HAPLOID PLANT REGENERATION THROUGH POLLEN CULTURE IN RICE (*Oryza sativa* L.)

BY
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CERTIFICATE

This is to certify that the thesis entitled, "HAPLOID PLANT REGENERATION THROUGH POLLEN CULTURE IN RICE (Oryza sativa L.)" submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of ***MASTER OF SCIENCE in GENETICS AND PLANT BREEDING,*** *embodies the result of a piece of bona fide research work carried out by* ***Mst. Shaila Begum,*** *Registration No.* ***06-02117*** *under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged by him.

11/03/2014

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Place: Dhaka, Bangladesh

DEDICATED TO MY BELOVED PARENTS AND FAMILY MEMBERS

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SOME COMMONLY USED ABBREVIATIONS AND SYMBOLS

The following abbreviations have been used in this thesis

Abbreviations	Full word
^0C	Degree Celcius
%	Percentage
1N	1 Normal
2, 4-D	2, 4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
AC	Activated charcoal
a.i	Active ingredient
Agril.	Agriculture
BA	Benzyl adenine
BAP	Benzyl Amino Purine
BBS	Bangladesh Bureau of Statistics
BR	Bangladesh Rice
BRRI	Bangladesh Rice Research Institute
Bt	*Bacillus thuringiensis*
BAU	Bangladesh Agricultural University
Cont'd	Continued
CRD	Completely Randomized Design
Cm	Centimeter
cv.	Cultivar
DAI	Days after inoculation
DDW	Double distilled water
DH	Double Haploid
DNA	Dioxyribo Nucleic Acid
DW	Distilled water
et al.	And others
etc.	Etcetera
F_1	First filial offspring
FAO	Food and Agricultural Organization
g	Gram
g/l	Gram per liter
GA_3	Gibberellic Acid
Gca	General combining ability
ha	Hectare
h	Hours
$HgCl_2$	Mercuric Chloride
IAA	Indole-3-Acetic Acid
IBA	Indole-3-Buteric Acid
i.e	id test (That is)
Interl.	International
IR	International Rice
IRRI	International Rice Research Institute
J.	Journal
kg/cm^2	Kilogram per square centimeter

SOME COMMONLY USED ABBREVIATIONS AND SYMBOLS (Cont'd)

Abbreviations	Full word
KI	Kinetin
LS	Linsmaier and Skoog
LSD	Least Significant Difference Test
mg	Milligram (s)
mg/l	Milligram per liter
ml	Milliliter
MS	Murashige and Skoog
Mt	Metric tons
µM	Micro mole
µm	Micro meter
n	Haploid number
N_6	Nitsch medium
NAA	Napthalene Acetic Acid
NaOH	Sodium Hydroxide
NL	Nitsch and Linsmaier medium
No.	Number
PAA	Phenyl Acetic Acid
PMC	Pollen mother cell
ppm	Parts per million
p^H	Negative logarithm of hydrogen ion concentration ($- \log[H^+]$)
Phys	Physiological
Plnt.	Plantarum
SAU	Sher-e-Bangla Agricultural University
sca	Specific combining ability
Sci.	Science
SDH	Spontaneous doubled haploids
Univ.	University
UV	Ultra violet
Viz.	Namely
v/v	Volume/Volume

HAPLOID PLANT REGENERATION THROUGH POLLEN CULTURE IN RICE (*Oryza sativa* L.)

BY

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ABSTRACT

An experiment was conducted at Biotechnology Laboratory, Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Sher-e- Bangla Nagar, Dhaka-1207, during the period from July 2011 to December 2012 to generate haploid plants of rice (*Oryza sativa* L.) through pollen culture. Pollens or anthers of F_1 hybrids obtained from the crosses of BR 21 x BRRI dhan28 and BR 26 x BRRI dhan29 were used as explant. Murashige and Skoog (MS) medium supplemented with eight hormonal combinations were used as treatment to identify the best hormonal combination for callus induction and plantlet regeneration. The minimum time (21.33 days) were required for callus induction in both F_1 hybrids on MS medium supplemented with 2.0 mg/l NAA + 2.0 mg/l 2,4-D + 0.5 mg/l KI. The highest callus induction frequency (13.63%) was obtained from BR 26 x BRRI dhan29 in same hormonal combination but the lowest callus induction frequency (1.11%) was recorded from same F_1 hybrid at 1.5 mg/l NAA. It was noticed that the genotype BR 26 x BRRI dhan29 showed the minimum time (21.67 days) for green plantlet regeneration. The maximum (40.60%) green plantlet was regenerated in F_1 hybrid of BR 26 x BRRI dhan29 on MS medium supplemented with 2.0 mg/l IBA + 2.0 mg/l BAP + 2.0 mg/l GA_3. But both F_1 hybrids did not produce any green plantlets at 1.0 mg/l BAP + 1.0 mg/l IAA hormonal doses. Green plants were transferred in pots with 55.55% survival rate. Present study suggested that BR 26 x BRRI dhan29 was a good F_1 hybrid for further studies.

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Chapter I

Introduction

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CHAPTER I

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most versatile and important cereal crops of Poaceae family cultivated for more than 10,000 years (Sasaki, 2005). It is a self-pollinated cereal crop having chromosome number 2n=2x=24 under the order Cyperales and class Monocotyledon. It is the world's most important food crop after wheat and maize. It is the staple food for 60% of the world population. More than 90% of rice is produced and consumed in Asia. It is grown under a wide range of agroclimatic conditions. Bangladesh is an over populated country and population is increasing day by day at rapid rate. The present population 145 million will increase to 233 million by 2030 requiring 48 million tonnes of extra food grain by that year. With increasing population pressure, the demand of cereal is increasing day by day. Rice is considered a major food crop in Bangladesh. Bangladesh ranks fourth among the rice producing countries of the world. At present, the rice growing areas are gradually decreased due to industrialization, housing and expansion of urban areas. Projected demand of the next 30 years clearly indicates the need for sustainable increase in production of rice and to meet the challenge of 21st century rice varieties are needed with higher yield potential and multiple resistances to diseases, insects and resistance to abiotic stresses. It is not possible to increase the production of rice horizontally due to lack of land. So we can improve the production of rice vertically. Production of rice will increase by proper using the land under three rice growing seasons (Aus, Aman and Boro). A considerable improvement has been done through traditional rice breeding in the past and even in the future. Bangladesh where 1,57,51,828 metric tons of rice is produced with average yield around 3.86 mt/ha; whereas world rice production 486 million tonnes and 441 million tonnes in Asia (BBS, 2013; FAO, 2012). This result indicates that average per hectare production of rice in Bangladesh is extremely low compared to other rice growing countries of the world. So there is no alternative to increase the yield of rice to fulfill the future national demand. Recent advances in cellular and molecular biology and rice biotechnology have produced new tools to increase the efficiency of rice production (Khush and Brar, 1998).

