo et al. 2009). They also found that in combination of 21 days cold (4°C) pre-treated spikes as explants with mannitol (0.3 M) produced the highest number of embryos per spike while the combination of cold with chemical + heat produced the lowest number. In the case of total and green plant regeneration, seven days cold pre-treatment + mannitol (0.3 M) showed more superior to other pretreatment factors in Iranian spring bread wheat (Shirdelmoghanloo et al. 2009). Pre-treatment consisted of incubation of anthers containing mid-late to late uninucleate microspores in 0.37 M mannitol solution for 4 days in the dark, at 25°C (Hoekstra et al. 1992). After pre-treatment, microspores were isolated by gentle blending in 0.37 M mannitol solution for 30 seconds using a commercial blender at medium power (Waring), filtered through 110 µm nylon meshes and collected by centrifugation at 800 rpm for 5 min. Roberts-Oehlschlager and Dunwell (1990) had reported that 4 day incubation of barley anthers in a medium containing 3.2% mannitol raised the pollen callusing response from 23% to 78% which was more than cold treatment alone or in combination with mannitol. According to these authors, mannitol stress improves sugar uptake causing build-up of glucose pool in the anther tissue. Similar results were reported by Ziauddin et al. (1992). Hoekstra et al. (1992) and Cistué et al. (1995) confirmed the importance of mannitol pre-treatment in barley anther culture responses.

In the latter case, application of 0.7 M mannitol for 3-5 days improved green plant regeneration significantly. Increase in mannitol concentration showed linear increase in the number of dividing microspores and an increase in the proportion of green plants regenerated. The optimum duration of treatment varied with the genotype. Hoekstra et al. (1993), who gave mannitol stress (440 mOs kg^{-1}) for 4 days and cultured the microspores on a medium of the osmolarity of 350 mOs kg^{-1} at a density of 2×10^4 microspores per

ml, obtained 320 embryo-like structures per × 10^4 microspores. The yield of green plants increased from 50% to 97%. Raina and Irfan (1998) reported that treatment of anthers in 0.4 M mannitol solution was essential to induce androgenesis in microspore cultures of indica and japonica rice cultivars. For indica cv., pre-treatment at 33°C was better than at 25°C, with respect to the number of embryo-like structures or calli formation. In the absence of cold treatment, mannitol treatment promoted androgenesis in anther cultures of cv. IR43 from 3% to 33.4 %; with cold treatment it had no promotory effect (Pande 1997). Under this study mannitol pre-treatment effect was found for both anther and isolated microspore culture along with cold and heat stress pre-treatment to improve embryo yield and regeneration efficiency in rice.

5.3.5.2 Application of sorbitol, PEG and mannitol

5.3.5.2.1 Materials and Methods

As variety BRRI dhan29 and medium SK-3 was considered to conduct this experiment. Three days cold pre-treated (4°C) spikes from BRRI dhan 29 were used for this study. Excised anthers were pre-treated at 26°C chamber for three days for sucrose starvation by **Sorbitol** (40 g/l, 60 g/l and 80 g/l), **PEG** (40 g/l, 60 g/l and 80 g/l) and **Mannitol** (40 g/l, 60 g/l and 80 g/l) and then transferred them to semi-solid induction medium (SK3). Other procedures are described in **Chapter III, Section 3.3.1.2.5.**

5.3.5.2.2 Results

Application of various chemical pre-treatment to harvested spikes, excised anthers or isolated microspores may influence the induction, regeneration and chromosome doubling efficiency in rice. Under this study as osmoticum agents three chemicals e.g. sorbitol, PEG and mannitol were added in the induction medium and for each case three dosages (40, 60 and 80 g/l) were applied and data were shown in **Table 38**. Here as induction medium SK3 was considered. Harvested spikes of BRRI dhan29 were pre-treated by cold and excised anthers were removed and cultured individually following

the dosages of chemicals. It was observed that out of three osmoticum agents mannitol showed better results (41.07%) on embryoids induction where 60 g/l was used. Here less number of embryoids (15.99%) was recorded in PEG (80 g/l). For all concentration mannitol also showed better performance on embryo yield (33.20%) than sorbitol (29.46%) and PEG (18.59%). For green plant regeneration better results (16.81%) also obtained with mannitol. In case of albino plant production less number of plants (4.21%) was recorded in mannitol (**Table 38**). For overall mean value on the basis of ELS and GRP (60 g/l) mannitol showed best performance (31.56%) than others. Analysis of variance (ANOVA) in comparison of three osmoticum agents (sorbitol, PEG and mannitol) showed significantly higher responses on ELS and GRP; and reducing albino plants with different dosages of chemicals are shown in **Table 38 & 39**.

Table 38: Sorbitol, PEG and mannitol's effects on anther culture responses and their productivity in rice

Stress pre-treatment (sugar starvation)	Dosages (g/l)	Per 100 anthers			Mean for
		ELS	GRP	ALP	ELS &GRP
Sorbitol (3 days)	40	29.48±0.45c	14.23±0.29c	5.65±0.50abc	21.86±0.37
	60	34.90±1.23b	17.93±0.76b	6.02±0.27abc	**26.42±1.00**
	80	23.99±1.10de	9.62±0.31de	6.54±0.42a	16.81±0.71
	Mean	**29.46±1.65**	**13.93±1.23**	**6.07±0.24**	**-**
Mannitol (3 days)	40	33.22±0.40b	14.07±0.96c	4.88±0.29abcd	23.65±0.68
	60	**41.07**±1.27a	22.04±0.61a	3.48±0.38d	**31.56**±0.94
	80	25.67±1.60d	14.31±0.96c	4.28±0.94cd	19.99±1.28
	Mean	**33.32±2.30**	**16.81±1.38**	**4.21±0.36**	**-**
PEG (3 days)	40	18.23±1.05fg	7.69±0.33e	4.60±0.36bcd	12.96±0.69
	60	21.56±1.18ef	11.55±0.50d	6.23±0.28ab	16.56±0.84
	80	**15.99**±1.59g	8.05±1.48e	4.38±0.94bcd	12.02±1.54
	Mean	**18.59±1.04**	**9.10±0.77**	**5.07±0.42**	**-**
F-value (Treat.)		50.152	43.278	3.511	-
Significance		0.000	0.000	0.016	-
F-value (Rep.)		1.099	2.866	0.780	-
Significance		0.357	0.086	0.475	-

Table 39: ANOVA for comparison of sorbitol, PEG and mannitol effects on anther culture responses and their productivity in rice (Data source Table 38)

Source of Variation	Dependent Variable	Sum of Squares	df	Mean Square	F-value	Sig.
Treatment	ELS	1630.621	8	203.828	50.152	0.000
	GRP	527.817	8	65.977	43.278	0.000
	ALP	25.877	8	3.235	3.511	0.016
Replication	ELS	8.932	2	4.466	1.099	0.357
	GRP	8.737	2	4.369	2.866	0.086
	ALP	1.437	2	0.718	0.780	0.475
Error	ELS	65.027	16	4.064	-	-
	GRP	24.392	16	1.524	-	-
	ALP	14.740	16	0.921	-	-
Total	ELS	21567.811	27	-	-	-
	GRP	5319.436	27	-	-	-
	ALP	749.025	27	-	-	-
Corrected Total	ELS	1704.581	26	-	-	-
	GRP	560.946	26	-	-	-
	ALP	42.055	26	-	-	-

5.3.5.3 Transferred of inoculated anthers to semi-solid induction medium

5.3.5.3.1 Materials and Methods

For Control, 90 g/l sucrose was used as carbon sources instead of any other osmoticum agent. For all treatments (T) and control 3 days pre-treated spikes from BRRI dhan 29 were used for anther (explants) sources. Excised anthers were pre-treated for 3 hrs with mannitol solutions for T_1 = 0.2 mM, T_2 = 0.3 mM, T_3= 0.4 mM, T_4 = 0.5 mM, T_5 = 0.6 mM and T_6 = 0.7 mM. Then transferred them to semi-solid induction medium (SK3). Other procedures are described in **Section 3.3.1.2.5**.

5.3.5.3.2 Results

In this case as osmoticum agent only mannitol was considered with different dosages (0.2 mM – 0.7 mM) to find out the suitable dosages of mannitol and their responses to anther culture of rice. For Control 90 g/l sucrose was considered instead of mannitol. Out of six concentration of mannitol T_2 (0.3 mM) showed highest embryo yield (43.06%) and very close embryos (42.07%) was recorded in T_3 (0.4 mM). It was observed that increasing the mannitol concentration the embryo yield was gradually decreased in T_4 (0.5 mM, 29.04%) and T_5 (0.7 mM, 16.86%). For green plant regeneration highest percentages of plants (24.74%) were recorded in T_2 that was nearly one fold higher (Control, 14.91%) and three times higher (8.06%) in comparison with T_6 (**Table 40**). So it was very clear effect of mannitol with different concentration on embryo yield as well as regeneration in rice anther culture. In this case lower percentages of albino plants (3.67%) were recorded in T_2 compared with control (4.07%) and other treatments (4.85% - 6.24%) of this study (**Table 40**). In case of overall mean value T_2 (33.90%) and T_3 (32.24%) showed better performances of ELS and GRP. By analysing the variances it was found that the effect of various dosages of manittol pretreatment were different significantly at $p < 0.01$ to yield embryo and regeneration in rice anther culture (**Table 40 & 41**).

Table 40: Anther induction and regeneration efficiency under different dosages of mannitol in rice

Mannitol stress (hrs)	Per 100 anthers			Mean for ELS & GRP
	ELS	GRP	ALP	
Cont.	29.71±1.00c	14.91±0.49d	4.07±0.32b	22.31±0.71
T_1 (0.2 mM)	35.90±1.79b	19.20±0.60c	3.67±0.23b	27.55±1.10
T_2 (0.3 mM)	**43.06±0.71a**	**24.74±0.55a**	4.85±0.68ab	**33.90±0.57**
T_3 (0.4 mM)	42.07±0.93a	22.40±0.40b	5.75±0.49a	32.24±0.50
T_4 (0.5 mM)	29.04±1.14c	20.63±0.36c	6.27±0.60a	24.84±0.65
T_5 (0.6 mM)	24.63±1.85d	14.73±0.74d	5.77±0.40a	19.68±1.26
T_6 (0.7 mM)	16.86±1.24e	8.06±0.51e	6.24±0.58a	12.46±0.74
Mean (SD)	31.6090 (9.15906)	17.8090 (5.42145)	5.2310 (1.22457)	-
F-value (Treat.)	46.337	119.188	4.246	-
Significance	0.000	0.000	0.016	-
F-value (Rep.)	0.123	1.458	0.591	-
Significance	0.885	0.271	0.569	-
Treat. $LSD_{0.05}$	2.800	1.045	1.026	-

Cont. = Without mannitol but with sucrose, 90 g/l. T_1 = 0.2 mM, T_2 = 0.3 mM, T_3= 0.4 mM, T_4 = 0.5 mM, T_5 = 0.6 mM and T_6 = 0.7 mM.

Table 41: Anther induction and regeneration efficiency under different dosages of starvation by mannitol pre-treatment (Data source Table 40)

Source of Variation	Dependent Variable	Sum of Squares	df	Mean Square	F-value	Sig.
Treatment	ELS	1606.985	6	267.831	46.337	0.000
	GRP	575.830	6	95.972	119.188	0.000
	ALP	19.765	6	3.294	4.246	0.016
Replication	ELS	1.421	2	0.711	0.123	0.885
	GRP	2.348	2	1.174	1.458	0.271
	ALP	0.917	2	0.458	0.591	0.569
Error	ELS	69.361	12	5.780	-	-
	GRP	9.663	12	0.805	-	-
	ALP	9.309	12	0.776	-	-
Total	ELS	22659.538	21	-	-	-
	GRP	7248.247	21	-	-	-
	ALP	604.612	21	-	-	-
Corrected Total	ELS	1677.768	20	-	-	-
	GRP	587.841	20	-	-	-
	ALP	29.991	20	-	-	-

5.3.5.4 Incubation of anthers in liquid induction medium

5.3.5.4.1 Material and Methods

Under this study as osmoticum agents 0.3 mM mannitol solutions was used as chemical pre-treatment agents to excised anthers for T_1 = 1 d, T_2 = 3 d and T_3 = 5 days. In this case pre-treated anthers at different days of 0.3 mM mannitol were incubated in liquid induction medium (SK3) for embryoids induction. And then embryoids were transferred to semi-solid regeneration medium. Other procedures are described previously in **Section 3.3.1.2.5**. In another study as osmoticum agents 0.3 mM mannitol solutions was used to excised anthers (T_1 = 1 d, T_2 = 3 d and T_3 = 5 d). Here, pre-treated anthers were incubated in semi-solid induction medium (SK3) for embryos production.

5.3.5.4.2 Results

An interesting experiment was conducted using liquid and semi-solid induction medium to evaluate their effect on embryo yield and regeneration efficiency and reducing albinism in case of rice anther culture. Here SK3 liquid induction medium was considered (**Table 42**). It was observed that increasing the incubation period to excised anthers in liquid induction medium for 3 days (T_2) showed significantly higher embryo yield (42.8%) induction than Control (26.71%) and T_3 (21.33%). The results were nearly doubled in comparison with T_3. For green plant regeneration T_3 showed best performance (21.09%) than Control (9.28%). The results indicate nearly two and half fold higher regenerating in T_2 (**Table 42**). Increasing the anther incubation period of cultured anther in induction medium albino plants was increased (4.66%) in comparison with Control (2.92%). For overall mean value T_2 also showed better performance on ELS and GRP than others (**Table 42**). Analysis of variance on the effect of incubation period in liquid medium was done and evaluated their responses on the basis of embryo yield and regeneration of mannitol shown in **Table 42 & 43**. The results were different significantly for different days tested, at $p < 0.01$ for ELS and GRP, and $p < 0.05$ for ALP in anther culture of rice.

Table 42: Effect of stress pre-treatment factors of mannitol using liquid induction medium for different days in anther culture (AC) of rice

Treatment	Per 100 anthers			Mean for ELS & GRP
	ELS	GRP	ALP	
Cont.	26.71±1.36c	9.82±0.88c	2.92±0.22b	18.26±1.03
T_1 (1 d)	32.38±1.01b	14.69±0.30b	3.88±0.33ab	23.53±0.66
T_2 (3 d)	42.86±1.74a	**21.09±1.07a**	3.67±0.29ab	**31.97±1.38**
T_3 (5 d)	21.33±1.23d	8.23±0.29c	4.66±0.50a	14.78±0.76
Mean (SD)	30.8200 (8.56528)	13.4558 (5.33683)	3.7792 (0.82748)	-
F-value (Treat.)	67.849	64.793	5.792	-
Significance	0.000	0.000	0.033	-
F-value (Rep.)	2.924	1.043	2.512	-
Significance	0.130	0.409	0.161	-
Treat. $LSD_{0.05}$	3.349	2.152	0.891	-

*Around 600 anthers were inoculated for AC, T_1 = 0.3 mM mannitol (1 day), T_2 = 0.3 mM mannitol (3 days), T_3 = 0.3 mM mannitol (5 days), Cont.: without mannitol and no pre-treatment durations, ELS = Embryo like structures, GRP = Green plants and ALP = Albino plants.

Table 43: ANOVA for the effect of stress pre-treatment factors of mannitol using liquid induction medium for different days (Data source Table 42)

Source of Variation	Dependent Variable	Sum of Squares	df	Mean Square	F-value	Sig.
Treatment	ELS	762.612	3	254.204	67.849	0.000
	GRP	300.788	3	100.263	64.793	0.000
	ALP	4.608	3	1.536	5.792	0.033
Replication	ELS	21.913	2	10.956	2.924	0.130
	GRP	3.227	2	1.614	1.043	0.409
	ALP	1.332	2	0.666	2.512	0.161
Error	ELS	22.480	6	3.747	-	-
	GRP	9.285	6	1.547	-	-
	ALP	1.591	6	0.265	-	-
Total	ELS	12205.473	12	-	-	-
	GRP	2486.013	12	-	-	-
	ALP	178.917	12	-	-	-
Corrected Total	ELS	807.004	11	-	-	-
	GRP	313.300	11	-	-	-
	ALP	7.532	11	-	-	-

5.3.5.5 Incubation of anthers in semi-solid induction medium

5.3.5.5.1 Material and Methods

Under this study as osmoticum agents 0.3 mM mannitol solutions was used as chemical pre-treatment agents to excised anthers for T_1 = 1 d, T_2 = 3 d and T_3 = 5 days. In this case pre-treated anthers at different days of 0.3 mM mannitol were incubated in semi-solid induction medium (SK3) for first 7 days at 14°C chamber and then incubated them at 25±1°C for embryoids induction. And then embryoids were transferred to semi-solid regeneration medium. Other procedures are described previously in **Section 3.3.1.2.5.**

5.3.5.5.1 Results

In this case 0.3 mM mannitol was added in the semi-solid induction medium and the anthers pre-treatment duration was 1-5 days. After discarding the liquid medium anthers were transferred to semi-solid induction medium (SK3) for first 7 days at 14°C chamber and then incubated them at 25±1°C for embryoids induction. The highest embryo yield (36.82%) was recorded in T_2 (3 days). Better regeneration was recorded in T_2 (23.83%) than Cont. (14.36%) and other treatments (T) of this study (**Table 44**). In comparison with both liquid and semi-solid induction medium along with mannitol pre-treatment to inoculated anthers semi-solid medium showed better performance on regeneration (23.83%). **Table 44** showed the best mean value on ELS and GRP for T_2 which was nearly two and half fold higher than others. Analysis of variance showed highly significant differences in the frequency of embryo yield and regeneration for the stress pre-treatment factors of mannitol using semi-solid induction medium for different days at $p < 0.01$ (**Table 44 & 45**).

Table 44: Effect of stress pre-treatment factors of mannitol using semi-solid induction media for different days in anther culture of rice

Treatment	Per 100 anthers			Mean for ELS & GRP
	ELS	GRP	ALP	
Cont.	19.30±1.08c	9.42±0.39c	4.22±0.30a	14.36±0.66
T_1 (1 d)	28.11±0.68b	14.67±1.00b	3.11±0.06b	21.39±0.76
T_2 (3 d)	36.82±1.08a	**23.83±0.28a**	4.64±0.26a	30.33±0.41
T_3 (5 d)	17.55±0.51c	8.04±0.09c	4.31±0.21a	12.80±0.23
Mean (SD)	25.4467 (8.13505)	13.9900 (6.52382)	4.0717 (0.69065)	-
F-value (Treat.)	82.218	177.620	7.311	-
Significance	0.000	0.000	0.020	-
F-value (Rep.)	0.184	1.284	0.439	-
Significance	0.837	0.343	0.664	-
Treat. $LSD_{0.05}$	2.935	1.609	0.738	-

*Around 600 anthers were inoculated for AC, T_1 = 0.3 mM mannitol (1 day), T_2 = 0.3 mM mannitol (3 days), T_3 = 0.3 mM mannitol (5 days), Cont.: without mannitol and pre-treatment durations, ELS = Embryo like structures, GRP = Green plants and ALP = Albino plants.