Application of pollen culture technique will provide a new avenue to us to develop and improve rice varieties for meeting the challenges of increase rice production. This allows rapid production of homozygous haploid lines from F_1 hybrids and incorporation of new genes into breeding materials. In China, a large number of rice varieties have been developed through anther culture and released for cultivation over several thousand hectares (Chen, 1986). However, number of green plants obtained through anther culture did not meet demand of practical exploitation through this technique for genetic improvement (Zhu *et al.*, 1990). Several influencing factors on anther culture have been studied such as genotype of explants (Shen *et al.*, 1982; Li, 1991), growth condition of donor plants (Chen, 1988), culture methods (Qu and Chen, 1983b) etc. Though different protocols have been proposed to improve efficiency of anther culture, the green plant regeneration frequencies still remained rather low, especially for indica cultivars and aromatic rice.

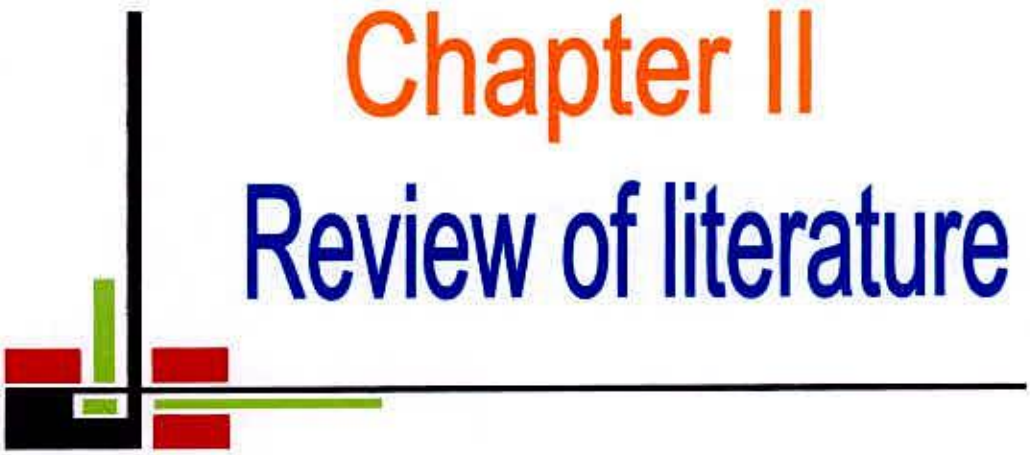
The development of pollen culture techniques for production of rice was a major advance in the field of rice breeding in the last few decades and pollen culture is one of the most intensively investigated areas of the *in vitro* culture methods of rice as haploid approach can effectively reduce the time required for varietal improvement (Herath and Bandara, 2011). The use of anther culture has been limited by many factors (Afza *et al.*, 2000). The production of callus and *in vitro* conservation of haploid cells might be considered as an option to extend the period of use of haploid culture, but when sub cultured, cells undergo various chromosomal and ploidy changes that may result in genetic changes in the cultures (D'Amato, 1977).

Several scientists (Niizeki and Oono, 1968; Hu Han, 1978; Wang *et al.*, 1979; Hakim *et al.*, 1991) have successfully produced callus and plants from anther cultures and isolated pollen. Several attempts for anther culture of local indica rice germplasm have also met with limited success (Kumari *et al.*, 2006; Silva and Achala, 2008; Rathieka and Silva, 2007). In rice production of haploid through anther culture has major obstacle with low regeneration rate and albino production (Lentini *et al.*, 1995). Many factors that affect the frequency of callus formation and green plant regeneration from *in vitro* anther culture of

rice. Among them composition of culture media, pretreatment of panicles, anther condition, genotype, growing conditions of the donor plants and development stage of microspores, effect of hormonal treatment (Yamada *et al.*, 1967; Chaleff and Stolarz, 1981; Pande and Bhojwani, 1999; Afza *et al.*, 2000; Sharmin and Bari, 2001; Trejo-Tapia *et al.*, 2002; Jacquard *et al.*, 2006; Cha-Um *et al.*, 2009.) are important.

Hence, the study was carried out to investigate the following objectives-

- To identify the best hormonal combination for pollen culture in rice,
- To establish haploid plant regeneration protocol in rice and
- To regenerate haploid plants from anthers or/ and microspores through *in vitro* culture.



Chapter II
Review of literature

CHAPTER II

REVIEW OF LITERATURE

Conventional techniques of crop improvement are lengthy and limiting for the crops. The technique of plant tissue cultures have been developed as a new powerful tool for crop improvement (Dorosiev, 1986; Razdan and Cocking, 1981; Carlson, 1975) and emphasized wide attention of modern scientists (Skirvin, 1978). Rice is the leading cereal crop in the world. Many studies on production of haploid rice plants have been carried out by many researchers in various countries of the world, but very few significant works have been yet done in Bangladesh. However, in recent time there has been rising consciousness among the researchers in our country to study about haploid rice plants. Some important studies those were conducted on production of haploid rice plants through different culture media are mentioned below-

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2.1 Concept of anther and pollen culture

A haploid is a plant with the gametic or *n* number of chromosomes. Androgenesis is the process by which haploid plants develop from the male gametophyte. When anthers are cultured intact, the procedure is called anther culture. Microspore culture involves isolating microspores from anthers before culture and is sometimes referred to as pollen culture.

The purpose of anther and pollen culture is the production of haploid plants by the induction of embryogenesis from repeated divisions of microspores or immature pollen grains (Dodds and Roberts, 1985).

Improved methods have been developed for anther and pollen culture by Gill *et al.* (2003) from diverse genotypes, including basmati and tropical japonica types, high-yielding indica x new plant type and *Bt* basmati. In general, the use of N_6 medium supplemented with 2,4-D (2.5 mg/l), proline (560 mg/l) and maltose (30 g/l) has resulted in better callusing. The use of antibiotics cefotaxime (500 mg/l) and cysteine (25 mg/l) induced pollen embryogenesis in some cases. Embedding of anthers in agarose was better

than culturing of anthers on semisolid medium or in liquid medium. Likewise, culturing of pollen on the filter membrane, overlying rice feeder cells or embedding of isolated pollen in agarose has resulted in a significant increase in the number of pollen calli in Hsinchu 64 and IR-65598-112-2. These methods are now being employed for producing homozygous *Bt* basmati through anther/pollen culture from transgenic basmati (T_1 plants) carrying *cry1A*(c).

Chowdhury and Mandal (2001) estimated on microscopic embryogenesis and fertile planet regeneration in a salt susceptible X salt tolerant rice hybrid. Shed microspore embryogenesis and fertile plantlet regeneration are observed in a salt susceptible X salt tolerant indica rice F_1 hybrid involving IR24 and CRM30. The *in vitro* culture response and regeneration of green plantlets in the hybrid are superior to those of the parents. Direct embryogenesis and plantlet regeneration with multiple tillers are observed in Shed microspore embryos. In intact anther culture, plantlet development from microspore involves a callus phase. The number of multiple tillers develop through secondary embryogenesis is almost equal in both the cases. However, the results indicate that regeneration of green plantlets was higher in case of Shed microspore culture in liquid medium containing the synthetic polymer Fi coll 400 than from intact anthers cultured on a semi-solid system.

Cho and Zapata (1990) conducted an experiment on isolated microspores of rice (*Oryza sativa* L.) cultivars IR 36 and IR 43, belonging to the recalcitrant indica subspecies were cultured. Two types of microspores were observed after isolation from the fresh anthers and from pre-cultured anthers one type consisted of vacuolated, larger sized grains, while the other was composed of microspores of smaller sizes with dense cytoplasm. Within few days in culture, all the smaller sized grains were dead and only the large grains were viable and produced pollen embryos. After 30 days from culture, micro calli were transferred to semisolid modified Murashige and Skoog medium containing 1 mg/liter each of kinetin and naphthalene acetic acid and kept under continuous light at 25°C. IR36 showed only cell division while IR43 gave 32 green plants from these experiments.

2.2 Callus induction and plant regeneration

A callus is an amorphous mass of loosely arranged thin-walled parenchymatous cells arising from the proliferating cells of parent tissue (Dodds and Roberts, 1990). Plant regeneration ability of cells of rice was closely correlated with water status of callus. Several researchers reported successful callus induction from different explants of various rice genotypes and plant regeneration in different combinations of growth regulators. In rice, plant regeneration capacity is affected by genotype (Seraj *et al.*, 1997; Abe and Futsuhara, 1986), age and plant types of explants (Haque and Mansfield, 2004), nutrient media such as basal media (Lin and Zhang, 2005; Lee *et al.*, 2002; Khanna and Raina, 1998), plant growth regulators (Lee *et al.*, 2002; Pons *et al.*, 2000) and culture conditions. Numerous efforts have been made to improve plant regeneration capacity of rice callus with varying degree of success. The most relevant literatures to callus induction plant regeneration is reviewed here:

2.2.1 Effect of genotype variability

The culturability of rice anther greatly differed among genotype, physiological status of donor plants, anther wall, pollen stage etc. In rice sufficient literature has now accumulated on various aspects of the genotypic variation. The genotype of the donor plant may be the most important factor in callus induction in rice. The success of crop improvement program depends on the extent of genotypic variability in *In vitro* culture, which helps to augments the new released varieties. The most important relevant literature is reviewed below:

There are occasions in which genotypes that show high callus induction have displayed poor regeneration ability and vice versa (Talebi *et al.*, 2007; He *et al.*, 1998).

Callus induction and plant regeneration are considered as two distinct phases in the anther culture process of rice. Also it has been reported that the rice cultivars that display high callusing ability show the best regeneration frequencies (Javed *et al.*, 2007; Shahnewaz *et al.*, 2003; Yan *et al.* 1996).

Carsono and Yoshida (2006a) reported that genotypes significantly differed in inducing high quality of calluses depending on medium and explants used.

Carsono and Yoshida (2006b) studied that Fatmawati, Ciapus, BP-23 and BP-360-3 genotypes would be useful for tissue-culture based research and for crop improvement, particularly for genetic transformation.

In vitro regeneration through anther culture was studied by Lannes *et al.* (2004) in two backcross populations of rice. Some 200 immature anthers from each genotype were inoculated into liquid NL medium for callus induction. After 40 days, the formed calluses were transferred to solid MS medium for plant regeneration. Each anther donor plant was used for DNA extraction and 7 random amplified polymorphic DNA primers were used to study the genomic regions associated with callus formation and plant regeneration. Callus formation ranged from 2.27 to 3.36%, whereas plant regeneration varied from 1.38 to 1.82%. Six linkage groups were recorded.

Haque and Mansfield (2004) carried out *in vitro* culture regeneration in four indica (Bangladeshi) rice genotypes from 3, 5, 7 and 9 days old root segments. Genotypic effects were observed in callus induction and subsequent plant regeneration. Moreover, the stage of development of the root explants also played a significant role callus induction and subsequent plant regeneration. Younger explants were more efficient in both callus induction and plant regeneration. Plants regenerated *in vitro* were successfully established in soil and produced fertile seeds.

Two F_4 lines derived from Hs-3 x EPS occasionally showed very high plantlet regeneration frequency in anther culture conducted by Huang *et al.* (2004) to promote homozygosity in these lines. To confirm whether the high plantlet regeneration in anther culture was heritable and to identify indica rice accessions with high regeneration capacity. A high variation in regeneration capacity was observed among the H_1 plants. The heritability of callus induction frequency, callus differentiation frequency and plantlet regeneration frequency reached 90.5, 81.8 and 88.3%, respectively.