Table 45: ANOVA for effect of stress pre-treatment factors of mannitol using semi-solid induction media for different days in anther culture (Data source Table 44)

Source of Variation	Dependent Variable	Sum of Squares	df	Mean Square	F-value	Sig.
Treatment	ELS	709.650	3	236.550	82.218	0.000
	GRP	460.754	3	153.585	177.620	0.000
	ALP	3.994	3	1.331	7.311	0.020
Replication	ELS	1.056	2	0.528	0.184	0.837
	GRP	2.220	2	1.110	1.284	0.343
	ALP	0.160	2	0.080	0.439	0.664
Error	ELS	17.263	6	2.877	-	-
	GRP	5.188	6	0.865	-	-
	ALP	1.093	6	0.182	-	-
Total	ELS	8498.363	12	-	-	-
	GRP	2816.803	12	-	-	-
	ALP	204.189	12	-	-	-
Corrected Total	ELS	727.969	11	-	-	-
	GRP	468.162	11	-	-	-
	ALP	5.247	11	-	-	-

5.3.5.6 Incubation of anthers in semi-solid induction medium

5.3.5.6.1 Materials and Methods

To evaluate the effect of mannitol (chemical) as osmoticum agent in combination with heat (physical) pre-treatments to anther culture responses in rice another experiment was done under this study. In this case for all treatments (T) spikes were pre-treated by cold at 4°C for 3 days. BRRI dhan 29 was used as explants sources of this study (**Table 46 and 47**). For Control, inoculated anthers were cultured in semi-solid induction medium (SK3) and incubated them at 26°C for embryos induction. For all treatment (T), T_1 = 3 h, T_2 = 6 h and T_3 = 12 hrs excised anthers were pre-treated by heat at 30°C with different duration in incubator where temperature was fixed at 30°C. Similarly for 33°C sealed petri dishes were incubated them for 3, 6 and 12 hrs. For all after the pre-treatment duration cultures were incubated at 14°C for 7 days. Then for both temperature (30°C and 33°C), cultures were transferred at 26°C chamber and incubated them for 3-6 weeks for embryos induction.

For all of the mentioned experiments under osmoticum agents harvesting spikes, preparation, sterilization procedure, stock solution and media preparation, inoculation, incubation at 14°C for 14 days and then culture incubation at 26°C until embryos induction, embryoids transferred to regeneration medium and other procedures are described in **Section 3.3.1.2.5**. For all cases (cont. and treatments) 3 replications were undertaken and in a column the mean values followed by same letter (s) are not significantly different at $p < 0.05$ according to DMRT.

5.3.5.6.2 Results

Under this study cold pre-treated spike of BRRI dhan29 was used. As osmoticum agent (chemical substances) mannitol and as physical stress heat pre-treatment at 30°C and 33°C with different hours was applied in combination to excised anthers to evaluate their callus induction and regeneration efficiency in rice anther culture (**Table 46**). Here 0.3

mM mannitol at 30°C for 3 h (T_1), 6 h (T_2), 12 h (T_3) and similarly for 33°C for 3-12 hrs applied to excised anthers. For both temperatures 3 hrs showed increasing embryo yield (30°C = 39.34%, 33°C = 43.12%) and regeneration (30°C = 17.40%, 33°C = 22.86%) compared to other treatments and Control. Temperature pre-treatment at 33°C showed best results for embryo like structures (43.12%) and green plant regeneration (22.86%) that was 2-3 times higher than Control (**Table 46**). It was observed that increasing temperature pre-treatment with various durations (h) the albino plant was increased (up to 8.28%) that were nearly 2.5 times higher than Control (3.84%). For overall mean value T4 (33°C, 3 h) showed the best results on ELS and GRP (32.99%). Analysis of variance under different durations of heat pre-treatment with 0.3 mM mannitol showed significant difference on ELS, GRP and ALP production (**Table 46 & 47**).

Table 46: Anther induction and regeneration efficiency under different durations of heat stress pre-treatment with 0.3 mM mannitol in rice

Treatment	Per 100 anthers			Mean for ELS & GRP
	ELS	GRP	ALP	
Cont. (26°C, 0 h)	26.48±1.20d	9.88±0.17d	3.84±0.18d	18.18±0.68
T_1 (30°C, 3 h)	**39.34±0.67b**	**17.40±0.71b**	4.26±0.33d	28.37±0.68
T_2 (30°C, 6 h)	31.95±2.11c	15.24±0.57c	6.95±0.34b	23.59±0.95
T_3 (30°C, 12 h)	17.40±0.60e	6.87±0.38e	7.71±0.36ab	12.14±0.34
T_4 (33°C, 3 h)	**43.12±0.65a**	**22.86±0.44a**	3.73±0.42d	**32.99±0.51**
T_5 (33°C, 6 h)	36.81±0.96b	17.44±0.34b	6.00±0.07c	27.13±0.65
T_6 (33°C, 12 h)	20.21±0.93e	8.83±0.31d	8.28±0.28a	14.52±0.52
Mean (SD)	30.7581 (9.40944)	14.0738 (5.48530)	5.8233 (1.85436)	-
F-value (Treat.)	72.401	187.254	56.699	-
Significance	0.000	0.000	0.000	-
F-value (Rep.)	0.744	2.015	4.047	-
Significance	0.496	0.176	0.045	-
Treat. $LSD_{0.05}$	2.316	0.846	0.509	-

Table 47: ANOVA for anther induction and regeneration efficiency under different durations of heat stress pre-treatment factors with various durations of 0.3 mM mannitol (Data source Table 46)

Source of Variation	Dependent Variable	Sum of Squares	df	Mean Square	F-value	Sig.
Treatment	ELS	1717.430	6	286.238	72.401	0.000
	GRP	593.304	6	98.884	187.254	0.000
	ALP	64.937	6	10.823	56.699	0.000
Replication	ELS	5.879	2	2.940	0.744	0.496
	GRP	2.129	2	1.064	2.015	0.176
	ALP	1.545	2	0.772	4.047	0.045
Error	ELS	47.442	12	3.953	-	-
	GRP	6.337	12	0.528	-	-
	ALP	2.291	12	0.191	-	-
Total	ELS	21638.020	21	-	-	-
	GRP	4761.284	21	-	-	-
	ALP	780.908	21	-	-	-
Corrected Total	ELS	1770.751	20	-	-	-
	GRP	601.769	20	-	-	-
	ALP	68.773	20	-	-	-

5.3.5.7 Effect of mannitol pre-treatment factors in rice anther culture

5.3.5.7.1 Materials and Methods

Under this study mannitol starvation was done to excised anthers before microspore isolation. For this study 3 days cold pre-treated spikes (4°C) of BRRI dhan29 was considered. For each treatment four dosages (0.2 mM, 0.4 mM, 0.6 mM and 0.8 mM) of mannitol was supplemented with induction medium of SK3. For Control no mannitol was added in the medium. For each treatment (T) inoculated anthers were incubated at 25±°C for 2, 4 and 6 days. Then liquid medium was removed and added same medium that was prepared without mannitol and incubated them at 25±1°C chamber for embryos induction.

5.3.5.7.2 Data recording and statistical analysis

For microspore isolation procedure data were recorded on the basis embryogenesis (number of regenerated embryoids, embryoids per 10^5 microspores from 100 anthers) and for regeneration e.g. total regenerated plantlets (TRP), green (GRP) and albino plants (ARP) per 100 embryos. Three replications were considered and 5×10^5 microspores were used from 500 anthers for all cases. In case of control 500 anthers were inoculated in induction medium (AMC). For each mean value 3 replications were used and in a column the mean values followed by different letter (s) are significantly differed at $p < 0.05$ according to DMRT.

5.3.5.7.3 Results (pre-treatment of anthers)

Under this study 3 days cold pre-treated spikes was used. Here excised anthers were pre-treated with different doses (0.2 M, 0.4 M, 0.6 M and 0.8 M) of mannitol for 2, 4 and 6 days. For Control not added mannitol in the induction medium. It was observed that out of four concentration of mannitol 0.6 M (T_3) showed highest embryo yield for all of the

days considered under this study (**Table 48**). We found four days pre-treatment with 0.6 M mannitol showed nearly two fold higher embryo yield (140.33%) than Control (72.33%). Mannitol concentration (0.4 M) showed second heights results on ELS and GRP for all durations. For average mean value 0.6 M (T_3) showed significantly higher embryo yield (122.67%) and green plant regeneration (71.33%) than Control and treatments. Remarkable results obtained when 4 days pre-treatment with mannitol concentration was 0.6 M for all of the dosages considered under this study. Here all of the dosages showed more or less embryoids induction and regeneration (**Table 48**). It was observed that higher dosages of mannitol (0.8 M) showed less embryo yield and regeneration and gradually the number was decreased (T_1 - T_3). For all cases more or less albino plants were recorded that was 13.67% to 26.67%.

Table 48: Effect of pre-culture duration with mannitol pre-treatment using explants as anthers before microspore isolation

Treatments (mannitol)	Days (incubation period)	ELS (%)	GRP (%)	ALP (%)
Cont.	0	72.33±2.91gh	47.67±2.60e	16.67±2.60def
T_1 (0.2 mM)	2	76.67±2.19fgh	49.33±2.96e	14.33±2.33fg
	4	87.33±2.91cd	56.67±2.60d	17.00±2.89de
	6	70.67±2.96h	47.67±2.40e	24.67±2.40a
	Mean	**78.22±4.87**	**51.22±2.76**	**18.67±3.10**
T_2 (0.4 M)	2	97.33±1.45b	58.00±2.89d	15.00±2.08
	4	84.67±2.33de	61.67±2.91c	17.33±2.33de
	6	78.67±3.28ef	50.33±2.91e	26.67±2.60a
	Mean	**86.89±0.92**	**56.67±0.01**	**19.67±0.26**
T_3 (0.6 M)	2	135.67±4.33a	**74.00±3.46b**	13.67±2.03
	4	**140.33±4.81a**	**82.67±3.18a**	20.00±2.31bc
	6	92.00±2.89bc	**57.33±3.18d**	21.33±2.91b
	Mean	**122.67±15.39**	**71.33±7.43**	**18.33±2.36**
T_4 (0.8 M)	2	63.67±2.96i	36.33±2.33g	16.67±2.03def
	4	74.00±3.06fgh	39.67±2.03f	14.33±2.40fg
	6	77.33±3.18fg	29.00±2.65h	18.00±1.73cd
	Mean	**71.67±4.11**	**35.00±3.15**	**16.33±1.07**
F-value (Sig.)	Treatment	7.743 (0.017)	39.425 (0.000)	0.746 (0.563)
	Days	1.609 (0.276)	11.712 (0.008)	8.96 (0.020)

Cont. = Without mannitol pre-treatment, T = Treatment, ELS = Embryo like structure, GRP = Green plants, ALP = Albino plants, per Treatment = 500 anthers was considered, 1 anthers = 10000 microspores (approx.).

5.3.5.8 Pre-treatment of rice floret

5.3.5.8.1 Material and Methods

For another study deep the rice floret (along with anthers lemma and palea were closed) in liquid SK3 medium that supplemented with various concentration of mannitol (0.2 mM, 0.4 mM, 0.6 mM and 0.8 mM) before microspore isolation. The incubation period was 2, 4 and 6 days at 25±1°C. In this case after the due duration for each treatment rice floret were placed in a sterilized microblender chamber containing 20-30 ml liquid WM. The microblender chamber covered with autoclaved aluminum foil during blending and anthers were blended with low speed 30-45 seconds. Then the debris was removed through a 100 μm stainless steel mesh sieve and microspores were collected following the procedures are described in **Section 3.3.2.1**.

5.3.5.8.2 Results

In this case another experiment was done where various concentration of mannitol (0.2 M - 0.8 M) was directly used in addition to the induction medium (AMC). The effect of mannitol dosages was observed for 2, 4 and 6 days on the basis of embryo yield, green plant regeneration and albino plant production. It was observed that the incubation period 4 days of rice floret in the induction medium showed significantly higher embryo yield in T_3 (163.67%). Without mannitol pre-treatment embryo yield showed only 92.67% in Control (**Table 49**). For green plant regeneration remarkable improvement were recorded also in T_3 (0.6 M mannitol) for all of the incubation periods (2 d = 70%, 4 d = 78.33% and 6 d = 71.67%). The regeneration frequency was decreased when higher concentration of mannitol was added in the medium for T_4 (46% - 67.33%). It was observed that in addition various concentration of mannitol to induction medium (T_1 - T_4) influences to increase embryo yield (94.44% - 140.89%) and green plant regeneration (48.56% - 73.33%) than Control (ELS = 92.67%, GRP = 54%). For all treatment and the incubation period showed more or less albino plants (16.22% - 37.22%). However, in case of albino plant production less concentration of mannitol (T_1) showed reduced albinism (13.67% - 18.33%) than Control (19.33%). Analysis of variance (ANOVA) showed significantly higher embryo yield and regeneration by the effect of various concentration of mannitol and the different incubation periods 2-6 days (**Table 50**).

Table 49: Effect of pre-culture duration with mannitol pre-treatment using explants as rice floret before microspore isolation

Treatments (mannitol)	Days (incubation period)	ELS (%)	GRP (%)	ALP (%)
Cont.	0	92.67±2.85j	54.00±2.08c	19.33±1.76ef
T_1 (0.2 mM)	2	103.33±4.33gh	43.67±1.76f	16.67±2.03g
	4	117.67±2.73d	53.33±1.76cd	13.67±1.45h
	6	100.67±4.70hi	48.67±2.60cdef	18.33±1.76efg
	Mean	**107.22±5.28**	**48.56±2.79**	**16.22±1.37**
T_2 (0.4 M)	2	126.33±4.91c	51.67±3.48cde	17.33±1.76fg
	4	111.00±3.46ef	50.00±2.08cdef	50.00±2.08a
	6	105.67±3.48fgh	44.33±2.03ef	44.33±2.03b
	Mean	**114.33±0.83**	**48.67±0.82**	**37.22±0.17**
T_3 (0.6 M)	2	145.67±4.10b	70.00±2.31b	17.67±2.03fg
	4	163.67±4.48a	78.33±3.18a	20.33±2.03e
	6	113.33±4.06de	71.67±2.03ab	23.67±1.76d
	Mean	**140.89±14.73**	**73.33±2.55**	**20.56±1.74**
T_4 (0.8 M)	2	81.67±4.63k	46.00±2.65def	29.67±2.60c
	4	95.00±3.79ij	67.33±2.96b	19.33±2.33ef
	6	106.67±2.60fg	49.67±3.18cdef	24.00±2.65d
	Mean	**94.44±7.22**	**54.33±6.59**	**24.33±2.99**
F-value (Sig.)	Treatment	4.467 (0.057)	15.823 (0.003)	2.552 (0.152)
	Days	0.902 (0.454)	4.202 (0.072)	0.594 (0.582)

Cont. = without mannitol pre-treatment, T = Treatment, T_1 = 0.2 M mannitol, T_2 = 0.4 M mannitol, T_3 = 0.6 M mannitol and T_4 = 0.8 M mannitol. For each treatment 10 spikes were considered, ELS = Embryo like structure, GRP = Green plants and ALP = Albino plants.

Table 50: Variance analysis (ANOVAs) of ELS, GRP and ALP (Data source Table 48 & 49)

Data source	Source of Variation	Sum of Squares	df	Mean Square	F-value	Sig.
ELS (Table 48)	Treat.	4654.523	3	1551.508	7.743	0.017
	Days	644.619	2	322.309	1.609	0.276
	Error	1202.258	6	200.376	-	-
	Total	6501.399	11	-	-	-
GRP (Table 48)	Treat.	2026.429	3	675.476	39.425	0.000
	Days	401.344	2	200.672	11.712	0.008
	Error	102.799	6	17.133	-	-
	Total	2530.573	11	-	-	-
ALP (Table 48)	Treat.	17.583	3	5.861	0.746	0.563
	Days	127.188	2	63.594	8.096	0.020
	Error	47.130	6	7.855	-	-
	Total	191.901	11	-	-	-
ELS (Table 49)	Treat.	3453.719	3	1151.240	4.467	0.057
	Days	465.129	2	232.565	0.902	0.454
	Error	1546.482	6	257.747	-	-
	Total	5465.330	11	-	-	-
GRP (Table 49)	Treat.	1236.616	3	412.205	15.823	0.003
	Days	218.929	2	109.464	4.202	0.072
	Error	156.302	6	26.050	-	-
	Total	1611.846	11	-	-	-
ALP (Table 49)	Treat.	737.554	3	245.851	2.552	0.152
	Days	114.415	2	57.208	0.594	0.582
	Error	577.936	6	96.323	-	-
	Total	1429.906	11	-	-	-

Sig.= Significance, df = Degrees of Freedom.

5.4 Discussion (Section 5.3.5.1 - 5.3.5.8)

Application of different chemical stress pre-treatment may influence the induction and regeneration efficiency in cereal and other crop plants. Mannitol is one of the suitable osmoticum agents that showed increasing embryoids and green plants in many cereals. Several reports have shown that carbohydrates, when used at elevated concentrations, stimulate plant differentiation in cultured cells. Barnabás (2003) found that culture medium with a higher concentration of sucrose improved plant regeneration in maize. In another study Kishor and Reddy (1986) reported that culture media in addition with carbohydrates, such as mannitol or sorbitol, induced high-frequency shoot formation in rice. Wojnarowiez et al. (2004) tested sugars and mannitol at different steps on anther culture in barley to elucidate their influence on both the overall embryo yield and the structure of plastids in relation to albinism. They obtained best results using mannitol (364 mOsm/kg) and found 139.7 percentages of green plants per 100 plated anthers. Pre-treatment with mannitol showed significant progress for microspore development in wheat (Slama-Ayed et al. 2010), rice (Raina and Irfan 1998) and maize (Pescitelli et al. 1990). Some stresses (cold, heat, carbon starvation and colchicines) are widely used and influences microspore embryogenesis in plants. In case of heat pretreatment usually carried out at 33°C - 37°C for a duration varying from several hours to several days, whereas cold treatment is carried out at 4°C - 10°C from some days to several weeks in rice (Khatun et al. 2010 and 2012, Rukmini et al. 2013). In barley, a mannitol pretreatment improved response (Ziauddin et al. 1992), whereas the inclusion of culture media macronutrients in the mannitol pretreatment were needed to obtain the microspore division and sprophytic development in wheat (Hu et al. 1995) and rice (Ogawa et al. 1994). In barley anther culture due to mannitol pretreatment up to 4,300 genes showed their expression and modification are reported by Muñoz-Amatriaín et al. (2006). They did transcriptome analysis of anthers before and after 4 days of stress pretreatment with 0.7 M mannitol and found the influence of development transition from gametogenesis towards embryogenesis. Hoekstra et al. (1992) and Cistué et al. (1995) confirmed the importance of mannitol pre-treatment in barley. Sugar and nitrogen starvation applied to

highly homogeneous population of immature pollen grains at the mid-bicellular stage allowed pollen development to follow sporophytic pathway directly, in plant species (Touraev et al. 1996b). In the latter case, application of 0.7 M mannitol for 3-5 days improved green plant regeneration significantly. Under this study as osmoticum agents three chemicals (carbohydrates) e.g. sorbitol, PEG and mannitol were added in the induction medium and for each case three dosages (40, 60 and 80 g/l) were considered. It was observed that mannitol showed better results (41.07%) on embryoids induction and green plant regeneration (22.04%) where 60 g/l was used. For barley, Wojnarowiez et al. (2004) found 139.7 percentages of green plants per 100 plated anthers. May be the embryo yield was different due to another crops, the dosages of mannitol, application mode and culture conditions. In this case an another study with different dosages (0.2 mM - 0.7 mM) of mannitol was considered and found that 0.3 and 0.4 mM mannitol showed highest embryo yield and green plant regeneration. So it was very clear effect with different concentrations of mannitol on embryo yield and regeneration in rice anther culture.

In another study different dosages of mannitol + heat pre-treatment at 30°C - 33°C for different hours to excised anthers were evaluated on the basis embryo yield and regeneration efficiency in rice anther culture. Here both temperatures showed increasing embryo yield and regeneration with 3 hrs incubation period. Temperature pre-treatment at 33°C showed the best results for embryo like structures (43.12%) and green plant regeneration (22.86%) that was 2-3 times higher than Control. Rukmini et al. (2013) reported that as heat pre-treatment at 33°C to 37°C for various durations showed increasing the embryo yield and regeneration. Under this study the heat pre-treatment duration was 3-12 h in addition 0.3 mM mannitol in medium. Oleszczuk et al. (2006) studies about the temperature effect at 32°C for 24 h + 0.3 mM mannitol pre-treatment and found regeneration efficiency were increased in barley. Here regeneration efficiency was also increased with high temperature at 33°C for 3 h that was different with the study of Oleszczuk et al. (2006).

Under this study an interesting experiment was done also using liquid and semi-solid induction medium to evaluate their effectiveness on embryo yield and regeneration. Here 3 days incubation period showed significantly higher embryo yield (42.8%) and regeneration (21.09%). This finding agreed well with Touraev et al. (2001) where he mentioned optimum incubation period in media containing non-metabolisable carbon sources, i.e. in mannitol containing media enhanced embryo yield and regeneration efficiency. Under this study it was observed that increasing the anther incubation period albino plants were increased. In this case 3 days incubation period was optimum. The results were varied with some other reports mentioned herein because due to other crop species and the duration of incubation period. It was observed that due to the prolonged incubation period albino plants were increased in rice anther culture. Here we found three days incubation period was best for both induction and regeneration and it is a clear message for further advance research for rice improvement through anther culture. Evaluating the embryo yield and green plant regeneration for overall mean value mannitol (60 g/l) showed the best performance (31.56%) than others. It is a clear message that the efficiency of mannitol for improvement of embryogenesis that directly affect on embryoids induction and regeneration in rice. Those results are agreed well with the findings of Ogawa et al. (1994), Raina and Irfan (1998).

Production of haploids and doubled haploids (DHs) through anther culture has major obstacle with low regeneration rate and albino plants. By application of different physical and chemical stress pre-treatment may improve induction and regeneration efficiency in cereal crops (Wędzony et al. 2000), Shariatpanahi et al. 2006a, Islam and Tuteja 2012). But the main problem in the case of anther culture, plants may originate from anther somatic tissue. Therefore, isolated microspore culture is the preferred method for haploid production in cereal crops (Jähne and Lörz 1995). Under this study as osmoticum agent various doses of mannitol (0.2 M, 0.4 M, 0.6 M and 0.8 M) were added in medium. Before microspore isolation excised anthers were incubated in liquid induction medium for 2, 4 and 6 days at 25±1°C. Under this study as osmoticum agent different doses (0.2 M, 0.4 M, 0.6 M and 0.8 M) of mannitol were added in medium for 2, 4 and 6 days.

Another study rice floret was used as microspore sources that were incubated in liquid induction media before microspore isolation. Here 4 days pre-treatment with 0.6 M mannitol showed higher embryo yield than Control. Mannitol concentration at 0.4 M showed second heights results on ELS and GRP for all durations considered of this study. Oleszczuk et al. (2006) found that the highest number of green plants was obtained after the treatment of anthers in 0.3 M mannitol at incubated them at 32°C for 24 h before microspore culture. The present findings showed also best resulst when anthers were incubated with mannitol with 0.4 M mannitol. For isolated microspore culture pre-treatment with mannitol showed significant progress for microspore development in wheat (Slama-Ayed et al. 2010), rice (Raina and Irfan 1998) and maize (Pescitelli et al. 1990). All of the previous reports showed mannitol has great effects on cllaus induction and regeneration. Another experiment was done where various concentration of mannitol (0.2 M - 0.8 M) was directly used in addition to the induction medium (AMC). The effect of mannitol dosages was observed for 2, 4 and 6 days on the basis of embryo yield, green plant regeneration and albino plant production. It was observed that the incubation period 4 days of rice floret in the induction medium showed significantly higher embryo yield (163.67%) than Control (92.67%). Roberts-Oehlschlager and Dunwell (1990) found that four days incubation of barley anthers in a medium containing 3.2% mannitol raised the pollen callusing response from 23% to 78% which was more than cold treatment alone or in combination with mannitol. Raina and Irfan (1998) have reported that treatment of anthers in 0.4 M mannitol solution was essential to induce androgenesis in microspore cultures of indica and japonica rice cultivars. These studies corroborate the findings of the present study.

DHs through *Agrobacterium*-mediated transformation

6. Production of DH fertile transgenic plants by *Agrobacterium*-mediated transformation

6.1 Introduction

Global warming leads to various climate changes resulting in new abiotic stresses in many agriculturally used regions in the world. The earth becomes less fertile as decreasing precipitations lead to drought problems in large areas, which in addition leads to salinization of soil. It is to be mentioned that sustainable agricultural development, SDGs-sustainable development goals and food security is not achievable without new technologies such as genetic engineering and biotechnology in Bangladesh. The population of rice eaters are increasing day by day and the number of rice consumers will probably two fold by the year of 2020 (Khush and Toennissen 1991). In many countries rice production area is threatened due to high salinity and drought stress factors resulting in the failure to achieve self-sufficiency in food (Boyer 1982). Those stresses adversely affect growth and productivity and trigger a series of morphological, physiological, biochemical and molecular changes in plants. However, there is a great need to exploit all genetic variability that can be used for stressful environments. Since plants are sessile, they have to perceive the changes in the environmental condition to survive and grow under stress conditions (Banu et al. 2014).

Bangladesh is a densely populated country and here the probability of increasing cultivable land is nearly zero. Agricultural genetics is one of the easier parts of the solution. The only alternative, therefore, is to increase productivity using suitable biotechnological approaches. Biotechnologies could provide us with useful tools in various sectors such as agriculture, fisheries, food production and industry (Purwoko et al. 2010). The application of biotechnology in combination with conventional breeding methods such as doubled haploid technology may help to increase food production properly (Weeks et al. 1993, Otani et al. 2005, Islam et al. 2013a). An endeavor for genetic improvement of this crop,

with respect to disease resistance, drought, heat and salinity tolerance with high yielding cultivars, may be helpful to boost up the rice production for developing countries. In order to respond quickly to their changing environment plants use an elaborate system of hormonal signals such as auxins (Creelman and Mullet 1995, Chini 2007), abscisic acid (ABA), JA (Jasmonic acid) and GA (gibberellins). The use of genetic engineering technology could lead to simpler and more effective gene-based approaches for improving crop tolerance (Fukuoka et al. 1998, Tuteja 2007, Chauhan and Khurana 2011).

The RNA helicases are ubiquitous enzymes that catalyse the unwinding of energetically stable duplex RNA secondary structures. They play an essential role in basic cellular processes regulating plant growth and development, such as DNA replication, repair, recombination, transcription and translation (Owttrim 2006). It seems therefore that helicase might be playing an important role in stabilizing growth in plants under stress by regulating stress-induced transcription and translation. A hallmark of most of the helicases (but not for all) is the existence of a set of highly conserved amino acid sequences called 'helicase motifs', which are clustered together for helicase function. Because of the presence of a DEAD motif, the family of these proteins is also called as DEAD box family of proteins (Tuteja 2007).

For genetic transformation of cereal crops have recalcitrant to recombinant techniques mainly because of problems in establishing regenerable cell and tissue cultures as well as efficient DNA delivery systems (Stöger et al. 1995, Obert et al. 2008, Shim et al. 2009). Microspore embryogenesis is an elegant system for genetic transformation and could provide a practical alternative for the production of transgenic doubled haploid lines in which regeneration from somatic cells is really very difficult, especially in the recalcitrant cereals (Datta et al. 1990, Bikash and Mandal 2001, Islam et al. 2001, Kumlehn 2009, Karasawa et al. 2016). This protocol may be used to overcome genotypic limitations of doubled haploid formation in cultivars that had previously been found to be recalcitrant in anther culture (Sopory et al. 1996, Zapata-Arias 2003, Cegielska-Taras et al. 2016). This

method offers unusual potential for reducing the time required for the production of homozygous progeny.

Agrobacterium tumefaciens is a gram negative soil inhabiting bacteria that causes crown gall disease in a wide range of dicotyledonous plants and its infection was first described by Smith and Townsend (1907). During infection process, a specific segment of the plasmid vector, T-DNA, is transferred from the bacterium to the host plant cells and integrates into the nuclear genome (Fig.13). The unique mode of action of *A. tumefaciens* has enabled this bacterium to be used as a tool in plant transformation.

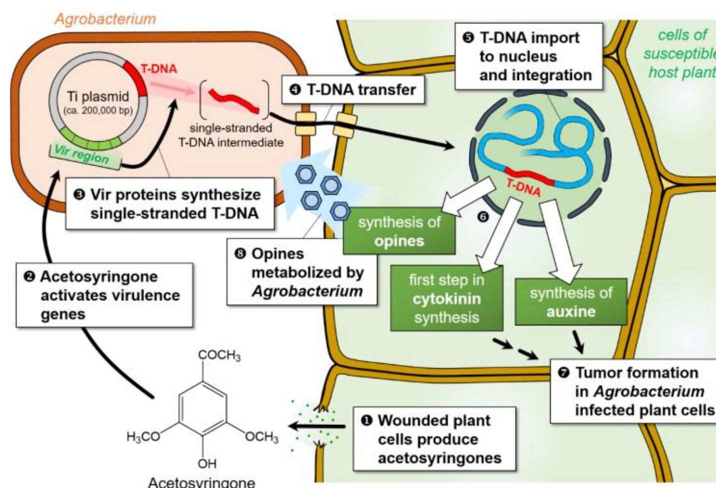


Fig. 13: General model of *Agrobacterium*-mediated transformation of a plant cell. (http://www.slideshare.net/annybaner/agrobacterium-tumefacienspptit-is-a-slide-presentation-on-interkingdom-gene-transfer).

Dr. Tuteja's Group preliminary work on this aspect shows that a *p68* gene is getting induced by drought and salinity stress in pea plants. The *p68* (Ddx5) gene is one of the prototypic members of the DEAD box family of proteins and were one of the first proteins to be shown to exhibit RNA helicase activity *in vitro*. Expression of *p68* was shown to be growth and developmentally regulated and to correlate with organ maturation and differentiation. Overall, *p68* is a multifunctional protein involved in RNA splicing, pre-rRNA processing, RNA-induced silencing, transcription initiation, transcriptional

repressor, etc. However, very little is known about *p68* protein in plant system. The role of *p68* in stress tolerance has not been reported so far for other crops. In an attempt to permit growth of plants in drought and/or high salinity stress, genetic transformation has been carried out with stress-induced *p68* gene using Bangladeshi rice cultivars in combination with androgenetic methods. Under this study *p68* (DB10) gene was successfully transformd using anther and microspore derived-embryoids of Bangladeshi rice cultivars as BRRI dhan29. This research work have been considered to find out the combination of *in vitro* androgenesis and *Agrobacterium*-mediated genetic transformation using targeted gene for the production of drought and/or salinity tolerant rice. The outcome of this research work is very much helpful for developing stress tolerant fertile transgenic plants which may contribute for food and environmental security and SDGs in Bangladesh.

6.2 Materials and Methods

6.2.1 Plant materials and media

As plant material embryoids/calli derived from anthers and or micropsores of BRRI dhan29 was considered for this study. The anther and micropsore derived calli were sub-cultured in the MS medium that contained MS basal salts and vitamins, L-proline 65 mg/l, Casein hydrolysate 30 mg/l, 2,4-D 2.5 mg/l, BAP 0.15 mg/l, Sucrose 30 g/l, Phytagel 3.0 g/l or agarose 6 g/l (callus induction media). Then the cultures were incubated at 26°C±2°C for 7 days and used for *Agrobacterium*-mediated genetic transformation.

6.2.2 Methods

6.2.2.1 Source of gene (p68/ DB10 for rice) - *In silico* analysis

P68 (AF271892)

NM_001048686
>gi|115434785|ref|NM_001048686.1| *Oryza sativa* (japonica cultivar-group)
Os01g0172200 (Os01g0172200) mRNA, complete cds
AATCACATTCTTCTCTTCTCATCGACCCCTCCGCCTCCTCTCCTCTCCTTTCCATTTTTTCCCCATCGCGCCC
TACTTAACCACGCGACGCCAAAAAAAGAAAAAGAGGAGAGAGCAAAACAAAGCGAAGGCTCCATTCATTCACT
CCATCTCCTCGCGTCCCCTCCCCGCTCCCCCCGCCGCAATCCGCATTCGCTAGGGG
TTG GGT TGG GAG **ATG** GCG TCG
GCGGCGGCGGCGGCGGCGACGGCGAGGGGGCCGCGGTACGCGCCGCCAGATCCGACGCTGCCCAAGCCGTGGA
GGGGGCTCATCGACGGCAACACCGGGTACCTCTACTTCTGGAACCCGGAGACCAAGGCCGTGCAGTACGACCG
CCCCACGGCGCCGCCGCCCTCCTCCCCCCCGGCGCAGCAGCCTCCGGAGAGGCCTAGGAACAGCGATCCTGCT
GAGTCGCAGGCGCAGGCTGGAGCGAGCCGAACGCAGAATGCTGCTCCTGCTGATGATAGGGCAAGGAATGATC
ATTTGAATGATCATTTCGAGCGGCGCACGGAAGCGGCGGGAAGTCATGCGCAGAACGTGCCTTTCACTGAGCA
AAATACTAGGAGCAACCCTTCTTCGCAGCCGTGCTCTGCAGCTGGAGTATACCCTGCACAGAATGTATTTTCA
GAGGCTGCCAGTGGAGACCGCACCTCACCAGAAGCGTATCGTGCTAAGCACGAGATCACTATTGTCGGAAATG
AGGCTCCAGCTCCATTCATGACATTTCAGTCCACAGGTTTCCCTCCAGAGATTCTAAGGGAG**GTA**AGTGCACA
CAATTTACATGACTATTTAATGCATTTTCTTGTATCCTTCTCGAAAGAAATTAGGAGGTCATTGTGCGTTCAT
ACTACGTATGTGATAACTACGCTGGATGTTCATCTCAGCAGGCTCTTCAAGTCAACAATCTATGTCAAGGTA**C**
AGCAAGCGGGATTCTCTGCACCAACTCCTATCCAAGCTCAATCATGGCCAATTGCTCTTAGGAATCGTGATAT
TGTAGCTGTGGCGAAGACAGGTTCCGGAAAAACACTGGGTTATCTCATTCCAGGGTTTATCCTTCTTAAGCGC
CTTCAACATAATTCAAGGGATGGTCCAACGGTATTAGTGCTTTCTCCAACAAGGGAATTGGCAACACAAATCC
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TCCTCAGCTAAGAGATCTAGAGCGTGGTGCAGACATTGTGGTTGCAACTCCTGGAAGATTGAATGACATTTTA
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TTGATCAAATCCTAAGGTCACAGGAACCTGGATCGAAAATCATAATATTTTGCTCCACA(FCST)AAGAGGAT
GTGTGATCAGCTGGCTCGAAACCTAGCACGGCAGTATGGTGCTTCTGCTATTCATGGTGATAAATCACAAGCC
GAGAGGGATTCTGTGTTGAGTGAATTTCGAAGTGGCAGATGCCCTATTCTTGTTGCTACTGATGTGGCTGCCC
GAGGCTTGGAC(RGLD)ATAAAGGATATCAGAGTTGTGGTCAACTACGATTTCCCAACAGGTGTTGAGGATTA
CGTCCATAGAATTGGGAGAACAGGACGG(HRIGRTGR)GCTGGAGCGACTGGAGTTGCCTATACATTCTTCTG
TGATCAGGATTCAAAGTATGCTTCAGATCTCGTGAAGATTTTGGAGGGTGCAAACCAGTCTGTTTCGCAACAG
TTGAGAGATATGGTCTCCCGTGGAGGGTATGGTGGAAGGTCACGGCGCTGGGCATCTTCAGATGACTCTTATG
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ACAACGATCAACCTGGTGATGGTCTCAGCTTTCATGCCAGGTTCTATAGCTCTTCTCGAGGCAGCGATCAGAG
CAGAACAAACAATGCTGGCTTTCGTGATAGAAGTAGGAGTCCTCCAAGCAATCGCAACCATGAAGATCCTGGG
TCC AAG GCT GTT GGT GTC TCC AAC TGG
TAAGTGCTAACTGCAAGATGCAAACAGCATCATGATGGTGACGAACAGAGTAGATGAAGCTGTTCTCTTACCA
AACTATACTATCGTGCGTGCTCCCCCTGAAGCAAAAGTGTGTCCCAGAACTTAACTAACATAGGATAAGACTG
TGGATCTTCTGCTGTGCAGATGTAGCGCACTTGATAATGTATGTCTGGTGTTAGTCGTTTGTCAGACCTGGGG
GCTCGGACTTCCTTGCTATCTATACCTGCTACCGCTATGATCATGTGGAAACAAACCATCTGAGGCATGCATT
TTGGACGAAAACATGCACTCTTAATCCTTATATGACTTGTAGATTATTTACCCCAGCGTCGTTTACATTGAAC
TATTTCC ALIGNED AREA WITH P68

6.2.2.1.2 DB10 protein

MASAAAAAATARGPRYAPPDPTLPKPWRGLIDGNTGYLYFWNPETKAVQYDRPTAPPPSSPPAQQP
PERPRNSDPAESQAQAGASRTQNAAPADDRARNDHLNDHFERRTEAAGSHAQNVPFTEQNTRSNPS
SQPCSAAGVYPAQNVFSEAASGDRTSPEAYRAKHEITIVGNEAPAPFMTFQSTGFPPEILREVSAH
NLHDYLMHFLVSFSKEIRRSLCVHTTYVITTLDVHLSRLFKSTIYVKVQQAGFSAPTPIQAQSWPI
ALRNRDIVAVAKTGSGKTLGYLIPGFILLKRLQHNSRDGPTVLVLSPTRELATQIQDEAKKFGRSS
RISSVCLYGGAPKGPQLRDLERGADIVVATPGRLNDILEMRRVSLHQVSYLVLDEADRMLDMGFEP
QIRKIVKQVQPKRQTLMFTATWPKEVRKIASDLLSNPVQVNIGNTDQLVANKSITQYVDVITPPEK
SRRLDQILRSQEPGSKIIIFCSTKRMCDQLARNLARQYGASAIHGDKSQAERDSVLSEFRSGRCPI
LVATDVAARGLDIKDIRVVVNYDFPTGVEDYVHRIGRTGRAGATGVAYTFFCDQDSKYASDLVKIL
EGANQSVSQQLRDMVSRGGYGGRSRRWASSDDSYGGRGYDSGYTSRSTDNYNSGYGSQSGNGSSFH
SSFHNSNSGNQFGDTSGFQTSFHNSSSNNQTSDNPSFHASSNNDQPGDGLSFHARFYSSSRGSDQS
RTNNAGFRDRSRSPPSNRNHEDPGSKAVGVSNW