Selection of high green-plant regenerating lines through rice anther culture was studied by He *et al.* (2003) in 32 crosses derived from indica × indica, indica × japonica, and indica × javanica were cultured. The number of inoculated anthers was about 71,600. All test materials could induce calli and regenerate green plants. Genotypic differences in anther culture ability were observed among the materials. Highly significant differences were observed in anther culture ability between indica × indica and indica × javanica. A significant negative relationship between callus induction and regeneration capacity was found. Both were positively correlated with green-plant yield. A new material from the indica/ japonica hybrid progeny was screened through anther culture. Its callus induction frequency was higher, with a green-plant regeneration frequency of up to 97%, while the albino plant frequency was less than 2.2%. Green-plant regeneration capacities of calli induced from different induction media were not significant.

Chen *et al.* (1999) showed that callus formation in the four genotypes (indica and japonica for first and second crop and their F_1 hybrids) varied significantly (0.42-20.13%), along with green plantlet differentiation (10.83-35.05%) and culturability (0.08-2.18%). A comparison between anther cultures of the three lines, conventional hybrids, and cultivated varieties (all are Hsien type) has shown that the average frequency of callus formation in three lines and hybrids was two to three and five to six times as high as in conventional intervarietal hybrids and in normal varieties respectively (Ling *et al.*, 1991). It has also been found that the strains or lines from pollen plants have a tendency to have a higher percentage of pollen callus formation and green plant regeneration than those from original varieties or hybrids (Xui and Liu, 1984).

Chen *et al.* (1999) obtained the following results for F_1s callus formation. Indica x japonica > japonica x japonica > japonica x indica > indica x indica; green plant differentiation ability followed the sequence: japonica x indica > indica x japonica > indica x indica; and culturability was: japonica x indica > indica x japonica > japonica x japonica > indica x indica.

Faruque *et al.* (1998) used all F_1 progeny from the crosses of Taipei 309 x.13R 7, Taipei x Binnotoa and Taipei x Rajashail and observed significantly increased callus induction compared to the indica parent although the values were lower than the mid-parent means.

Rohilla *et al.* (1997) observed a high frequency of androgenic calli formation and green plant regeneration in the indica breeding line IET 12012.

Yan *et al.* (1996) reported that both additive and maternal effects significantly contributed to the genotypic and phenotypic correlations between callus induction and green plant regeneration. Genotypic differences in anther response have also been observed in rice (Oono, 1975; Guha-Mukherjee, 1973; Niizeki and Oono, 1968). In general, anthers of the cultivars of japonica subspecies are much more responsive than those of the indica subspecies (Lu *et al.*, 1998; Mikami and Kinoshita, 1988; Woo and Chen, 1982).

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Mandal and Gupta (1996) observed that uninucleate microspores with dense cytoplasm from rice hybrid (Pankaj x FR-13A) responded to form more androgenic calli than binucleate pollen grains. Several factors may cause a change in the optimal responsive stage. In rice, the maximum response shifts from the mid-uninucleate to an early-uninucleate stage when sucrose concentration of the culture medium was increased from 6 to 9% (Chen, 1978). Little work has been reported on the use of morphological markers such as panicle length, spikelet position, spikelet color, anther color and anther position in the spikelet on subsequent callus induction. Based on the study by Mercy and Zapata (1986), the distance between the flag leaf and the subtending leaf as well as the late uninucleate and early binucleate pollen stage has been used as markers for callus induction although with inconsistent success. Shahjahan *et al.* (1992) observed that the best microspore stage for highest callus induction in four indica rice varieties was the mid-uninucleate stage and this corresponded with spikelets of yellowish green color and anthers feaching the middle of the spikelet.

Anderson and Al-Khayri (1996) showed that genotype influenced plant regeneration.

Zou *et al.* (1995) showed that rate of callus formation and plantlet regeneration were higher under a short photoperiod than under a long photoperiod. The effect of the anther

wall on androgenesis and callus formation has not been extensively studied but it was observed that the electrophoretic pattern of soluble proteins in the anther wall changed considerably after 5 days of culture. The changes also varied with the varieties used, which was recognized as one of the reasons to generate varietal difference in rice anther culture.

Callus formation and green plant regeneration varied among genotypes and no matter what kind of growth regulator and sugar were used in the culture medium (Xie *et al.*, 1995). Xie *et al.* (1994) reported that green plant regeneration dependent on genotype.

Guiderdoni *et al.* (1992) examined on anther culture of tropical japonica X indica hybrids of rice (*Oryza sativa L.*). Nine japonica X indica F_1 hybrids of rice involving 6 indica and 3 japonica tropical varieties are large scale anther cultured. The frequency of callusing anthers average 18.7%. The microspore-derived calli produced green plants with a mean frequency of 8.7%. Albino plants represent 61% of the shoot forming calli. Monitoring of the green and albino plant regenerating capabilities of calli arising between week 4 and week 8 of incubation of the anthers showed no increase of the albino/ green ratio and a slow decrease of the shoot forming ability of the transferred calli after the sixth week of culture.

Intraspecific variability, reflected in the genotype-dependent response of *in vitro* cultures, necessitates empirical determination of suitable plant regeneration conditions for individual cultivars (Al-Khayri *et al.*, 1991; Pierik, 1987).
Davoyan (1987) showed that the genetic factors controlling callus formation and regeneration capacity were independent. Abe and Sasahara (1982) observed that calli induced from F_1 seeds exhibited a clear resemblance to the low callus formation rate parent and suggested that factors suppressing callus formation were dominant.

Yeh and Tsay (1987) reported that low temperature (15 / 20^{0}C for night/day) under which the rice donor plants were grown affected callus formation more severely than did the high temperature (30-35^0 C). Anther culture ability of photo-thermo-sensitive genic male sterile rice in the sterile period was significantly lower than in the fertile period observed by Lu *et al.* (1997).