6.2.2.1.3 T7 forward (950 bp)

NNNAANTNNNATGCTTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTGGATCCTTGGGTTGGGAG**ATG**GCGT
CGGCGGCGGCGGCGGCGGCGACGGCGAGGGGGCCGCGGTACGCGCCGCCAGATCCGACGCTGCCCAAGCCGTGG
AGGGGGCTCATCGACGGCAACACCGGGTACCTCTACTTCTGGAACCCGGAGACCAAGGCCGTGCAGTACGACCG
CCCCACGGCGCCGCCGCCCTCCTCCCCCCCGGCGCAGCAGCCTCCGGAGAGGCCTAGGAACAGCGATCCTGCTG
AGTCGCAGGCGCAGGCTGGAGCGAGCCGAGCGCAGAATGCTGCTCCTGCTGATGATAGGGCAAGGAATGATCAT
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AGGGATGGTCCAACGGTATTAGTGCTTTCTCCAACAAGGGAATTGGCAACACAAATCCAAGATGAAGCGAAAAG
TTTGGGAGATCGTCAAGAATATCGTCTGTTTGTTTATATGGAGGTGCTCCTAAAGGTCCTCA

6.2.2.1.4 SP6 reverse

NNNANTCAAACGGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGATTGGAT
CCCCAGTTGGAGACACCAACAGCCTTGGACCCAGGATCTTCATGGTTGCGATTGCTTGGAGGACTCCTACTTCT
ATCACGAAAGCCAGCATTGTTTGTTCTGCTCTGATCGCTGCCTCGAGAAGAGCTATAGAACCTGGCATGAAAGC
TGAGACCATCACCAGGTTGATCGTTGTTGCTGCGGGCGTGGAAGCTTGGATTGTCACTGGTTTGATTGTTGCTG
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ACTATGAAAGCTAGAACCATTCCCAGACTGACTGCCATAACCACTGTTATAGTTGTCAGTCGACCTTGAAGTAT
AGCCTGAATCATATCCTCGACCACCATAAGAGTCATCTGAAGATGCCCAGCGCCGTGACCTTCCACCATACCCT
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AGCATACTTTGAATCCTGATCACAGAAGAATGTATAGGCAACTCCAGTCGCTCCAGCCCGTCCTGTTCTCCCAA
TTCTATGGACGTAATCCTCAACACCTGTTGGGAAATCGTAGTTGACCACAACTCTGATATCCTTTATGTCCAAG
CCTCGGGCAGCCACATCAGTAGCAACAAGAATAGGGCATCTGCCACTTCGAAATTCACTCAACACAGAATCCCT
CTCGGCTTGTGATTTATCACCATGAATAGCAGAGCACCATACTGCCGTGCTAGGTTTCGAGCCAGCTGATCACA
CATCCTCTTTGTGGAGCAAATATTATGATTTTCGATCCAGGTTCCTGTGACCTTAGGATTTG

6.2.2.1.5 Primers

Designing Primers for DB10 genes (full length and truncated part)
DB10F (BamHI) **GGA TCC** TTGGGTTGGGAGATGGCGTCG (27 bp) Tm = 80.9
DB10R (BamHI) **GGA TCC** CCAGTTGGAGACACCAACAGCCTT (30 bp) Tm = 79.1
DB10Del (BamHI) **GGA TCC** TATTATGATTTTCGATCCAGG (27 bp) Tm = 67°C

6.2.2.2 GenBank accession (for indica rice cultivar)

```
GenBank accession GQ267545-GQ267546
LOCUS       bankit1209181          2127 bp    mRNA    linear   PLN 15-APR-2009
DEFINITION O. sativa (indica group-cultivar) ATP-dependent RNA helicase DB10.
SOURCE      Oryza sativa
  ORGANISM  Oryza sativa
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Ehrhartoideae; Oryzeae; Oryza.
REFERENCE   1  (bases 1 to 2127)
  AUTHORS   Islam,S.M.S., Umate,P. and Tuteja,N.
  TITLE     ATP-dependent RNA helicase DB10
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 2127)
  AUTHORS   Islam,S.M.S., Umate,P. and Tuteja,N.
  TITLE     Direct Submission
  JOURNAL   Submitted (15-APR-2009) Plant Molecular Biology, International
            Center for Genetic Engineering and Biotechnology, Aruna Asaf Ali
            Marg, New Delhi 110 067, India
FEATURES             Location/Qualifiers
     source          1..2127
                     /organism="Oryza sativa"
                     /mol_type="mRNA"
                     /sub_species="indica"
                     /db_xref="taxon:4530"
BASE COUNT      561 a    502 c    563 g    501 t
ORIGIN
        1 atggcgtcgg cggcggcggc ggcggcgacg gcgaggggc cgcggtacgc gccgccagat
       61 ccgacgctgc ccaagccgtg gaggggctc atcgacggca acaccgggta cctctacttc
      121 tggaacccgg agaccaaggc cgtgcagtac gaccgcccca cggcgccgcc gccctcctcc
      181 cccccggcgc agcagcctcc ggagaggcct aggaacagcg atcctgctga gtcgcaggcg
      241 caggctggag cgagccgaac gcagaatgct gctcctgctg atgatagggc aaggaatgat
      301 catttgaatg atcatttcga gcggcgcacg gaagcggcgg gaagtcatgc gcagaacgtg
      361 cctttcactg agcaaaatac taggagcaac ccttcttcgc agccgtgctc tgcagctgga
      421 gtataccctg cacagaatgt attttcagag gctgccagtg gagaccgcac ctcaccagaa
      481 gcgtatcgtg ctaagcacga gatcactatt gtcggaaatg aggctccagc tccattcatg
      541 acatttcagt ccacaggttt ccctccagag attctaaggg aggtacagca agcgggattc
      601 tctgcaccaa ctcctatcca agctcaatca tggccaattg ctcttaggaa tcgtgatatt
      661 gtagctgtgg cgaagacagg ttccggaaaa acactgggtt atctcattcc agggtttatc
      721 cttcttaagc gccttcaaca taattcaagg gatggtccaa cggtattagt gctttctcca
      781 acaagggaat tggcaacaca aatccaagat gaagcgaaaa agtttgggag atcgtcaaga
      841 atatcgtctg tttgtttata tggaggtgct cctaaaggtc ctcagctaag agatctagag
      901 cgtggtgcag acattgtggt tgcaactcct ggaagattga atgacatttt agaaatgcga
      961 agagtgagcc tacatcaagt atcttatctt gttcttgatg aggctgaccg catgcttgat
     1021 atgggttttg agccacaaat aagaaaaatt gtgaaacaag tacaacccaa acgacagact
     1081 cttatgttca ctgccacttg gccaaaagag gtgaggaaaa tagcctcaga tttgctgtcc
     1141 aatccagtcc aagttaacat tgggaacact gatcaactgg ttgccaataa gtcaatcact
     1201 cagtatgtgg atgttatcac acctccggag aaatcgaggc ggcttgatca aatcctaagg
     1261 tcacaggaac ctggatcgaa aatcataata ttttgctcca caaagaggat gtgtgatcag
     1321 ctggctcgaa acctagcacg gcagtatggt gcttctgcta ttcatggtga taaatcacaa
     1381 gccgagaggg attctgtgtt gagtgaattt cgaagtggca gatgccctat tcttgttgct
     1441 actgatgtgg ctgcccgagg cttggacata aaggatatca gagttgtggt caactacgat
     1501 ttcccaacag gtgttgagga ttacgtccat agaattggga gaacaggacg ggctggagcg
     1561 actggagttg cctatacatt cttctgtgat caggattcaa agtatgcttc agatctcgtg
     1621 aagattttgg agggtgcaaa ccagtctgtt tcgcaacagt tgagagatat ggtctcccgt
     1681 ggagggtatg gtggaaggtc acggcgctgg gcatcttcag atgactctta tggtggtcga
     1741 ggatatgatt caggctatac ttcaaggtcg actgacaact ataacagtgg ttatggcagt
     1801 cagtctggga atggttctag ctttcatagt agcttccata acagcaacag tggcaatcag
     1861 tttggcgata cttctggctt ccaaaccagc tttcataaca gcagcagcaa caatcaaacc
     1921 agtgacaatc caagctttca tgccagcagc aacaacgatc aacctggtga tggtctcagc
     1981 tttcatgcca ggttctatag ctcttctcga ggcagcgatc agagcagaac aaacaatgct
     2041 ggctttcgtg atagaagtag gagtcctcca agcaatcgca accatgaaga tcctgggtcc
     2101 aaggctgttg gtgtctccaa ctggtaa
```

6.2.2.3 Bacterial strains and vectors

Under this study as bacterial strains- *Escherichia coli* (DH5α), Invitrogen, Carlsbad, USA, *Agrobacterium tumefaciens* (LBA4404) - ICGEB, New Delhi, India and as vectors pGEMT, pRT100 and pCAMBIA1301 were used.

6.2.2.3.1 pGEM-T vector

The pGEM-T vector is a high-efficiency TA cloning vector which contains multiple cloning sites as shown below. The pGEM-T vector is 3.0kb in size and contains the ampicillin resistance gene for selection. The coding sequence was inserted by TA cloning. Most commercially available competent cells are appropriate for the plasmid, e.g. TOP10, DH5α, and JM109. pGEMT-T (3000 bp)- Promega, Madison, WI, USA.

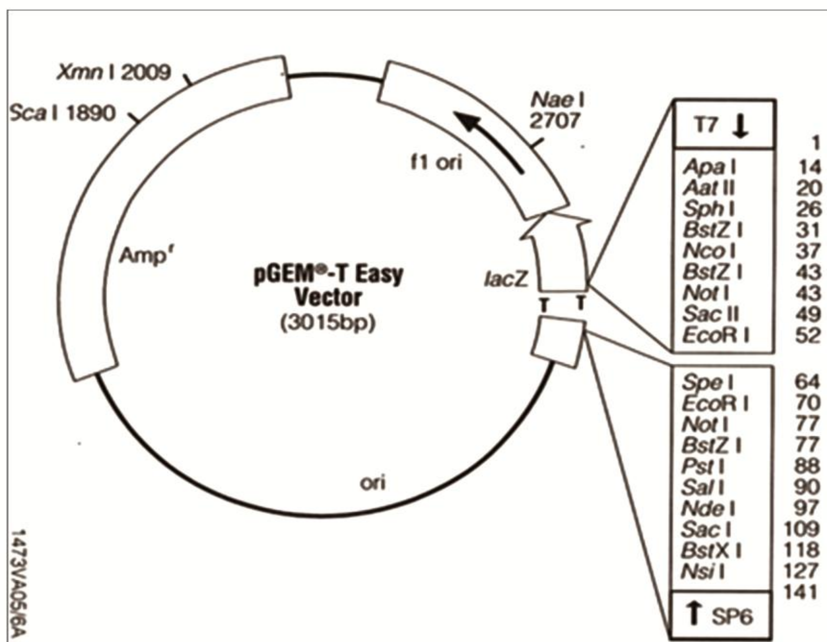


Fig. 14: pGEMT-T Easy empty vector with targeted cloning sites.

6.2.2.3.2 pRT100

The plasmids pRT100-pRT104 carrying the 35S promoter and the polyadenylation signal of CaMV strain Cabb.

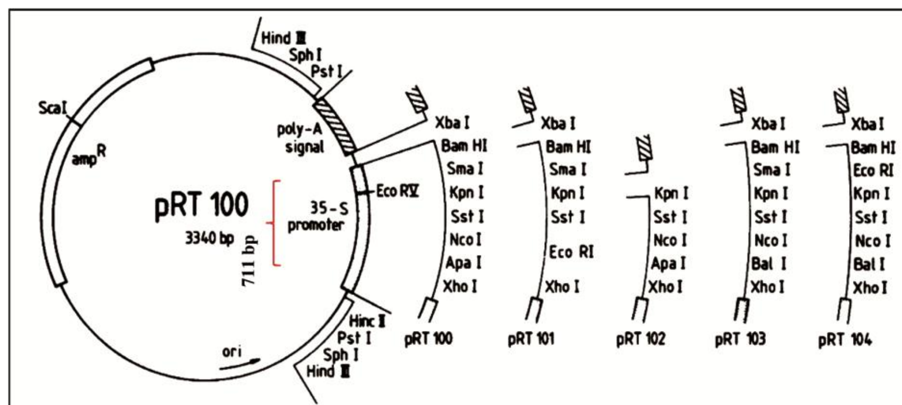


Fig. 15: Empty vector of pRT100 (sub cloning of gene in EcoRI site).

6.2.2.3.3 pCAMBIA 1301

The plant transformation vector pCAMBIA1301 contains strong, nominally constitutive, 35S promoter from cauliflower mosaic virus, kanamycin and hygromycin resistance gene for bacterial and plant selection, respectively. The pCAMBIA1301 vector offers- i) high copy number in *E.coli* for high DNA yields, ii) pVS1 replicon for high stability in *Agrobacterium,* iii) small size, Restriction sites designed for modular plasmid modifications and small but adequate poly-linkers for introducing your DNA of interest, iv) bacterial selection with kanamycin, v) plant selection with hygromycin B, and vi) Simple means to construct translational fusions to *gusA* reporter genes.

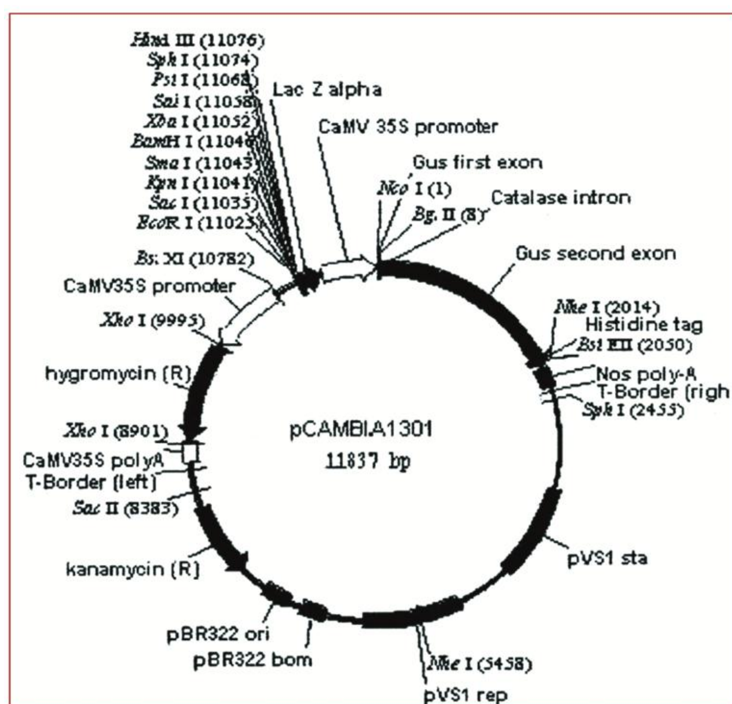


Fig. 16: Empty vectors with multiple cloning site of targeted gene for plant transformation (pCAMBIA1301).

6.2.2.4 Markers

1kb DNA Marker- Fermentas, Canada

6.2.2.5 Restriction enzymes

Fermentas, Canada Calf intestinal alkaline phosphatase (CIP)

New England Biolabs Inc.- MA, USA

Taq Polymerase, *Pfu* Polymerase- Fermentas, Canada

6.2.2.6 Plasmid isolation kits

Mini Prep plasmid kit- Fermentas, Canada; Qiagen, Hilden, Germany

Midi Prep plasmid kit- Fermentas, Canada; Qiagen, Hilden, Germany

PCR/ gel extraction kit, Fermentas, Canada; Qiagen, Hilden, Germany

6.2.2.7 General chemicals

Sigma Chemical Company, St. Louis, USA; Serva, Heidelberg, Germany; USB Amersham International plc.), Buckinghamshire, UK; Amersham Biosciences, United Kingdom; Himedia, India; Promega Life Science, Madison, WI, USA.

6.2.2.8 Isolation of total RNA by Trizol method

Total RNA was isolated from young leaves of 7 days old rice (*Oryza sativa* L.) seedling **(Fig. 13a)** of Swarna (Indica rice cultivars) by Trizol method.

6.2.2.9 Quantification and quality checking of RNA and cDNA preparation

Spectophotometric quantification of RNA and purity of RNA was confirmed by measuring standard method for obtaining high quality and intact RNA which was the first as often the most critical step in performing many fundamental molecular biology experiments and RT-PCR, cDNA library construction, etc. The isolated RNA was also checked in 1% agarose formaldehyde denaturing gel electrophoresis. If two strong RNA bands were visible (28 S rRNA and 18 S rRNA), then RNA can be used for cDNA synthesis (Sambrook and Russell 2001). Quality of RNA was checked by gel electrophoresis **(Fig. 13b).** Complementary DNA (cDNA) was made from the total RNA by reverse transcription (RT-PCR) using a cDNA by First Sand Synthesis Protocol with M-MuLV Reverse Transcriptase (New Englad Biolabs).

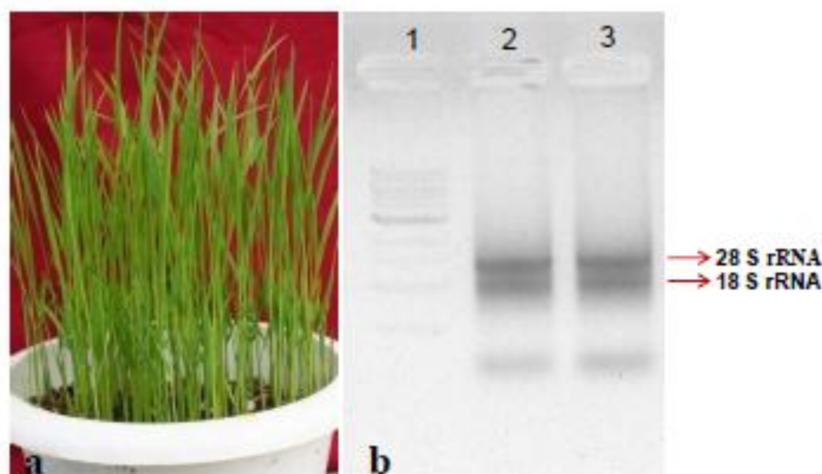


Fig. 17 (a-b): RNA isolation and cDNA preparation. a) 7 days old rice seeding, b) Quality checking of RNA by spectrophotometer (L1= 1 kb ladder, L 2 & 3: RNA (28S rRNA and 18S rRNA).

6.2.2.10 Reverse transcription (RT-PCR) and cDNA library construction

Step 1: In a sterile microfuge (eppendorf) tubes (in addition)

RNA solution 0.5-2.0 µg total RNA (or 50-100 ng polyA-selected RNA)	1.0 µl /as per quantification
Primer (Oligo-dT) Random Hexamer primers	1.0 µl
dNTP Mix	1.0 µl
Nuclease free D.W to final volume of 16 µl	12.0 µl
	16.0 µl

Step 2: Heat for 3-5 minutes at 65-80°C. Spin briefly and placed promptly on ice.

Step 3: Add 10X RT Buffer 2.0 µl + RNAse inhibitor 1.0 µl + M-MuL V Reverse Transcriptase 1.0 µl

Final volume = 20 µl

N.B. For Control = add water in a tube but without Reverse Transcriptase.

Step 4: Incubate at 42°C for one hour.

Step 5: Inactivate enzyme at 90°C for 10 minutes.

Step 6: Store products at -20°C or proceed to next steps.