The genotype of the donor plants has a profound effect on pollen plant formation (Wu and Chen, 1 987; Fabian and Gomoiu, 1982). Not only the species within a genus but also the cultivars of the same species often show varied responses (Maheswaran and Rangaswamy, 1988; Mostafa, 1987; Tomes and Collins, 1976; Guha-Mukherjee, 1973; Nitsch, 1969).

Tsay *et al.* (1986) indicated that the epidermis and endothecium of the cultured rice anther can accumulate and transport lipid and polysaccharides to the microspores at certain stages during the cultured period. Mil (1976) pointed out that the wall of a browned anther has harmful effects on pollen grains that have not yet attained the critical stage of development. Tsay (1982) reported higher concentration or longer application of sterilant accelerated the browning culture rice anthers and reduced the ability for callus induction.

Miah *et al.* (1985) observed marked differences in callus induction ability of indica and japonica rice varieties. They reported that frequency of callus induction was 41.9% in Taipei 309 a japonica variety whereas the frequency of indica varieties ranged from 0 to 7.9%.The indica cultivars (Suphanburi and Chainat) showed very low rates of callus induction 1-4.4% but the japonica cultivar (Notohikari) had a higher frequency of induction (13.9-17%) (Prapa-Sripichitt *et al.*, 2000).

Xui and Liu (1984) analyzed the genetic effects of callus formation rate by diallel analysis and found significant differences in general combining ability (gca) specific combining ability (sca), indicating a strong genotype effect. Navapainch and Sahavacharin (1980) cultured 6 indica varieties and found variation among them in callus induction.

Chen and Tsay (1984) showed that the frequency of callus formation was significantly lower in anthers collected from the tertiary tillers than in those from the main culms and primary and secondary tillers. So the booting stage of rice plant is (lie dine to sample panicles for anther culture. Panicles are collected when the distances between pulvinuses of flag leaves and second leaves are 7-12 cm; this criteria is based on relationships between morphological characters and progress of pollen development (Mercy and

Zapata, 1986). The effects of photoperiod, light intensity and temperature under which the donor plants are grown have been little investigated for rice anther culture.

Ding *et al.* (1983) evaluated the anther culturability of 38 indica varieties and reported differences in callus formation rates ranging from as low as 0.35% to a high of 28.4%.

Zapata *et al.* (1983) reported that anthers containing mid-uninucleate to early binucleate stage showed better response than those planted at early and late stage of development. The frequency of callus induction from bi- and tri-nucleate rice pollens were low (Genovesi and Magill, 1979; Chen and Lin, 1976; Yin *et al.*, 1976).

Sheng *et al.* (1982) suggested that anther culturability differed between major types of rice in the following order: glutinous rice > japonica > indica / japonica hybrids > hybrid rice > indica. The culturability of *Oryza sativa* was higher than that of *O. perennis* (annual type) and wild rice produced only albino plants (Wakasa, 1982). Similar findings were obtained by Woo and Huang (1980) where interspecific crosses of *O. sativa* with *O. perennis* and *O. sativa* with *O. glaberrima* showed reciprocal differences in anther culturability.

Hu *et al.* (1978 b) observed that the highest frequency of callus induction was obtained with the anthers from the plants grown under sufficient sunshine at 18.5-20 ° C. Continuous cloudy and rainy weather or lower (16-18 ° C) and higher (26-28 ° C) temperature would apparently decrease the rate of callus formation. The anther culture from the plants subjected to the higher temperature also increased the albino plantlet regeneration (Huang *et al.* 1983).

Dunwell (1976) stated that hormone balance of the anther wall played an important role in embryo development from tobacco anther culture. In rice anthers forming pollen callus, the protoplasm in the cells of the epidermis and mid-layer tissue of the anther wall became dense and dark- stained with large nuclei, while there was no such change in the anthers without MPG (Sung *et al.*, 1978).

Yin *et al.* (1976) using a japonica cultivar observed that the best microspore stage for callusing was the late- nucleate stage when spikelets were yellowish green in color and

the length of stamen was 1/3 to 1/2 of the glume. Afza *et al.* (2000) showed that the callusing abilities of anthers from different spikelet positions were significantly different, the percentage of anthers forming calli was 20% in the basal part, 12% in the middle part and 8% in the top part.

The productivity in anther culture also varies with the physiological status of anther-donor plants. Chen and Lin (1976) observed that anthers collected and cultured at the beginning of the flowering period were more productive than those harvested at the end of this period.

The pollen stage appears critical for successful anther culture. Microspores (pollen) can manipulate to bypass their normal way of development if cultured within a certain stage of development (Nitsch, 1969).This exact period or stage varies from species to species. For example, the critical stage of anthers for Datura, Nicotiana and Petunia, is just before or after the pollen mitosis (Bhojwani and Razdan, 1983); but in cereal it is in the early to mid-uninucleate stage (Sunderland, 1978). In tomato, the ideal stage for callus induction is in the meiosis-1 (Sundarland, 1974; Gresshoff and Doy, 1972). In rice, nuiximum response occur when anther is cultured at uninucleate stage to mid-uninucleate stage and calli recovered from anther at that stage produced maximum green plants with minimum number of albino plants. Calli arising from microspores in the late uninucleate stage appeared less capable for plant regeneration. The microspores at bi-nucleate stage produced more albino plants than those of the uninucleate stage (Miah *et al.*, 1985; Oono, 1981; Chen, 1977; Sunderland, 1974; Guha-Mukherjee, 1973; Guha *et al.*, 1970).

The nutritional requirements for optimal growth of a tissue *in vitro* may vary with the varieties. Even tissues from different parts of the same plant may have different requirements for satisfactory growth (Murashige and Skoog, 1962).

2.2.2. Effect of explants

In vitro culture technique of rice depends on age and the types of explants. Various explants such as embryo, anther, pollen, scutellum, shoot and root tips etc. with different

ages have been used for regeneration of plants. A differential in *in vitro* response of explants were reported by a number of authors, some of which are reviewed here:

Low temperature shock has been reported to enhance the androgenic response in several species including rice (Sen *et al.*, 2011; Gueye and Nidr, 2010; Silva and Ratnayke, 2009; Pande, 1997; Ogawa *et al.*, 1995). Zapata-Arias (2003) recommended a treatment at 8-10° C for 8 days.