6.2.2.11 Make PCR to amplify the full length gene of DB10 (2.2 kb)

Amplification of 2.2 kb DNA product by DB10 F & R primers and the eluted of PCR product by kits. The cDNA (1:50 dilution) was used as template for amplification in PCR. The PCR reaction was-

10X PCR Buffer with $MgCl_2$	5.0 µl
10 mM dNTP Mix	5.0 µl
Forward primer (10 µM)	1.0 µl
Reverse primer (10 µM)	1.0 µl
Taq DNA polymerase (5 U/ µl)	0.4 µl
DNA template (DB10) 1:50 cDNA	1.0 µl
Nuclease free D.W	**to 50 µl**

6.2.2.12 Purification of DNA extraction from agarose gels

For cloning the PCR or digestion products were resolved by electrophoresis on 0.8 - 1% agarose gel. The desired fragment was identified using standard molecular weight marker (1 kb ladder) and purified using the following techniques of Quick gel extraction using Qiagen or GFX (Amersham) columns. To the cut pieces of agarose gel containing the desired DNA fragment, 3 volumes of QG buffer, as supplied with Qiaquick gel extraction kit, (Qiagen GmbH, Hilden, Germany) was added and dissolved by heating at 50°C for 10 min. The mixture was loaded onto Qiaquick spin column and spun briefly 10,000 rpm for 30 sec. The flow through was discarded and the column was washed twice with PE buffer. The purified DNA fragment was eluted with 50 µl of 10 mM Tris-Cl, 1 mM EDTA pH 8.0. For PCR purification, PCR product was mixed with 5 volumes of QG buffer and the mixture was loaded onto Qiaquick spin column and spun briefly. The flow through was discarded and the column was washed twice with wash buffer PE. The purified DNA fragment was eluted with autoclaved MQ water.

6.2.2.13 Preparation of competent cells (DH5α)

A single colony of *Escherichia coli* (DH5α) was picked up and inoculated into 5 ml Luria-Broth (LB) medium and grown overnight at 37°C. One ml of this was inoculated freshly into 100 ml LB and grown at 37°C till an OD600 of 0.4-0.5 was obtained (2-3 hours). The cells were harvested by centrifugation at 3,000 X g for 10 min. The pellet was re-suspended in 40 ml of sterile ice-cold 100 mM $CaCl_2$ solution, centrifuged and the pellet was re-suspended in 4.0 ml of sterile ice-cold 100 mM $CaCl_2$. All these steps were carried out in a laminar flow hood under sterile conditions. The cells were then kept on ice for at least 4 h (for better efficiency the cells can also be kept on ice till 12 h). The cell suspension was mixed with 25% of glycerol and aliquoted (0.1 ml) into eppendorf tubes, frozen immediately in liquid nitrogen and stored them at -80°C.

6.2.2.14 Cloning of DB10 gene into pGEM®-T vector by ligation method and transformation in DH5α

Ligation of eluted PCR product into pGEM®-T and pGEM®-T East Vectors Systems (Promega, USA). Transformation of ligated product in competent *E. coli*. Cloning of DB10 gene into pGEMT the both restriction site was *Bam*HI. The DNA fragment (DB10 full length gene) was ligated at room temperature for 1 hour or at 4°C for overnight to the appropriate vectors of pGEM®-T (in the ratio 3:1 by using T4 DNA ligase). Then transformation procedures were done as per the standard protocol of Promega (Technical Manual, www.promega.com).

6.2.2.15 Selection of positive colonies (blue-white selection)

Under this study for blue and white selection, 10.0 µg IPTG and 1.0 µg X-gal per plate were spread prior to plating the cells. The plates were incubated overnight at 37°C. As a negative control DH5α competent cells (pGEM®-T Vector System I) were also plated on plates containing the selection media.

6.2.2.16 Colony PCR for screening of positive colones

Rapid amplification of DNA fragments was carried out using *Taq* DNA polymerase and a set of gene specific primers of DB10 F & R. The reaction mixture of 50 µl contained 10-50 ng DNA template, 3 ng of each primer, 200 µM of each dNTP, 5 µl 10 X Taq buffer and 2.5 units of *Taq* DNA polymerase. PCR could also directly used for screening of transformed cells containing recombinant plasmids. For this, a single colony was re-suspended in 10 µl of water and boiled for 5 min. 5 µl of the supernatant was used as template for PCR using gene specific or vector specific forward and reverse primers with the same reaction conditions as described above.

6.2.2.17 Confirmation of 2.2 kb fragment insertion by PCR and restriction enzyme (RE) analysis

Mini prep for isolation of plasmid DNA from positive clones and restriction enzyme (RE) analysis was done by *Bam*HI and PCR confirmation was done gene specific primers. Reconfirmation of 2.2 kb fragment was also done by internal cutter of *Sph*I (RE).

6.2.2.18 DNA Sequence identification

Analysis regarding an unknown sequence was performed using NCBI's Basic Local Alignment Search Tools (BLAST), which enables researchers to find regions of similarity between sequences of interest and those available in the database. BLAST analysis allows researchers to compare a query sequence with a library or database of sequences and calculates statistical significances between matches (Britton Jr. et al. 2007). http://blast.ncbi.nlm.nih.gov/Blast.cgi.

6.2.2.19 Cloning of DB10 gene into pRT100

Ligation of eluted PCR product into pRT100 vector for because the plasmid pRT100 carrying the 35S promoter and the polyadenylation signal of caMV strain Cabb. This part is describes the sets of vectors that are derivatives of the expression vector cassette pRT100, which uses the CaMV 35S RNA promoter in combination with various reporter and selectable marker genes.

6.2.2.20 Cloning of DB10 gene into pCAMBIA 1301

The ligation reaction was carried out using appropriate vectors (pRT100 and pCAMBIA1301) and PCR amplified product or digested fragment from another vector. Insert and vectors were digested with appropriate restriction enzymes and electrophoreses on 0.8% agarose gel. Then the transformation procedures were done as per the standard protocol of Promega (Technical Manual, www.promega.com).

6.2.2.21 Preparation of *Agrobacterium tumefaciens* competent cells and transformation

A. tumefaciens competent cells were prepared and the recombinant plasmid was transformed to *Agrobacterium* by freeze-thaw method. Transformation of constructs into *Agrobacterium* was done by mixing 1.0 µg of plasmid DNA with competent cells followed by immediate freezing in liquid nitrogen. Subsequently cells were thawed by incubating the eppendorf tube at 37°C for 5 min. Thereafter 1.0 ml of YEM liquid medium was added to the tube and incubated at 28°C for 6 h at 200 rpm. Cells were spread on a YEM agar plate supplemented with 50 µg/ml kanamycin, 25 µg/ml streptomycin, 20 µg/ml rifampicin and incubated at 28°C. Colonies appeared after 2-3 days were analyzed by PCR and the positive colonies were confirmed by PCR or restriction digestion of the purified recombinant plasmid.

6.2.2.22 Transformation and regeneration of rice plantlets

Under this study DB10-pCAMBIA1301 plasmid construct into *A. tumefaciens* was made. *Agrobacterium tumefaciens* strain LBA4404 harboring DB10 gene was grown overnight in YEM medium containing 50 mg/l kanamycin, 50 mg/l streptomycin, and 10 mg/l rifampicin. Culture was diluted with liquid RM medium to OD600 = 0.2. Anther and miscrospore derived calli were used for infection to *Agrobacterium* for 20 min with gentle shaking. For transformation various media were used are mentioned in **Table 51 & 52**. The infected explants (anther and microspore derived embryoids) were blotted on sterile filter papers and placed on N6D-AS medium for co-cultivation in dark, at 26°C for 2 days. After co-cultivation, explants were cultured in petri dishes containing N6D-Hygro medium (MS + 1 mg/l BAP + 0.1 mg/l NAA + 3% sucrose + 500 mg/l carbenicillin + 30 mg/l hygromycin) 16 h (in light): 8 h (in dark) at 26°C for selection/regeneration (**Table 51 & 52**). After 5-6 weeks, the individual well developed shoots were excised and transferred to bottle containing MS medium + 3% sucrose + 30 mg/l hygromycin + 500 mg/l carbenicillin for rooting. For disinfections of *Agrobacterium* and screening of transformed of treated embryos/calli were washed properly with Claforan (250 mg/l). Blot dry calli on sterile filter paper and transferred to selection medium and incubated in dark at 28ºC for 4-6 weeks. Transformed embryoids/calli will be transferred to N6-REM medium and incubated at 28ºC for 2-3 weeks in light for regeneration. To promote root and shoot development of regenerated plantlets will be transferred to N6-HFM medium.

Table 51: Name of major media and chemicals for rice transformation

Components	Media			
Component (final concentration)	**2N6**	**2N6AS**	**2N6TCH**	**RGH6**
N6 major, minor salts and vitamins (1x)	Yes	Yes	Yes	Yes
Sucrose (30 g/l)	Yes	Yes	Yes	Yes
Casamino acids (1 g/l)	Yes	Yes	Yes	-
2,4-D (2 mg/l)	Yes	Yes	Yes	-
L-proline (0.5 g/l)	Yes	-	-	Yes
L-glutamine (0.5 g/l)	Yes	-	-	Yes
Glucose (10 g/l)	-	Yes	-	-
Acetosyringone (100 µM)	-	Yes	-	-
Casein enzymatic hydrolysate (0.3 g/l)	-	-	-	Yes
6-Benzylaminopurine 3 mg/l	-	-	-	Yes
Naphthalene acetic acid 0.5 mg/l	-	-	-	Yes
Cefotaxime (250 mg/l)	-	-	Yes	-
Timentin (200 mg/l)	-	-	Yes	-
Hygromycin-B (50 mg/l)	-	-	Yes	Yes
pH	5.8	5.2	5.2	5.8
Phytagel (g/l)	2.5	3.5	3.5	6.0

Table 52: Briefly described about media composition for callus induction, regeneration and rooting of rice transformation

Components	mg/l					
	CI				Regeneration	Rooting Medium
	N6D	2N6-AS	AAM	N6-HYG	N6-REM	N6-HFM
Macronutrients						
KNO_3	2830	2830	-	1900	1900	-
$(NH_4)_2 SO_4$	463	463	-	-	-	-
$MgSO_4 .7H_2O$	185	185	250	370	370	296
$NaH_3PO_4. 2H_2O$	-	-	150	-	-	1300
KCl	-	-	3000	-	-	-
K_2HPO_4	-	-	-	-	-	3000
KH_2PO_4	400	400	-	170	170	-
$CaC1_2. 2H_2O$	166	166	150	440	440	10-
NH_4Cl	-	-	-	-	-	1000
NH_4NO_3	-	-	-	1650	1650	-
Micronutrients						
$MnSO_4 .4H_2O$	4.4	4.4	10	22.3	22.3	-
$ZnSO_4.7H_2O$	1.5	1.5	2.0	8.6	8.6	
$CuSO_4 .5H_2O$	-	-	0.025	0.025	0.025	-
$CoC1_2 .6H_2O$	-	-	0.025	0.025	0.025	-
K1	0.8	0.8	0.75	0.83	0.83	-
H_3BO_3	1.6	1.6	3.0	6.2	6.2	-
$Na_2M_OO_4 .2H_2O$	-	-	0.25	0.25	0.25	-
Iron						
$FeSO_4 .7H_2O$	37.3	37.3	-	37.3	37.3	-
Na_2 EDTA	27.8	27.8	-	27.8	27.8	2.5
Fe-EDTA	-	-	40	-	-	-
Vitamins & Amino acids (Organic componenets)						
Pyridoxine HCl	0.5	0.5	1.0	0.5	0.5	-
Nicotinic Acid	0.5	0.5	1.0	0.5	0.5	-
Thiamine HCl	1.0	1.0	10.0	0.1	0.1	-
Myo-Inositol	100	100	100	100	100	-
L-glutamine	-	-	-	900	-	-
L-proline	2878	-	-	-	-	-
L-Aspartic acid	-	-	-	300	-	-
L-Arginine	-	-	176.7			
Casamino acid	300	300	500	2000	-	-
Glycine	2.0	2.0	7.5	2.0	2.0	-
Hormones						
2, 4-D	2.0	2.0	-	-	-	-
Kinetin	-	-	-	2.0	-	-
NAA	-	-	-	0.02	-	-
Acetosyringone	-	10~20	10~20	-	-	-
Carbon source						
Sucrose	30000	30000	68500	30000	30000	-
Sorbitol	-	-	-	30000	-	-
Glucose	10000	36000	-	-	-	5000
pH	5.8	5.2	5.2	5.8	5.8	7.2

6.2.2.23 Isolation of plant genomic DNA

Plant tissue (0.5 g) was ground in liquid nitrogen, 500 µl of grinding buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 250 mM NaCl and 15% w/v sucrose) was added to the grounded tissue and nuclei were collected as pellet after centrifugation at 5000 rpm at 4°C for 10 min. Pellet was resuspended in 0.3 ml of suspension buffer (20 mM Tris-HCl, pH 8.0, 10 mM EDTA). To this 20µl of 20% SDS was added and mixed. Solution was incubated at 70°C for 15 min and 150 µl of 7.5 M ammonium acetate was added. This solution was placed on ice for 30 min and centrifuged at 15000 rpm for 5 min, and the supernatant was collected. To this 0.6 volume of isopropanol was added and incubated at room temperature for 15 min. Nucleic acids were recovered after centrifugation at 14000 rpm for 5 min at 4°C and dissolved in 250 µl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). 2µl of RNaseA (10 mg/ml) was added and incubated for 15 min at 37°C. To this, 250 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed by vortexing for 30 sec. The whole mixture was centrifuged at 10,000 rpm for 5 min at room temperature and the upper aqueous phase was transferred to another tube. To this ammonium acetate was added to a final concentration of 0.3 M and two volumes of chilled ethanol was added, and incubated at –80°C for 1 h. DNA was collected by centrifugation at 15,000 rpm for 15 min at 4°C. The pellet was rinsed in 70% ethanol, dried in vacuum and dissolved in 200 µl TE.

6.2.2.24 Confirmation of transgenic plant by PCR

Genomic DNA was isolated from the putative transgenic lines. This DNA was used as template in PCR and reaction was carried out using gene specific forward and reverse primers of DB10. Wild type plant DNA was used as a negative control and the gene cloned into plant transformation vector as positive control.

6.2.2.25 Confirmation of transgenic plant by histochemical GUS assay

The binary vector pCAMBIA1301 contained GUS as the reporter gene. This method was used for confirmation of integrity of T_1 transgenic plants, for expression of β-glucuronidase (GUS). This gene encodes for β-glucuronidase enzyme which cleaves the chromogenic agent X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide) to develop blue colouration. The different tissues (root, node, leaf sheath, mature leaf) from T_2 generation of transgenic rice were incubated overnight in GUS assay buffer solution containing 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide) (Fermenats) in 100 mM of NaH_2PO_4 buffer (pH 7.0), 10 mM EDTA, 1 mM K_3Fe (CN)6 0.1% triton X-100 solution at 37°C overnight in darkness. After staining, tissues were rinsed extensively in 70% ethanol to remove chlorophyll and mounted on microscope slide. Histochemical staining of GUS expression in leaf samples was performed as described by Jefferson et al. (1987). The transformation efficiency was calculated as per the formula given below (Zaidi et al. 2006). GUS positive plants together with the wild type (WT) plants were analyzed by PCR for confirmation of transgenics.

$$\text{Transformation efficiency (\%)} = \frac{\text{Number of GUS positive plants}}{\text{Number of calli inoculated with } \mathit{Agrobacterium}} \times 100$$

6.2.2.26 Testing of DHs to drought and salt

Testing of doubled haploid (DH) transgenic plants for drought and salinity stress tolerence and plant were grown in pot for DHs seed collection. The whole plants were transferred to soil pots in the greenhouse. Along with the transformation experiments, control experiments were performed where leaf disc were not infected with *Agrobacterium* but cultured on selection/regeneration medium along with the transformed leaf disks. Leaf disc assay was performed with slight modification of method described by Tuteja (2010). Healthy and fully expanded leaves from control and transgenic plants were detached and washed briefly in deionised water. Leaf discs of 1 cm diameter were cut and floated in 6 ml of 200 mM sodium chloride solution for 0-72 h. The treatment was carried out under continuous white light at 25°C+2°C. The effects of this treatment on leaf discs were assessed by observing phenotypic changes.

6.2.2.27 Checking of ploidy levels

Ploidy levels of transgenics plants were determined by flow cytometry methods. If haploids the whole plants need to transfer to soil pots for seeds collection.

6.3 Results

The part of this work has been carried out mainly to focus on studying the rapid development of drought and salinity stress tolerance homozygous plants through androgenetic (anther and microspore culture) and transgenic approaches (*Agrobacterium*-mediated genetic transformation). Under this research programme we have screening out some agronomically important androgenetic varieties of rice in Bangladesh (**Table 18, Chapter V**). For successful transformation work the p68 gene [AF271892, *Oryza sativa* (japonica cultivar-group) Os01g0172200 (Os01g0172200), DB10 for rice, **Chapter VI, Section 6.2.2.1**] was identified through *in silico* analysis. For this work the gene construction into pGEMT, pRT100 and pCAMBIA 1301 vectors were provided by SMS Islam (GenBank accession GQ267545-GQ267546, Prof. Dr. Narendra Tuteja group, ICGEB, New Delhi, India on 2014). A suitable protocol has been standardized to develop drought and/or salinity stress tolerant transgenic rice cultivars using DB10 gene. Before starting the transformation work all previous cloning genes were re-checked by PCR, restriction enzyme and sequence analysis. For the cloning strategy the DB10 gene was first cloned in pGEMT Easy vector is 3.0 kb in size contains the ampicillin resistance gene for the selection (**Fig. 14a-d**).

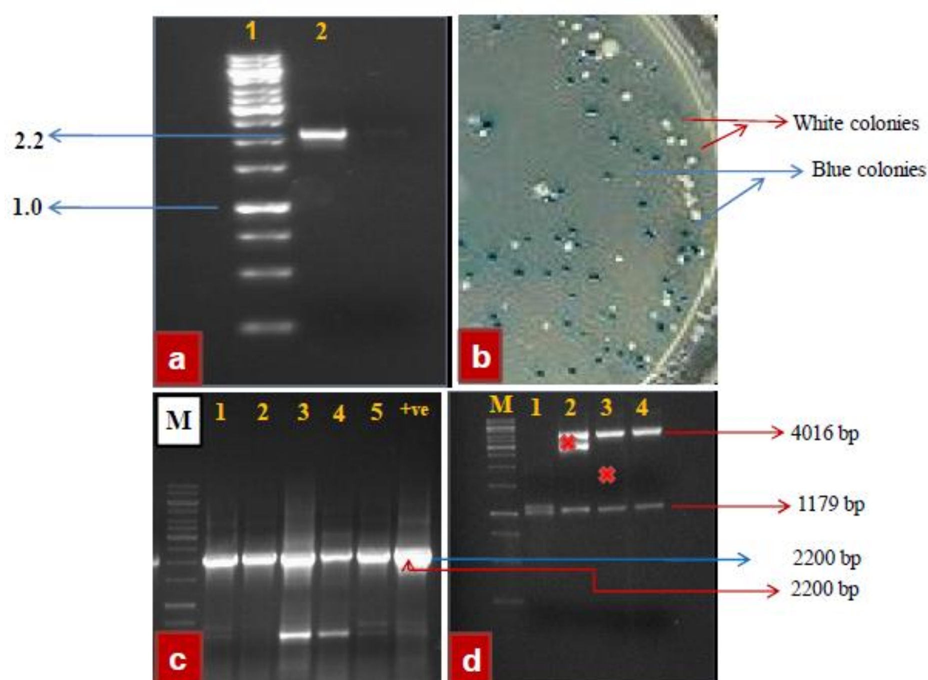


Fig. 18 (a-d): Amplification, cloning and RE analysis of DB10 full length (2.2 kb) gene. a) Full length of amplified gene, b) Transformation of gene to DH5α (*E. Coli*), blue white colony screening, c) Colony PCR with gene specific primers of DB10 F&R, d) Restriction enzyme analysis by *Sph*I and confirmation of cloning to pGEMT-T easy vector, V: 3015 bp, G: 2200 bp, Internal cutter *Sph*I = 1179 bp (2200 bp - 1179 = 1021 bp + 3015 bp = 4016 bp (fall out).

The plasmids pRT100 carrying the 35S promoter and the polyadenylation signal of CaMV strain Cabb. It introduces a set of expression vectors for enhanced gene expression in monocotyledonous plants, especially in rice, wheat and barley. These vectors have been constructed as basic tools applicable for transient gene expression, as well as for stable integration of foreign genes into plant genomes. Ligation of DB10 into pRT100 vectors and confirmation of cloning by restriction enzyme (RE) analysis was done and re-checked (**Fig. 15 a-e**).

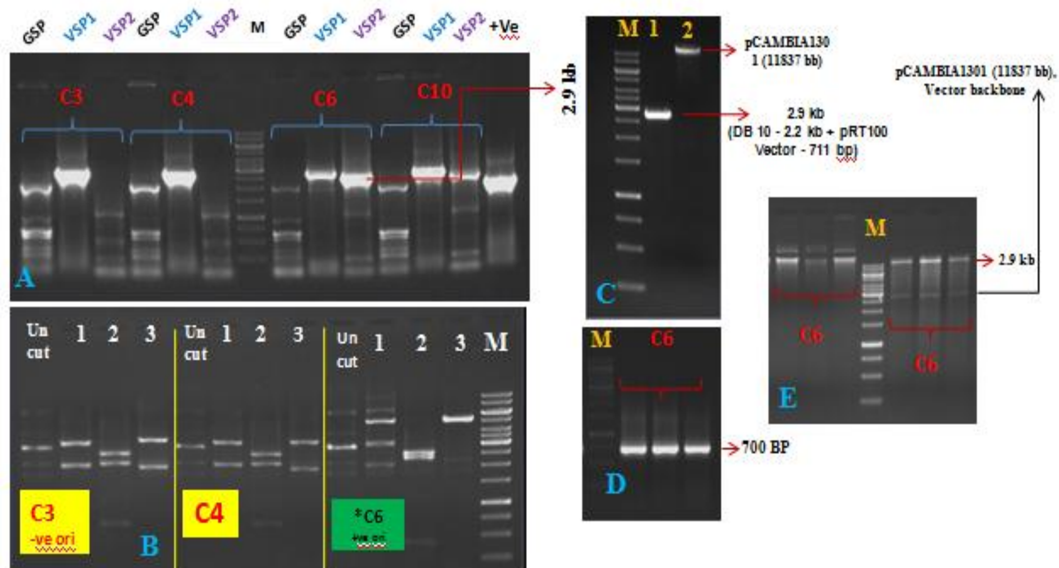


Fig. 19(a-e): Cloning of DB10 full length (2.2 kb) gene to pRT100 and pCAMBIA 1301 vectors for rice transformation. a) Cloning of gene to pRT100 for CaMV 35S promoter site, b) RE analysis of full laigth gene (2.9 kb) + Ve and -Ve orientation, c) Cloning of full length gene (2.9 kb) to pCAMBIA1301 and RE analysis, d) *Vir* gene checking (700 bp) by *Vir* F & R primers after transformation to LBA4404, e) RE analysis wilh *Eco*RI, C = Clone, Uncut = Without RE, M= 1 kb ladder, VSP = Vector specific primers and GSP = Gene specific primers.

For plant transformation the p68 (DB10) gene cloned into pCAMBIA 1301 vector and for that transformation to LBA4404 *Agrobacterium* strain was used. The sets of vectors that are derivatives of the expression vector cassette pRT100, which uses the CaMV 35S RNA promoter in combination with various reporter and selectable marker genes. Schematic representation of strategy used for cloning DB10 gene in the plant transformation vector of pCAMBIA 1301. The DB10 ORF was cloned as a *Sal*I fragment into pRT100 vector. Following which the entire cassette (35S promoter + Gene + Terminator) was taken out as an *Eco*RI fragment and cloned in pCAMBIA 1301 vector (**Fig. 16**).

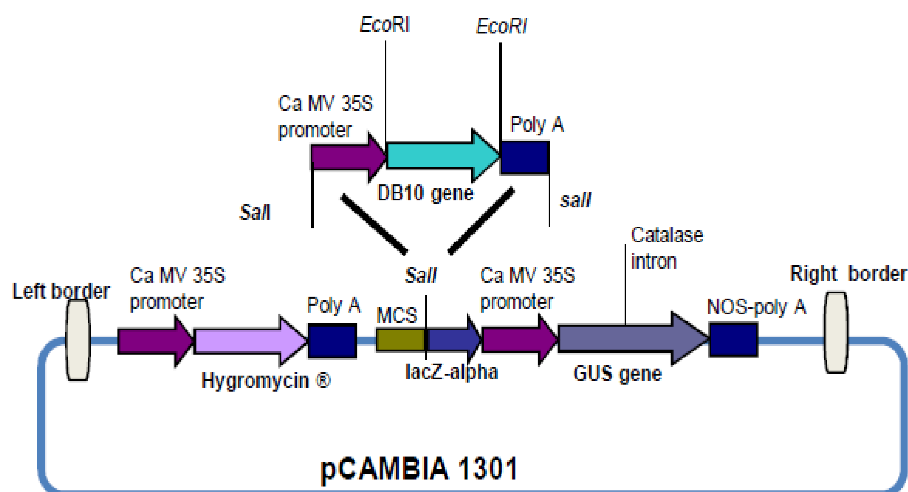


Fig. 20: Schematic representation of strategy used for cloning DB10 gene in the plant transformation vector of pCAMBIA 1301. The DB10 ORF was cloned as a SalI fragment into pRTl00 vector. Following which the entire cassette (35S promoter + Gene + Terminator) was taken out as an EcoRI fragment and cloned in pCAMBIA 1301 vector.

For rice transformation the standard protocol was followed by Hiei et al. (1994). The gene was cloned for vector control (VC = Vector control, without gene) sense and antisense (C1) and transformed to *indica* rice calli. We found greenish calli and plantlets in **Fig. 17 A-F**. Genomic DNA of transformants with p68 were extracted from the putatively transgenic plants and control untransformed plants using modified CTAB method and found that out of 17 plants two (02) showed positive results on transgenics (**Fig. 18**). Then the T0 plants were transferred to pots and grwon in greenhouse for molecular confirmation and for further resaerch on fertile transgenic development (**Fig. 19 A-D**).

The binary vector pCAMBIA1301 contained GUS as the reporter gene and under this study confirmation of integrity of calluses expression of β-glucuronidase (GUS) was done. The GUS reporter gene activity was determined by spectrofluorometric method according to the protocol described by Jefferson et al. (1987). Histochemical localization of GUS was

carried out by incubating the tissue samples overnight at 37°C in histochemical buffer. The GUS activity was found to be positive in nearly all the tissues examined, including roots, nodes, internodes, leaf, floret and young embryos of immature seeds of transgenic plants.

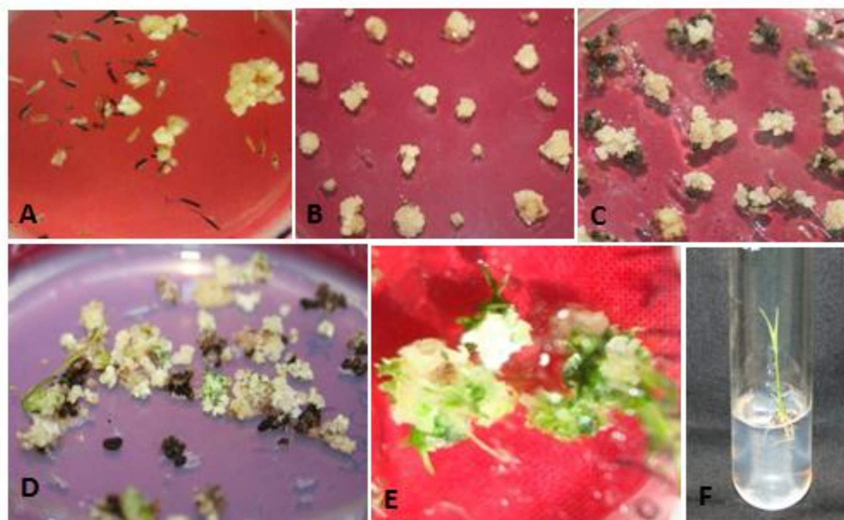


Fig. 21 (A-F): *Agrobacterium*-mediated genetic transformation in rice using anther and microspore derived embryoids (callus sub-culture for transformation work and regeneration of transgenic plants). A & B: Callus sub-culture when it was 3-4 weeks old to the induction medium, C) *Agrobacterium*-mediated transformation and infected callus were transferred to the selection medium in addition with Hygro-H, D) Hygromycin resistant plants are growing in medium, E) Green plantlets and F) Transgenic plants with good root and shoots.

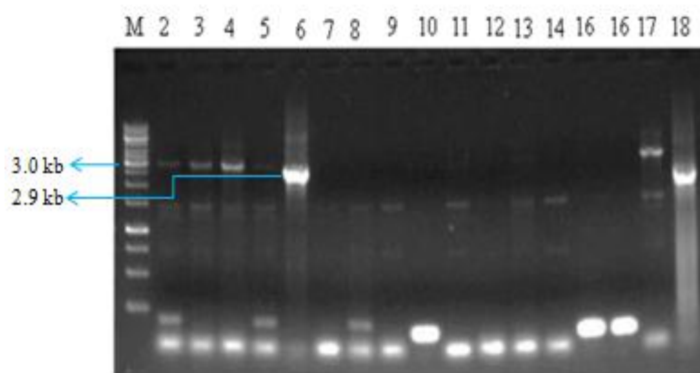


Fig. 22: PCR analysis of genomic DNA. DNA isolation from T0 plants and amplified with gene specific primers (DB10 F & R). Out of 17 plants only two showed positive results (Lane 6 and 18). Lane 1- 1 kb Ladder. Lane 6&1S: Transgenic with p6S gene, Lane 2-5: and 7-17: Non- transgenic.

Fig. 23 (A-D): PCR confirmation plants were transferred to pot after hardening. A) Transgenic plants in hardening (*p68* gene) stage before transferring to field greenhouse, B) Plants were transferred to pots, C) Transgenic plants were grown in ICGEB greenhouse (New Delhi, India).

Under this study leaf disc assay for both transgenic and non-transgenics were performed following the method described by Tuteja (2010). The presence of the transgenic and its effect was tested by the experiment on leaf disk senescence (LDS) using 200 mM salt. During the assay, the pieces leaf disc of wild type BRRI dhan29 plant (Control) showed dark brown stripes with necrotic tissues after 7 days immersed in 200 mM NaCl level **(Fig. 20)**. On the other hand the leaf of the transgenic plants remained greeninsh and healthy in comparison with control. Therefore, the transgenic plants might be carried the targeted gene while they were been able to hold the chlorophyll or other pigments into their mesophyll tissues and showed tolerance to salt stress (**Fig. 20**). Plants tolerant to drought and salinity implemented a series of adaptations to morphological, physiological and biochemical changes and these changes include increases in the root ratio and in the chlorophyll content in addition to changes in the leaf anatomy that ultimately lead to preventing leaf ion toxicity, thus maintaining the water status in order to limit water loss and protect the photosynthesis process. Under this study transgenic and control plants obtained from anther and microspore derived calli were evaluated on the basis of root production. It was observed that uprooting plants of transgenic showed very compact roots while the Control plants showed very loose type of roots (**Fig. 21 a-b**). It seems that the plants may able to show tolerance in drought in rice.

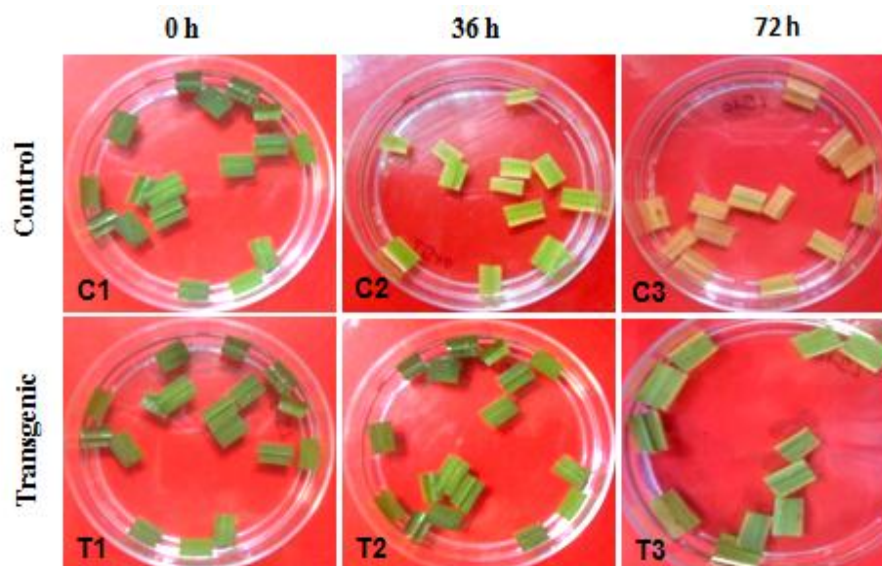


Fig. 24: Leaf disk senescence assav with 200 mM NaCl of T0 plants. Control (C1 -C3): Control plants were treated with 200 mM for 0 h, 36 h and 72 h. Transgenics (T0: T1-T3) were also treated with 200 mM for 0 h; 36 h and 72 h.

Fig. 25 (A-B): Morphology of drought stress tolerance in rice. A) Less number of root showing drought sensitive plants, and B) Compact root showing phenotypically stress tolerance.

Under this study plants obtained from anther or microspore derived embryoids were checked by flow cytometry (**Fig. 22 A-B**) and haploids (n) plants were pre-treated by colchicine. For chromosome doubling colchicines was used for diplodization and fertile homozygous transgenic plants (2n) were grown in the pot/field for seed collection. For chromosome doubling regenerated haploid plants (after flow cytometry screening) with good root were immersed in colchine and DMSO (dimethylsulfoxide) and transferred to soil.

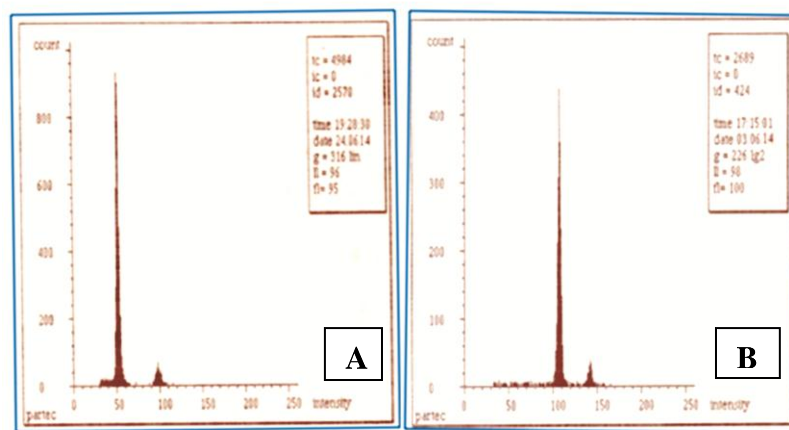


Fig. 26 (A-B): Transgenic (T0) haploid (n) and doubled haploids (2n) plants (anther & microspore derive embiyoids were used as explants sources for mediated genetic transformation). A. Flow cytometric analysis of haploid (n) and B. Doubled haploid homozygous (2n) plants.

6.5 Discussion

The *in vitro* production of doubled haploids through androgenesis (anther & microspore culture) is an efficient system for production of fully homozygous plants rapidly. Traditionally, plant breeders can achieve homozygosity by using self-fertilization or backcrossing, a time consuming process. Significant advantage is that the system is not only speed up the advance to homozygosity, but also to increase selection efficiency. We have identified the elite genotypes that showed good performance to gametic embryogenesis, and a protocol has been standarized for developing drought and/or salinity

stress tolerant transgenic rice cultivars using *p68* (DB10) gene. This work is a combination of *in vitro* androgenesis and *Agrobacterium*-mediated gene transfer for the production of drought and/or salinity tolerant rice cultivars. For anther culture of rice regeneration potentials the BRRI dhan29 was assessed on the basis of anther response, embryo induction, plantlet regeneration and production of green and albino plantlets. Embryoids were obtained from BRRI dhan29 on media containing specific amino acids and different combination of phytohormones in SK3 medium. It was observed that *Agrobacterium*-mediated transformation here out of 17 plants only two showed positive response on putatively transgenic plants were regenerated which analyzed by PCR. Those plants were grown and the transgenics (T_0) showed tolerance to NaCl stress at 200 mM level when LDS was tested. Hence, the plants would be considered for further molecular analysis related to gene expression. However, the standardized protocol of plant transformation for BRRI dhan29 may helpful to advance biotechnological research to develop highly efficient transgenics with expected agronomical traits. Because of time limitation, the advance related experiments couldn't be conducted in this study. Further works however, would be taken based on the present investigations.

Chauhan and Khurana (2011) used of doubled haploid technology for development of stable drought tolerant bread wheat through *Agrobacterium*-mediated genetic transformation. They used haploids embryoids for their studies and found to be extremely responsive to T-DNA delivery as indicated by transient GUS assay. Holiloglu et al. (2004) also reported wheat anther culture derived embryo transformation via electroporation; however, only albino plants were regenerated on the selection medium. In the present study, *Agrobacterium*-mediated transformation was attempted and regeneration of stable green haploid plants obtained that confirmed by flow cytometry analysis. Then plants were grown in potted soil, and genomic DNA extracted from the leaf tissue. PCR analyses of the T1 transgenic DH plants revealed the presence of transgene in the treated but not in control plants. The homozygosity was achieved in the first generation, and future generations were meant only for sufficient seed production for stress tolerance analysis. In a previous study,

Massiah et al. (2001) found that transgene inheritance in haploid plants followed the pattern of normal seed–derived transgenic plants. The present study was conducted to use anther and microspore derived embryoids from BRRI dhan29. This type of attempts was done first using anther and microspore derived embryoids and standardized a suitable protocol. Some more attempts using other varieties also needed for further investigation and molecular and ploidy confirmation of transgenic plants. That procedure will be speeding up the breeding technology for rapid development of fertile transgenic plants.

Under this study we have successfully standardized the anther and isolated microspore culture procedure, optimized media and other culture conditions. That is very important part for success for doubled haploid production. We have optimized the transformation protocol with our limitations and rice transgenic has been development. Under this study we have standardized the transformation protocol and rapid development of drought and salinity stress tolerant DHs transgenics that is very important to develop transgenic homozygous lines shortly to mitigate the challenges of climate change for sustainable agriculture.

Chapter VII
General Discussion

7. General Discussion

Sustainable agricultural development for food and environmental security is not achievable without new technologies such as biotechnology and genetic engineering. *In vitro* techniques like embryo culture (mature and immature) and gametic embryogenesis (DHs production) are very efficient tools for crop improvement. Somatic or gametic cells are able to differentiate into embryos *in vitro* by the application of plant growth regulators or various physical and chemical stress pre-treatment factors. The *in vitro* somatic embryogenesis is the process where plant embryos developed from somatic cells (George et al. 2007). Major advances of somatic embryogenesis in plants are- clonal propagation, elimination viruses causing diseases, suitable explants source for advance biotechnological research, plants can be developed from single cells like protoplasts and synthetic seed technology.