Bishnoi *et al.* (2000) investigated that pathways of pollen callus development in cultured anthers of *L. temulentum*, *F. pratensis* and the *L. multiflorum* x *F. pratensis* hybrid Elmet. In all 3, development from the vegetative cell was the predominant pathway. However, there were characteristic differences in the behaviour of the generative cell. In *L. temulentum*, it remained attached to the pollen wall and degenerated, whereas in *F. pratensis* it divided. In Elmet it became detached from the pollen wall and remained undivided. Both polarized and unpolarized partitioned calluses were observed. Development of the fusion product of the vegetative and generative nuclei was also observed in anthers of *L. temulentum*. Anomalous grains were not found to be the major source of pollen calluses. Sections of anthers of *L. temulentum* were used to investigate the origin of pollen grains, the small pale-staining grains which denote pollen dimorphism. Such grains were found to be formed when PMCs are out of contact with the tapetum and are therefore determined before or during meiosis (i.e. before harvest of anthers for culture). Information is also presented from sections on the influence of the duration of pretreatment at 7^0 C on the development of the middle layer of the anther wall.

2.2.3. Effect of regeneration ability

The capacity of rice pollen callus to differentiate into plants is largely dependent upon the quality of the callus before its transfer to the regeneration medium. Callus with compact texture and light green is more likely to generate green plantlets and the loose and semi-transparent callus is difficult to redifferentiate (Zhang, 1982).

Ranjan *et al.* (1998) observed that green plant regeneration frequency from 0 to 26.86% in indica cultivars (Basmati-370, Tulsi and Tetep) depending upon the genotype and the constituents of the media used. From the genetic analysis of plant regeneration ability in anther culture of rice. Sutimoto and Takeoka (1998) proposed that different genes are involved in regeneration ability from callus formation ability and that genes or effects of genes for regeneration ability vary according to the cultured media.

Mandal and Bandyopadhyay (1997), Marrassi *et al.* (1996) and Lenka and Reddy (1994) reported that the best medium for plant regeneration was MS media with different concentrations of IAA + NAA or Kinetin + BAP (benzyladenine) or IBA. Huang *et al.* (1986) reported that calli formed in the presence of NAA were more capable of plant regeneration than those initiated in media with 2,4-D. The addition of 2-5 mg/1 ABA is effective in maintaining the regeneration ability of callus for a long period (Narayanan *et al.*, 2000).

Zhuo *et al.* (1996) observed that the anther-derived calli of all genotypes regenerated shoots directly on the callus induction medium containing PAA.

Yoshida (1995) estimated the relationship between callus size and plant regeneration in Nipponbare and Akenohoshi rice cultivars. He observed that the number of haploid green plants regenerated from calli increased when the callus was over 2.0 mm size in diameter. However, if the calli were too small in size (<1 mm), their growth was generally very slow and gradually they became necrotic after transfer onto the differentiation medium. The suitable spacing for callus to undergo differentiation was about 2 calli / 20 mm (Courtois, 1992). Narrowing the callus spacing resulted in a decrease of differentiation ability (Chen *et al.*, 1984).

Mandal and Gupta (1995) observed that maximum green plant regeneration was obtained in calli induced in the presence of ABA and later transferred to regeneration medium supplemented with 0.5 mg NAA and 2 mg Kinetin/l. Green plantlets could grow directly from the anthers cultured on the medium with NAA alone (Hsu and Hung, 1978). Further studies showed that 2,4-D at lower concentration (about 0.01 mg/l) used in combination with 3 mg/1 NAA and 3-4.5 mg/l Kinetin was very helpful for pollen plantlet

regeneration directly from the culture anthers (Lin *et al.,* 1983 and 1984).

Huang (1993) and Maeda *et al.* (1988) observed plantlets differentiation on R4 medium in presence of 2 mg /1 Kin and 0.5 mg/1 NAA is significantly different in the presence of the same growth regulators on the other media. Chaleff and Stolarz (1981) used modified MS medium to determine plant regeneration frequency and observed a high rate of regeneration (70%) in modified MS medium supplemented with NAA and Kin.

The callus of the japonica cultivars exhibited extremely poor shoot and root regeneration, whereas the indica cultivars showed good ability to regenerate shoot and roots. A remarkably high frequency of 80-100% shoot regeneration was obtained from indica cultivar ANT 39 (Lu and Pan, 1992).

Higuchi and Maeda (1991) reported the favorable effect of high-level sucrose on the regeneration potential in rice (cv. Nipponbare) callus. Huang *et al.* (1978) showed that with 2% and 4% sorbitol as a carbon source, explants survived for more than 25 days and regeneration rates of green plants were 52.6% and 90%, respectively. The green plant regeneration rate per anther was higher in the medium with increased KNO_3 (Daigen *et al.,* 2000; Raina and Zapata, 1997).

Chen *et al.* (1978) indicated that rice callus induced in early stage, e.g., 30-50 days after anther inoculation, has high differentiation for green plants. Because pollen callus of rice loses its morphogenetic potential very rapidly during culture, an early transfer to the plant regeneration medium has been suggested (Chen, 1977; Oono, 1975): Callus age, i.e., days from callus induction to transference affects plant regeneration. The most suitable age for the transfer to plant regeneration medium was approximately 10- 15 days after callus induction; transfer before this usually results in death of the callus (Chen *et al.*, 1984; Wang *et al.*, 1974); more than 20 days old and show high frequencies of albino (Chuang *et al.*, 1978).When the pollen calli (1-3 mm in size) were transferred, adventitious shoot and roots gradually differentiated (Shen *et al.*, 1982).

2.2.4. Albinism

The recovery of albino plants from microspore derived calli in rice especially in *indica* rice varieties has been a formidable obstacle to the utilization of rice anther culture for *indica* rice improvement (Chowdhury and Mandal, 2001; Sripichitt *et al.*, 2000; Raina and Zapata, 1997; Chen *et al.*, 1991).

Woo and Huang (1992) considered that the presence of the cytoplasm from wild species was related to the high frequency of albino plants in anther culture of wild rice and its hybrids. Vaughn *et al.* (1980) suggested that formation of albino plantlets was due to a physical alteration of the organelles during *in vivo* microsporogenesis

Cai (1984) reported that 100% of regenerated pollen plants were albino and subsequently green plant frequency was very low. From the experiments on anther cultures of hybrids between wild and cultivated rice.