The *in vitro* production of doubled haploids (DHs) through androgenesis is an efficient system for rapid development of fully homozygous plants. Significant advantage is that the system is not only speed up the advance to homozygosity, but also to increase the selection efficiency. Doubled haploid plants are genetically normal and phenotypically stable. Different abiotic stresses including drought and high salinity showed adverse effects on the growth of plants and the productivity of crops, thus resulting in significant economic losses worldwide. Minimizing these losses is a major concern for all countries by suitable biotechnological and breeding techniques. Under these study biotechnological approaches such as somatic embryogenesis, androgenesis and transgenic techniques have been employed to develop stress tolerance in rice. Various abiotic stresses (physical and chemical) such as cold, heat, drought, starvation, salt, colchicine were applied for androgenetic improvement for both anther and isolated microspore culture. Here an important strategy have been undertaken also to develop homozygous fertile transgenic plants rapidly with enhance tolerant to drought and salinity by *Agrobacterium*-mediated genetic transformation system.

Experiments on somatic embryogenesis (**Section 3.0**) using mature seeds of a total number of twenty indica rice varieties and two local varieties were cultured in four media *viz*. MS, N6, SK-3 and B5 to evaluate their efficiency on callus induction and regeneration. The studied rice genotypes were selected into three major groups such as Boro, Aus and Aman where the Aus group gave maximum callusing. Out of four media N6 was the best for callus induction (78.67%) in BRRI dhan29. The medium B5 performed with the lowest value of callusing (37.33%) for BRRI dhan56. Aananthi et al. (2010) tested two basal media (MS and N6) for callus induction using five indica rice cultivars *viz.* ASD 16, White Ponni, Pusa Basmati-1, Pusa Sugandh-4 and Pusa Sugandh-5 where they obtained highest callus induction in White Ponni (88.75%) with MS (2.0 mg/l 2,4-D) and Pusa Basmati-1 (84.97%) also in MS + 2.5 mg/l 2,4-D. They described the MS performed better than N6 medium. In this case almost varieties showed better callusing in N6 medium.

To examine the effect of plant growth regulators on regeneration, six (T_1-T_6) different hormonal concentration and combinations were added to the N6 medium (**Section 4.1.2.2.2**). It was observed that the genotype BRRI dhan29 gave highest plant regeneration (72%) with BAP (2 mg/l) + Kin (1 mg/l) + NAA (0.5 mg/l). Four basal media such as, MS, ½MS, N6 and RRM were tested to evaluate their efficiency on rooting. The medium RRM showed maximum rooting (64-90.67%) for BRRI dhan29 and the Kalijira genotype was worst in N6. In a previous report Evangelista (2009) found 26.10% well developed root in half MS medium from LX278 as an elite line of japonica × indica cross. Alam et al. (2012) reported the highest number of roots for BRRI dhan29 (3.66) and lowest for BINA dhan7 (3.11) in ½MS medium. From our findings it may be concluded that for further experiments is required to increase the regeneration ability of BRRI dhan24, BRRI dhan29 and BRRI dhan42 that would be very much helpful for advance research in the field of biotechnology and genetic engineering for rice improvement in Bangladesh.

To evaluate the effect of genotypes in anther culture responses (**Section 5.1.1**) 23 rice cultivars from BRRI and IRRI and two local cultivars were cultured in four induction media (FHG, AMS3, N6 and SK3). It was observed that among the responding genotypes BRRI dhan29 (22.36%), BRRI dhan30 (27.85%) and IR-43 (47.07%) showed better results

on embryoids induction in SK3 medium. All of the genotypes showed more or less (0.94-24.59%) albino plants. The success in anther culture depends on first genotype and then duration of cold pre-treatment, culture medium and plant growth regulators (PGRs) was reported by Ponitka and Ślusarkiewicz-Jarzina (2007), Niroula and Bimb (2009) and Kahrizi et al. (2011). It appears that optimum culture medium and pre-treatment factors vary from genotype to genotype. Here we found almost the tested genotypes showed more or less callusing in SK3 medium. May be the differences comes genotypes sources, media composition, PGRs and culture condition that was not same with other findings reported by some other scientist.

Four different types of induction media *viz*. FHG, AMS3, N6 and SK3 were tested and found their effect on callus induction in **Section 5.1.2**. For plant tissue culture, MS medium is widely used for major cereal crops to anther culture. Under this study AMS3 used as induction medium that modification with MS (Islam 2000). Chu et al. (1975) reported a suitable medium (N6) for rice anther culture. It was observed that N6 medium gave better responses for embryoids induction than AMS3 and FHG. The present results agreed well with the finding of Chu et al. (1975). Ogawa et al. (1995) mentioned that amino acids induced a higher degree of plantlet regeneration than the medium containing alanine or no amino acid. Raina (1997) reported that in addition of maltose to the induction medium enhanced plants regeneration in rice.

Albino plant production in cereal anther and microspore culture is a great problem and till it has no agronomic value. The **Section 5.1.3** was conducted for a suitable regeneration system and reducing albino plant production by early transferring methods. In this case anthers were transferred with or without embryo like structures (ELS) to regeneration medium (SK3) with different time points (5-25 days). Results indicated that transfer of anthers with or without ELS from induction to regeneration medium posed a significant impact on improving regeneration potentiality and reducing albinisms. Inoculated anthers from liquid induction to regeneration medium within 10-15 days showed significantly higher percentages of green and less number of albino plants in this study. In a previous report early transfer of maize anthers (21 days of inoculation) with or without ELS to

regeneration medium gave promising results on induction and regeneration (Barloy and Beckert 1993, Saisingtong 1998). The present results agreed well with the previous findings done by Barloy and Beckert (1993) and Saisingtong (1998). Barloy and Beckert (1993) mentioned that quality of embryos might influences regeneration and reported that embryos quality might also be modified by media composition and timing of anther transferring methods.

For isolated microspore culture procedure (**Section 5.2**) various isolation procedures were conducted for rice. 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 an important technique (Coumans and Zhang 1995, Ferrie and Caswell 2011). Under this study for suitable microspore isolation procedures was conducted using six isolation methods (**Section 5.2.1**). Six rice genotypes (BR-4, BR-11, BRRI dhan29, IR-43, IR-54 and Kaljira) was considered for this study. Out of six rice cultivars IR-43 (71.71%), BRRI dhan29 (61.29%) and BR-11 (53.29%) showed best performance on embryoids induction. For green plant regeneration BR4 (32.66%) and IR-43 (30.15%) showed best results than other cultivars. Less number of albino plants (9.58%) was recorded for BR4. For microspore isolation procedure glass rod was much easier and simplest where 65-123% of embryoids obtained from 100 anthers. Highest percentage of embryoids (177%) was recorded in P_4 where anthers were squeezed by homogenizer. The system for homogenizer (polytron) and blending isolation techniques gave highest embryo yield than others. Microspore isolation by homogenizer and blending also gave quite similar results with the system reported by Olsen (1991). Regeneration from microspores obtained by microblending was described for wheat (Mejza et al. 1993), barley (Olsen 1991) and maize (Pescitelli et al. 1990). Gustafson et al. (1995) compared four microspore isolation methods e.g. blending, stirring, maceration and floating and obtained highest initial microspore viability (75%) with blending isolation technique. They reported that blender isolation without mannitol conditioning and an initial density 2×10^5 microspore ml^{-1} was best for continued microspore viability. Under this study microspore isolation by sterile glass rod and homogenizer showed quite better performances for reducing albino plants compared with control and other microspore isolation procedures.

May be some physiological changes happen during slow microspore isolation procedure and it may be concluded that for high embryo yield and reducing albinisms in microspore isolation by homozeginer and glass rods are better and more faster techniques.

Under this study harvested spikes were pre-treated by cold at 4°C chamber for 1-15 days (**Section 5.3.1.1**). It was observed that three days cold pre-treated spikes (T2) showed highest embryo yield (28.85%) than Control (17.83%). Highest percentages of regeneration (15.78%) also found in T2. We found that due to long duration of cold pre-treatment (11-15 days) green plant regeneration rate was decreased gradually. Here 3 days cold pre-treated showed less (2.91%) albino plant production and that is an important message for plant biotechnologist for further advance research in this area. On the basis of ELS, TRP and GRP, for overall mean value showed significantly higher in T_2 (19.19%), T_3 (16.56%) and T_4 (17.56%) than Control (10.92%). Bajaj (1983) reported that cold pre-treatment used to increase the frequency of androgenic responses for anther and isolated microspore culture. Lazar et al. (1985) reported that variation of intensity and duration of cold pre-treatment increased the embryoids yield and regeneration. Chaleff and Stolarz (1981) reported about the effect of cold treatment in rice and they found more androgenic response occurred when the anthers were pre-treated at 7°C for 3 days. Pan et al. (1975) showed the variation in intensity and duration in cold pre-treatment lead to significant differences for callus induction. Under this study 3 days cold pre-treatment showed highest embryoids yield as well as regeneration. These results agreed well with the previous report of Chaleff and Stolarz (1981) and Pan et al. (1975). Pande (1997) observed that cold pre-treatment was found to be essential for anther culture responses in Indica cv. IR-43. He also mentioned that with longer pre-treatment duration than 11 days showed more albino plant production. About albinism Torp and Andersen (2009) reported that for reducing the frequency of albino plant production avoid prolonged cold pre-treatment duration. Here we found that longer duration of cold pre-treatment (11-15 days) also showed increasing the number of albino plants. These results agreed well with the findings of Torp and Andersen (2009).

To determine the effect of drought stress pre-treatment factors in different durations (hrs) of drought stress pre-treatment was done to excised anthers and evaluated their responses on the basis of embryo yield and green plant regeneration (**Section 5.3.2**). Interesting results were obtained of simple exposure of drought stress pre-treatment of excised anthers brought about a remarkable progress in improving anther culture responses in rice. The drought stress pre-treatments varied from 2-12 hrs. Here T_2 (3 h) proved to be more suitable for embryo yield and green plant regeneration in rice. In case of albinism less number of plants (2.87%) was recorded with short duration of drought stress pre-treatment factors (2 h). Whereas increasing drought stress pre-treatments duration higher percentages of albino plants (T_3-T_6) were increased. There are some reports on successful application of abiotic stress pre-treatment for crop improvement (Maraschin et al. 2006, Shariatpanahi et al. 2006b, Seguí-Simarro and Nuez 2007, Ochatt et al. 2009, Żur et al. 2009). Zoriniants et al. (2005) reported that a variety of pre-treatments have been used such as applying as stress to the developing microspores at a critical stage, causing a block or delay in their development (Clément et al. 2005, Obert et al. 2009). Ben-Gal et al. (2009) mentioned that drought stress usually leads to oxidative stress due to stomatal closure which causes the over-reduction of photosynthetic electrons. Drought tolerance is a difficult trait to define as it encompasses a wide range of characteristics involving multiple genetic, physiological, cellular and biochemical strategies in the plant. The mechanisms underlying the stress-induced switch from gametophytic to sporophytic pathway during transition to the embryogenic state have been reported by Shariatpanahi et al. (2006a). Under this study drought stress has been considered as parallel to the thermal shock treatment. We found that drought exposure for a prolonged time gradually decreased the embryo yield as well as regeneration in rice anther culture. It is very interesting to note that drought stress for a longer duration caused to increase the albino plant production. It was observed that application of drought stress pre-treatment (3 h) to excised anthers prior to culture in liquid induction medium was very effective for embryoids induction as well as green plant regeneration in rice.

Combined effect with drought and heat stress pre-treatment was evaluated for anther culture responses in rice (**Section 5.3.3**). Here three hours drought with shorter duration of heat stress enhanced embryoids yield as well as green plant regeneration. In this case the highest embryo yield (38.99%) was recorded when excised anthers were pre-treated at 27°C for 1 hr. Touraev et al. (1996b) found that high temperature (33°C - 37°C) with starvation showed a positive effect on embryo yield and regeneration in tobacco microspore culture. Ho et al. (1978) obtained high embryo yield and plantlets in wheat anther culture following heat stress pre-treatment at 28°C - 32°C. Cao et al. (1994) provided three different temperature treatments, such as at 25°C (continuous) and 33°C for 1-3 days to *Brassica* anther culture and obtained highest embryo yield per bud at 33°C for one day. Here we found the combined effect with drought (3 hrs) + shorter duration of heat stress enhanced embryo yield as well as green plant regeneration in rice. Very effective results were also obtained with drought stress pre-treatment factors either single uses or in combination for both embryoids induction and regeneration. Results from the previous studies agreed well with the present findings in rice anther culture. But in this case the application of drought stress either single or in combination the methods were not similar. Previously some works reported their findings on tobacco and *Brassica* anther culture using stress effect. But here we applied those stress factors only for rice anther culture so why the results showed differences due to crop and application modes was not similar.

From gametophytic to sporophytic development to switch microspores some chemicals had a positive effect also on androgenesis responses in cereal and other crops are reported by many scientist. The effect of chemicals as stress pre-treatment factors to anther culture of rice was evaluated in **Section 5.3.4**. Three antimitotic agents such as azetidine, colchicine and pronamide were added to the induction medium. Here three different concentrations (100, 150 and 250 mg/l) of each agent was considered and cultured anthers were incubated at 26°C for three days in the induction medium. After antimitotic treatment duration's the explants (anthers) were re-suspended in fresh induction medium, and kept them in dark at 26°C around 3-4 weeks for embryos formation. It was observed that out of three dosages 150 mg/l azetidine showed highest embryo yield (30.51%) than others. For colchicine 150 mg/l also showed higher embryo yield (35.24%). Redha (1998) used

colchicine in the induction medium for 3 days and found higher percentage of doubled haploid (DH) plants in wheat anther culture. Direct application of colchicine to the induction medium increasing the frequency of fertile plants up to 76% in some cereals crops are reported by Obert and Barnabás (2004) and Redha et al. (1998). Under this study using colchicine also showed the most effective chemicals for chromosome doubling (6.99%) than azetidine (5.72%) and pronamide (4.34%). The overall mean performance 150 mg/l colchicine showed significantly higher embryo yield and regeneration (25.89%) and here the fertile plant was 7.95%. In the present investigation, it was observed that higher concentration (250 mg/l) of colchicine showed less doubling efficiency (15.07%) and increasing albino plants (7.41%). The present results showed quiet similar effect with the findings of Navarro-Alvarez et al. (1994). Here the variety, medium and culture conditions were different than the reported findings. The present evidence is also agreed well with the findings of Barnabás et al. (1991) and Hansen and Andersen (1998). Out of three antimitotic agents less number of doubling efficiency was recorded in pronamide (150 mg/l) that was nearly half in comparison with colchicine. As per the report of Vaughn and Vaughn (1987) mentioned that pronamide is less toxic than azetidine. Here we found higher embryo yield in azetidine than pronamide and that may happen for the effect of high toxicity. Wang et al. (1991) reported that oryzaline can be less phytotoxic but less effective than colchicine. The results on embryo yield azetidine (250 mg/l) showed better but for chromosome doubling efficiency colchicine showed best results to developed fertile plants that are very important for crop improvement.

Application of different chemical as stress pre-treatments factors may influences induction and regeneration efficiency in cereal and other crops. Three osmoticum agents as sorbitol, PEG and mannitol with different concentration were applied to anther and also isolated microspore culture of rice (**Section 5.3.5.2**). For each cases three concentrations (40 g/l, 60 g/l and 80 g/l) was considered. It was observed that out of three osmoticum agents mannitol (60 g/l) showed better results (41.07%) on embryoids induction. Less number of embryoids (15.99%) was recorded in PEG (80 g/l). All concentrations of mannitol showed better performance on embryo yield (33.20%) than sorbitol (29.46%) and PEG (18.59%). In case of albino plant production less number of plants (4.21%) was recorded in mannitol.

For overall mean value on the basis of ELS and GRP, 60 g/l mannitol showed best performance than others. They tested sugars and mannitol at different steps on anther culture in barley to elucidate their influence on both the overall embryo yield and the structure of plastids in relation to albinism. Wojnarowiez et al. (2004) found best results using mannitol (364 mOsm/kg) and found 139.7 percentages of green plants per 100 plated anthers in barley. Pre-treatment with mannitol showed significant progress for microspore development in wheat (Slama-Ayed et al. 2010), rice (Raina and Irfan 1998) and maize (Pescitelli et al. 1990). May be the embryo yield was different due to variation of crops, different osmoticum agents, dosages, application mode and culture conditions.

Another study (**Section 5.3.5.3**) was conducted with different concentration (0.2 mM - 0.7 mM) of mannitol and found that 0.3 and 0.4 mM mannitol showed better on embryo yield and regeneration. For Control, 90 g/l sucrose was used instead of mannitol and as induction medium semi-solid medium SK3 was used. Results indicated that 0.3 mM mannitol showed higher embryo yield (43.06%). We found increasing the mannitol concentration the embryo yield was decreased. ANOVA showed significantly higher embryo yield and regeneration with the effective dosages and its application in rice anther culture. Pre-treatment consisted of incubation of anthers containing mid-late to late uninucleate microspores in 0.37 M mannitol solution for 4 days in the dark, at 25°C (Hoekstra et al. 1992). Oleszczuk et al. (2006) found that the highest number of green plants was obtained after the treatment of anthers in 0.3 M mannitol at incubated them at 32°C for 24 h before microspore culture. In this case three days incubation period of cultured anthers in liquid medium gave better results. The incubation period and temperature pre-treatment duration were varied under this study.

Under this study an interesting experiment was conducted using liquid and semi-solid induction medium to evaluate their effectiveness on embryo yield and regeneration (**Section 5.3.5.4**). Here 3 days incubation period showed significantly higher embryo yield (42.8%) and regeneration (21.09%). For ELS and green plant regeneration T3 showed best performance than Control. This finding agreed well with Touraev et al. (2001) where they mentioned optimum incubation period in media containing non-metabolisable carbon sources, i.e. in mannitol containing media enhanced embryo yield and regeneration

efficiency. Under this study it was observed that increasing the anther incubation period albino plants were increased. Here we found three days incubation period was best for both induction and regeneration and it is a clear message for further advance research for rice improvement through anther culture. Evaluating the embryo yield and green plant regeneration for overall mean value, mannitol 60 g/l showed best performance (31.56%) than others. It is a clear message that the efficiency of mannitol for improvement of embryogenesis that directly affected on embryo yield and regeneration in rice. These results agreed well with the findings of Ogawa et al. (1994), Raina and Irfan (1998).

In another study 0.3 mM mannitol was added in the liquid induction medium (SK3) and the anthers were pre-treatment for 1-5 days (**Section 5.3.5.5**). After discarding the liquid medium anthers were transferred to the same semi-solid induction medium and first 7 days incubated them at 14°C chamber and then kept at 25±1°C for embryoids induction. The highest embryo yield (36.82%) was recorded in T_2 where 3 days pre-treatment was done. In comparison with both liquid and semi-solid induction medium along with mannitol pre-treatment to inoculated anthers semi-solid medium showed better performance on regeneration (23.83%). Here ELS and GRP for T_2 (3 d) which was nearly two and half fold higher than others. Analysis of variance showed highly significant differences in the frequency of embryo yield and regeneration for the stress pre-treatment factors of mannitol using semi-solid induction medium for different days at $p < 0.01$.