Temperature is most critical in albino plant regeneration. Pretreatment of rice panicles at higher temperature (35^0 C) for 3 or 5 days increased the albino plants. If rice panicles were pretreated at room temperature (26^0 C), all the regenerated plantlets were albinos; however, the pretreatment at lower temperature (10^0 C) increased the rate of green plants (Qu and Chen, 1983 b).

Huang *et al.* (1983) also showed that the frequency of albinos increased with rise in temperature, i.e., they were 13.9, 22.5 and 34.7% for 25, 35 and 38 ° C respectively. However, the frequency of green plants followed the opposite sequence: 15.0, 9.2 and 2.8% for the same series of temperature treatments.

Chen *et al.* (1982) reported that increasing the iron concentration to 0.2 mM showed toxicity to callus formation and green plant production. In this connection, it is noted that the frequency of albinos is influenced by factors such as incubation temperature (Wang *et al.*, 1974), kind and concentration of media compounds (Lee and Chen, 1982), pollen stage (Chen, 1977) and genotype (Cai, 1984).

Albinism has been found to be particularly prevalent in pollen-derived plants of interspecific hybrids (Woo and Huang, 1982) and intraspecific hybrids between japonica

and indica subspecies (Tsay *et al.*, 1982) and also from wild rice species.

In rice, albino frequencies ranged from 10% (Genovesi and Magill, 1979; Wang *et al.*, 1978) to 100%. Production of albino plantlets is a common phenomenon in anther culture of cereals (Clapham, 1973).

There was no relation between the basic formula of the media and the production oh albinos, but high levels of 2,4-D 20 mg/l, (Wang *et al.*, 1978) sucrose (9%) (Chen, 1978) in the callus induction medium enhanced the formation of albino plantlets after the pollen calli were transferred onto the differentiation medium.

2.2.5. Effect of culture media

Thuan *et al.* (2001) studied on anther culture of F_1 plants from crosses between aromatic and improved rice cultivars. Anthers of F_1 plants derived from four crosses of aromatic and improved rice cultivars were cultured in N_6 and MS media supplemented with 2, 4-D (0.5mg/L) + NAA (1.0mg/L)+ BAP (0.5mg/L) for callus induction. The anther-derived calli were subsequently sub cultured in MS and N_6 media supplemented with BAP (1.0mg/L) and NAA (1.0mg/L) for plant regeneration. Frequency of callus formation was better in N_6 medium as compared to MS medium (11.9% and 7.95%, respectively). Anther-derived calli from the cross of Khao Hom Suphanburi and DS15 exhibit the highest response to the N_6 medium in plant regeneration (7.57% with 37 green plants). Green plants can be regenerated in N_6 eight times higher than in MS medium.

Ogawa *et al.*(2000) observed that callus induction frequency was best on the medium containing one-fourth of the KNO concentration N_6 medium, which was the same as (lie N concentration in KSN medium). MS and N_6 were the most commonly used basal media (Toki, 1997; Rashid *et al.*, 1996; Pandey *et al.*, 1994).

Tyagi (1997) observed that microspore-calli derived from a medium of low level of $(NH_4)_2SO_4$ (1.75 mM) showed greater regeneration potential than those derived from a higher level (3.5 mM).

Raina and Zapata (1997) reported that combinations involving KNO_3 (31-34 mM) and $(NH_4)_2SO_4$ (2.0-2.5 mM) were superior not only for achieving; higher anther response but also for subsequent green plant regeneration.

QiFeng and ShuYe (1995) investigated that to determine the microelement requirement of cell suspension cultures of rice (*Oryza sativa*, Norin No. 16). The requirements for iron, manganese, zinc, copper, boron and molybdenum were assessed. Omissions of iron and zinc from the culture solution caused the most remarkable decreases in the growth of rice cells as well as its seedlings. The need for manganese by the cells was much lower than that observed with whole plants. In iron-deficient cultures, aminobutyric acid, alanine and amides accumulated both in the cells and the culture medium. Under conditions of zinc deficiency, the cells contained less protein and accumulated alanine and amides. The respiratory activity in terms of oxygen uptake was reduced in iron- and zinc-deficient cultures.

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Yeh and Tsay (1988) reported that inorganic salts of N_6 medium were inure elective in delaying the browning and concomitantly accelerating (lie growth of callus as compared to those of MS medium. Another observations that the indica varieties gave better results when N_6 medium was supplemented with 3.0 mM ammonium compared to MS, Blayde's, SK-8 and R2 media (Dhaiwal *et al.*, 1997).

Lin and Tsay (1984) reported that the pretreated rice inflorescence at 8°C for 1 week followed by dissection of anthers and inoculated in N_6, inorganic salts and MS organic substances supplemented with NAA and kinetin. It was found that these anthers formed callus four times higher than those cultured on MS medium with the same plant growth regulators. This demonstrated that there was an additive effect between basal medium and cold shock.

Both the B_5 and LS medium (Chaleff and Stolarz, 1981) also have an increased concentration of nitrate-nitrogen and reduced concentration of ammonium- nitrogen and have been found to give good results (Zapata *et al.*, 1982; Chaleff and Stolarz, 1981).

Subsequently, Chu *et al.* (1975) developed the N_6 medium which was characterized by having a low concentration of $(NH_4)_2SO_4$ and a high concentration of KNO_3. This medium has proved to be very efficient for rice anther culture (Chen *et al.*, 1982; Tsay *et al.*, 1982; Genovesy and Magill, 1979; Chu, 1978) and other cereals (Nitsch *et al.*, 1982; Miao *et al.*, 1978; Chu, 1978). Most of the inorganic nitrogen supplied as ammonium source appears generally favorable for rice tissue culture over nitrate. A concentration of 3.4mM ammonium in the culture medium was seen optimal for *indica* rice anthers culture (Huang *et al.*, 1978).

Clapham (1973) first bound that the high concentration of ammonium ion in the LS medium (Linsmaier and Skoog, 1965) was inhibitory to callus formation from barley microspores.