Different dosages of mannitol + heat pre-treatment at 30°C - 33°C for different hours to excised anthers were applied and evaluated the embryo yield and regeneration efficiency (**Section 5.3.5.6**). Here both temperatures showed increasing embryo yield and regeneration with 3 hrs incubation period. Temperature pre-treatment at 33°C showed the best results for embryo like structures (43.12%) and green plant regeneration (22.86%) that was 2-3 times higher than Control. Rukmini et al. (2013) reported that as heat pre-treatment at 33°C to 37°C for various durations showed increasing the embryo yield and regeneration. Under this study the heat pre-treatment duration was 3-12 h in addition 0.3 mM mannitol in medium. Oleszczuk et al. (2006) studies about the temperature effect at 32°C for 24 h + 0.3 mM mannitol pre-treatment and found regeneration efficiency were

increased in barley. Here regeneration efficiency was also increased with high temperature at 33°C for 3 h that was different with the study of Oleszczuk et al. (2006).

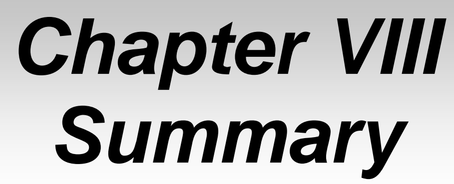
Production of doubled haploids through anther culture has major obstacle with low regeneration rate and albino plants. Isolated microspore culture is the preferred method for haploid production in cereal crops (Jähne and Lörz 1995). Under this study (**Section 5.3.5.7**) as osmoticum agent various doses of mannitol (0.2 M, 0.4 M, 0.6 M and 0.8 M) were added in the medium before microspore isolation. Here excised anthers were incubated in liquid induction medium for 2, 4 and 6 days at 25±1°C. It was observed that out of four concentration 4 d pre-treatment with 0.6 M mannitol showed nearly two fold higher embryo yield (140.33%) than Control (72.33%). We found higher dosages of mannitol (0.8 M) showed less embryo yield and regeneration and gradually the number was decreased (T_1 - T_3). For all cases more or less albino plants were recorded that was 13.67% to 26.67%. Here all dosages showed more or less embryoids induction and regeneration. But 4 days pre-treatment with 0.6 M mannitol showed higher than Control. Mannitol concentration at 0.4 M showed second heights results on ELS and GRP for all durations considered of this study. Oleszczuk et al. (2006) found that the highest number of green plants was obtained after the treatment of anthers in 0.3 M mannitol at incubated them at 32°C for 24 h before microspore culture. The present findings showed also best results when anthers were incubated with 0.4 M mannitol. For isolated microspore culture pre-treatment with mannitol showed significant progress for microspore development in wheat (Slama-Ayed et al. 2010), rice (Raina and Irfan 1998) and maize (Pescitelli et al. 1990). All of the previous reports showed mannitol had a great effect on callus induction and regeneration.

Another experiment (**Section 5.3.5.7**) was conducted where various concentration of mannitol (0.2 M - 0.8 M) was directly used in addition to the induction medium (AMC) for microspore culture. The effect of mannitol dosages was observed for 2, 4 and 6 days on the basis of embryo yield, green plant regeneration and albino plant production. It was observed that the incubation period 4 days of rice floret showed significantly higher embryo yield (163.67%) than Control (92.67%). Roberts-Oehlschlager and Dunwell (1990) found that four days incubation of barley anthers in a medium containing 3.2%

mannitol raised the pollen callusing response from 23%-78% which was more than cold treatment alone or in combination with mannitol. Raina and Irfan (1998) reported that treatment of anthers in 0.4 M mannitol solution was essential to induce androgenesis in microspore cultures of indica and japonica rice cultivars. These studies corroborate the findings of the present study.

Under this study (**Section VI**) the *p68* gene (DB10 for rice) was successfully transformed using anther and microspore derived-embryoids using Bangladeshi rice cultivars of BRRI dhan29. Here we identified the elite genotypes that showed good performance on androgenetic responses, and a suitable protocol has been standarized for developing drought and/or salinity stress tolerant transgenic rice cultivars. This work was in a combination of *in vitro* androgenesis and *Agrobacterium*-mediated gene transfer for the production of drought and/or salinity stress tolerant rice cultivars. For anther culture of rice regeneration potentials the BRRI dhan29 was assessed (**Section 5.1.1.3.1)** previously on the basis of anther response, embryo induction, plantlet regeneration and reducing albino plants. For successful transformation work DB10 gene was identified through *in silico* analysis. For this work the gene construction into pGEMT, pRT100 and pCAMBIA 1301 vectors were cross checked by PCR, restriction enzyme and sequence analysis for all stages are mentioned in **Section 6.2**. It was observed that out of 17 plants from anther and microspore derived embryoids where only two showed positive response on putatively transgenic plants. Those plants were analyzed by PCR with gene specific primers. Plants are grown and the transgenics (T_0) showed tolerance to NaCl stress at 200 mM level when LDSA-leaf disk senescence assay was tested. Hence, the plants would be considered for further molecular analysis related to gene expression. However, the standardized protocol of plant transformation for BRRI dhan29 may helpful to advance biotechnological research to develop highly efficient transgenics characterized of expected agronomical traits. Chauhan and Khurana (2011) used doubled haploids technology for development of stable drought tolerant bread wheat through *Agrobacterium*-mediated genetic transformation. They used haploids embryoids for their studies and found to be extremely responsive to T-DNA delivery as indicated by transient GUS assay. Holiloglu et al. (2004) also reported wheat anther culture derived embryo transformation via electroporation; however, only

albino plants were regenerated on the selection medium. In the present study, *Agrobacterium*-mediated transformation was attempted and regeneration of stable green haploid plants obtained that confirmed by flow cytometry analysis. Then plants were grown in potted soil, and genomic DNA extracted from the leaf tissue. PCR analyses of the T1 transgenic DH plants revealed the presence of transgene in the treated but not in control plants. The homozygosity was achieved in the first generation, and future generations were meant only for sufficient seed production for stress tolerance analysis. In a previous study, Massiah et al. (2001) found that transgene inheritance in haploid plants followed the pattern of normal seed–derived transgenic plants. In the present study conducted to use anther and microspore derived embryoids from BRRI dhan29. This type of attempts was done first using anther and microspore derived embryoids and standardized a suitable protocol. Some more attempts using other varieties also needed for further investigation and molecular and ploidy confirmation of transgenic plants. That procedure will be speeding up the breeding technology for rapid development of fertile transgenic plants. We have standardized also the transformation protocol and rapid development of drought and salinity stress tolerant DHs transgenics that is very important to develop transgenic homozygous lines shortly to mitigate the challenges of climate change for sustainable crop development.



Chapter VIII
Summary

8. Summary

Under these study biotechnological approaches such as somatic embryogenesis work has been done using mature and immature embryos derived from seeds, doubled haploid production through androgenesis (anther and microspore culture) and transgenic approaches have been conducted to standardize a suitable protocol and to develop plants for stress tolerance in rice. An important strategy have been undertaken to develop homozygous fertile transgenic plants rapidly that enhanced tolerant to drought and salinity. Here the targeted gene of DB10 was transferred using anther and microspore derived-embryoids with Bangladeshi rice cultivars. The work mainly focused on the following objectives- i) *in vitro* somatic and gametic embryogenesis for crop improvement, ii) application of various stress pre-treatments factors to enhance androgenetic responses and increasing doubled haploid plants in rice, iii) to develop highly efficient gametic embryogenic system of getting haploids (n) or doubled haploid (2n) plants through androgenesis using elite rice genotypes in Bangladesh, and iv) to develop anther and microspore derived embryoids for *Agrobacterium*-mediated transformation system in rice.

Present study **(Section 3.0)** provides a simple protocol for somatic embryogenesis to produce plants through *in vitro* with high frequency of callus induction and its subsequent regeneration system for indica rice genotypes. Mature seeds of a total number of twenty indica rice varieties were considered for this study and cultured them in four media *viz.* MS, N6, SK-3 and B5 to evaluate their efficiency on callus induction and regeneration. The studied rice genotypes were selected into three major groups such as Boro, Aus and Aman where the Aus group gave maximum production of callusing. Out of four media N6 was showed best callusing (78.67%) for BRRI dhan29. For callus induction six different concentration and combinations of plant growth regulators were tested where 3.0 mg/l 2,4-D + BAP 1.5 mg/l + Kin 1.0 mg/l in addition to the induction medium showed the most effective combination. The variety BRRI dhan29 showed supremacy on the others genotypes for both callusing and regenerating plants. Four basal media (MS, ½MS, N6 and RRM) were tested to evaluate their efficiency on rooting and found that RRM showed maximum rooting (64-90.67%) for BRRI dhan29 and Kalijira was worst in N6. Therefore,

for further experiments could be conducted to increase the regeneration ability of BRRI dhan29, BRRI dhan24, BRRI dhan42 would be very much helpful for advance research in the field of biotechnology and genetic engineering for crop improvement in Bangladesh.

We evaluated (**Section 5.1.1**) twenty three rice cultivars for their responses on anther culture. Four induction media (FHG, AMS3, N6 and SK3) were used and here we found almost genotypes showed more or less callusing in all media but SK3 showed better than others. Responses on embryos induction and regeneration the differences come may be on genotypes sources, media composition, PGRs and culture conditions. All of the genotypes showed more or less (0.94 - 24.59%) albino plants. It appears that optimum culture medium and pre-treatment factors may vary from genotype to genotype. For a suitable regeneration system and reducing albino plant production by early transferring methods with or without embryo like structures from liquid to semi-solid induction medium was used (**Section 5.1.3**). Results indicated that transfer of anthers with or without ELS from induction to regeneration medium posed a significant impact on improving regeneration potentiality and reducing albinisms. Inoculated anthers from liquid induction to regeneration medium within 10-15 days showed significantly higher green and less number of albino plants.

For suitable microspore isolation procedure (**Section 5.2.1**) it was conducted using six isolation procedures using also six rice genotypes. Out of six rice genotypes IR-43 (71.71%), BRRI dhan29 (61.29%) and BR-11 (53.29%) showed best performance on embryoids induction. For green plant regeneration BR4 (32.66%) and IR-43 (30.15%) showed best results than other cultivars. For microspore isolation procedure glass rod was much easier and simplest where 65-123% of embryoids were found from 100 anthers. Microspore isolation by sterile glass rod and homogenizer showed quite better performances for reducing albino plants than control. It might be conclude that for high embryo yield, improved regeneration efficiency and reducing albinisms in microspore isolation system by homozeginer and glass rods are showed better and faster techniques than others.

In **Section 5.3.1.1** harvested spikes were pre-treated by cold at 4°C chamber for 1-15 days. It was observed that three days (T2) cold pre-treatment to spikes prior to culture anthers showed highest embryo yield (28.85%) than Control (17.83%). Highest percentages of regeneration also found in T2. Long duration of cold pre-treatment (4°C) to harvested spikes showed worse response on ELS and green plant regeneration. Here 3 days cold pre-treated showed less albino plant production. Whereas increasing the cold-pretreatment duration albino plants were increased. It is an important findings and message for plant biotechnologist for further advance research in these areas. Besides cold pre-treatment another physical stress pre-treatment drought pre-treatment was also considered to improve androgentic responses in rice. To determine the effect of drought stress pre-treatment factors (**Section 5.3.2**) it was applied to excised anther with different durations (2-12 hrs). Evaluation procedures were done on the basis of their responses on embryo yield and green plant regeneration. Interesting results obtained of simple exposure of drought stress pre-treatment of excised anthers brought about a remarkable progress in improving anther culture responses in rice. Here three hours drought stress pre-treatment proved to be more suitable for embryo yield and green plant regeneration in rice. In case of albino plant production less number of plants was recorded with short duration (2 h) of drought stress pre-treatment factors. It is very interesting to note that drought stress for longer duration caused to increase the albino plant production. However, application of drought stress pre-treatment for three hours to excised anthers prior to culture in liquid induction medium was very effective for embryoids induction as well as green plant regeneration in rice. Combined effect with drought and heat stress pre-treatment was also applied to evaluate their efficiency on androgenetic responses in rice anther culture (**Section 5.3.3**). Interesting results obtained when three hours drought with shorter duration of heat stress applied to excised anthers that enhanced embryoids yield and green plant regeneration. In this case the highest embryo yield (38.99%) was recorded when excised anthers were pre-treated at 27°C for 1 hr. Very effective results obtained with drought stress pre-treatment application either single or combined with heat and that showed enhancement of embryo yield and regeneration in rice.

The effect of chemicals as stress pre-treatment factors to anther culture of rice was evaluated in **Section 5.3.4**. Three antimitotic agents *viz.* azetidine, colchicine and pronamide were considered in addition to the induction medium. Here three different concentrations (100, 150 and 250 mg/l) of each agent was considered and incubated the cultured anthers at 26°C for three days. It was observed that out of three dosages of azetidine 150 mg/l showed highest embryo yield (30.51%) than others. For colchicine 150 mg/l also showed higher embryo yield (35.24%) than other dosages. But the colchicine showed the most effective chemicals for chromosome doubling than azetidine and pronamide. The overall mean performance 150 mg/l colchicine showed significantly higher embryo yield and regeneration and here fertile plants were 7.95%. The results on embryo yield azetidine (250 mg/l) showed better but for chromosome doubling colchicine showed best results to developed fertile (2n) plants.

As chemical stress pre-treatments factors three osmoticum agents e.g sorbitol, PEG and mannitol with different dosages were applied to anther culture medium. For each cases three concentrations (40-80 g/l) was considered (**Section 5.3.5.2**). It was observed that out of three osmoticum agent's 60 g/l mannitol showed better results on embryoids induction. Less number of embryoids was recorded in PEG (80 g/l). All concentrations of mannitol showed better performance on embryo yield (33.20%) than sorbitol (29.46%) and PEG (18.59%). In case of albino plant production less number of plants was recorded in mannitol. For overall mean value on the basis of ELS and GRP, 60 g/l mannitol showed best performance than others. Application of different concentration of mannitol (0.2 mM - 0.7 M) for 3-5 days improved green plant regeneration significantly in **Section 5.3.5.3**. Here for Control, 90 g/l sucrose instead of mannitol and semi-solid medium SK-3 was used for induction. Results indicated that 0.3 mM mannitol showed highest embryo yield (43.06%). It was observed that increasing the mannitol concentration in medium the embryo yield was decreased. ANOVA showed significantly higher embryo yield and regeneration with the effective dosages and its application in rice anther culture. In this case three days incubation period of cultured anthers in liquid medium gave better results. However, the incubation period and pre-treatment duration with heat was not same and that was varied in this study.

An interesting experiment was conducted using liquid and semi-solid induction medium to evaluate their efficiency on embryo yield and regeneration (**Section 5.3.5.4**). It was observed that increasing the anther incubation period albino plants was increased. Here we found three days incubation period was best for both induction and regeneration and it is a clear message for further advance research to rice improvement through anther culture. Evaluating the embryo yield and green plant regeneration for overall mean value, mannitol 60 g/l showed best performance (31.56%) than others. In conclusion it is a clear message that the efficiency of mannitol for improvement of embryogenesis directly affected on embryo yield, green regeneration and reducing albino plants in rice.

Under this study (**Section 5.3.5.5**) 0.3 mM mannitol was added in the liquid induction medium (SK-3) and the anthers were pre-treatment for 1-5 days. The highest embryo yield (36.82%) was recorded in T_2 where 3 days pre-treatment was done. In comparison with both liquid and semi-solid induction medium along with mannitol pre-treatment to inoculated anthers semi-solid medium showed better performance on regeneration (23.83%). Analysis of variance showed highly significant differences in the frequency of embryo yield and regeneration for the stress pre-treatment factors of mannitol using semi-solid induction medium for different days at $p < 0.01$. Different dosages of mannitol + heat pre-treatment at 30°C - 33°C for different hours were applied to excised anthers and evaluated their efficiency on the basis embryo yield and regeneration (**Section 5.3.5.6**). Under this study the heat pre-treatment duration was 3-12 h in addition 0.3 mM mannitol in medium. Here both temperatures showed increasing embryo yield and regeneration with three hours incubation period. Temperature pre-treatment at 33°C showed the best results for embryo like structures (43.12%) and green plant regeneration (22.86%) that was 2-3 times higher than Control.

Production of doubled haploids through anther culture has major obstacle with low regeneration rate and albino plants. Isolated microspore culture is the preferred method for haploid production in cereal crops. Under this study as osmoticum agent various doses of mannitol (0.2 M, 0.4 M, 0.6 M and 0.8 M) were added in medium before microspore isolation (**Section 5.3.5.7**). Here excised anthers were incubated in liquid induction

medium for 2, 4 and 6 days at 25±1°C. It was observed that out of four concentration four days pre-treatment with 0.6 M mannitol showed nearly two fold higher embryo yield (140.33%) than Control (72.33%). We found higher dosages of mannitol (0.8 M) showed less embryo yield and regeneration and gradually the number was decreased (T_1 - T_3). For all cases more or less albino plants were recorded. Here all dosages showed more or less embryoids induction and regeneration. The present findings showed best results when anthers were incubated with 0.4 M mannitol before microspore isolation. Another experiment was conducted where various concentration of mannitol (0.2 M - 0.8 M) was directly used in addition to the induction medium (AMC) for microspore culture (**Section 5.3.5.7**). It was observed that the incubation period 4 days of rice floret showed significantly higher embryo yield (163.67%) than Control (92.67%).

In **Section VI**, the *p68* gene (DB10 for rice) was identified through *in silico* analysis and successfully cloned and the transformation procedures were done using anther and microspore derived-embryoids using Bangladeshi rice cultivars of BRRI dhan29. Here we identified the elite genotypes that showed good performance on androgenetic responses, and a suitable protocol has been standardized for developing drought and/or salinity stress tolerant transgenic rice cultivars. This work was in a combination of *in vitro* androgenesis and *Agrobacterium*-mediated gene transfer for the production of drought and/or salinity stress tolerant rice cultivars. It was observed that out of 17 plants from anther and microspore derived embryoids, where only two showed positive response on putatively transgenic plants. Those plants were analyzed by PCR with gene specific primers. Plants are grown and the transgenics (T_0) showed tolerance to NaCl stress at 200 mM level when LDSA-leaf disk senescence assay was tested. Hence, the plants would be considered for further molecular analysis related to gene expression. However, the standardized protocol of plant transformation for BRRI dhan29 may helpful to advance biotechnological research to develop highly efficient transgenics characterized of expected agronomical traits.

In the present study, *Agrobacterium*-mediated transformation was attempted and regeneration of stable green haploid plants obtained that confirmed by flow cytometry analysis. Then plants were grown in potted soil, and genomic DNA extracted from the leaf

tissue. PCR analyses of the T1 transgenic DH plants revealed the presence of transgene in the treated but not in control plants. This type of attempts was done first using anther and microspore derived embryoids and standardized a suitable protocol. Some more attempts using other varieties also needed for further investigation and molecular and ploidy confirmation of transgenic plants. That procedure will be speeding up the breeding technology for rapid development of fertile transgenic plants. Here we standardized the anther and isolated microspore culture procedure, optimized media and other culture conditions. That is very important part for success for doubled haploid production. We have standardized also the transformation protocol and rapid development of drought and salinity stress tolerant DHs transgenics that is also very important to develop transgenic homozygous lines shortly to mitigate the challenges of climate change for sustainable crop development.