2.2.6. Effect of growth regulators

Best regeneration response on MS media with same hormonal combination (1mg/l NAA and 2mg/l BAP) was found by Agarwal *et al.* (2006) and Rachmawati and Anzai (2006). Kim *et al.* (1992) reported that in presence of NAA at low concentrations and 2 - 10 mg/l kinetin, green calli were induced from excised embryos. They also observed that chlorophyll contents increased as kinetin concentrations increased but callus induction decreased.

Islam *et al.* (2005) reported that the effect of different concentrations of 2, 4-D in the presence and absence of calcium silicate on rice seed culture was investigated in three rice genotypes. Callus induction medium was supplemented with the following concentrations of growth regulator: 2, 4-D 1 mg/l + Calcium Silicate 60 mg/l, 2, 4-D 2.5 mg/l, 2, 4-D 2.0 mg/l and 2,4-D 1.5 mg/l. For plant regeneration MS medium fortified with Casein Hydrolysate 4 g/l, NAA 1 mg/l and Kinetin 3 mg/l was applied. Both genotype and growth regulators significantly affected callus induction and plant regeneration. The variety Pajam and the medium containing 2, 4-D 1 mg/l + Calcium Silicate 60 mg/l and 2, 4-D 2.5 mg/l were found most efficient for callus induction.

Variety Kalizira showed better performance in plant regeneration; calli of this variety, derived from the medium fortified with 2, 4-D 2.5 mg/l produced 80% regenerated plants.

Islam *et al.* (2004) conducted an experiment to find the effects of different concentrations and combinations of growth regulators viz. 2, 4-D, IAA, α-NAA and Kinetin on callus induction from the anthers of a commercial hybrid rice line IR-69690, developed by Bangladesh Rice Research Institute and subsequent plant regeneration. N_6 medium was used as basal medium. Callus induction frequencies in different media combinations ranged from 1.2 to 35.5%. The medium supplemented with 2, 4-D 1.0 mg/l, α-NAA 2.0 mg/l and Kinetin 1.0 mg/l was found most effective for callus induction (35.5%). Regeneration of plants from the callus on agarified MS medium supplemented with α-NAA 0.5 mg/l and Kinetin 3.0 mg/l was also variable ranged from 16.7 to 69.3%. Calli derived from the media supplemented with2, 4-D 1.0 mg/l, α-NAA 2.0 mg/l and Kinetin 1.0 mg/l also showed better performance for plant regeneration (69.3%) and among these plants 56.14% were green. However the callus induction medium containing α-NAA 1.0 mg/l and Kinetin 1.0 mg/l directly produced green regenerated plants in higher frequency (70%), without transferring the calli on to the regeneration medium; but rate of callus induction in this medium was very low (6%). *In vitro* complete plant regeneration was investigated on MS medium supplemented with two combinations of growth regulators i.e., NAA and BAP (1:2mg/l and 1:4mg/l). Combinations of auxin and cytokinin along with the effect of basal salts played an important role for plant regeneration (Lee *et al.*, 2002; Prodhan *et al.*, 2001).

Huang *et al.* (1998) observed that addition of kinetin to the induction medium was more favorable for high callus induction and green seedling induction than addition of BA (benzyl adenine). Wakasa (1982) substituted BA for kinetin in differentiation media and observed a high rate of green plantlet regeneration.

Sohn *et al.* (1996) reported that ABA in callus formation medium was most effective for inducing pollen embryogenesis of rice. The maximum frequency (10.8%) of embryo formation was obtained at 0.5 ppm ABA. The effects of 2,4-D, NAA and kinetin on callus formation and plant regeneration in rice anther culture have been critically examined by Huang *et al.* (1985). They observed that the optimal concentration of NAA

in the callus medium appears to be 2 mg/1. At this concentration, callus formation and plant regeneration Increase with the increase in the concentration of kinetin. However, a too high concentration of kinetin (2mg/1) may result in the production of more albino plants. Thus, the best hormone combination among seemed to be 2 mg/1 NAA. Organic Substances are usually added to the anther culture medium to provide an organic source of nitrogen or to furnish necessary but unidentified components.

The most crucial constituents in the rice anther culture medium are plant hormones (auxins and cytokinins).Various combinations of auxins and cytokinins in the culture medium have been tested for their effects on both callus induction and plant regeneration in rice (Anderson and Al- Khayai, 1996; Wang *et al.*, 1974; Chen *et al.*, 1974; Guha-Mukherjee, 1973; Harn, 1969; Niizeki and Oono, 1968).

For obtaining callus of high morphogenetic potential, cytokinin and ABA (abscisic acide) are also needed (Torrizo and Zapata, 1986; Inoue and Maeda,1981; Chen, 1977). The two-step culture technique (ABA in the preculture medium and kinetin in the last culture media) leads to a high frequency of shoot formation and plant regeneration in rice callus culture (Inoue and Maeda, 1981).ABA increases the embryogenecity of callus and regeneration percentage (Narayanan *et al.*, 2000).

Cytokinins in particular kinetin, appear to help trigger morphogenic differentiation of callus into plants. Regeneration rates as high as 78% were obtained in anther culture when kinetin and NAA were used in the medium (Lee and Chen, 1982; Zhang, 1982; Chaleff and stolarz, 1981; Chen, 1977).

Challef and Stolarz (1981) used modified Murashige and Skoog (1962) medium supplemented with NAA and produced callus from rice anthers. Combining NAA with Kinetin resulted in significant increases in callus induction and plant regeneration rates (Chen *et al.*, 1982; Hu and Liang, 1979).

Although the induction phase of androgenesis may be nutrient independent, continued division of the induced microspores to the formation of embryos or callus require the presence of appropriate nutrients in the culture medium (Wang *et al.*, 1974; Nitsch, 1969).

In the early 1970s, a 2,4-D concentration of 9m showed suitable for callus induction (Chen *et al.*, 1974). For callus induction in rice, a different concentration of 2,4-D was most widely used in MS medium for sufficient callus induction (Brisibe *et al.*, 1990).

Niizeki and Oono (1971) reported that NAA (naphthalene acetic acid) was superior to 2,4-D in differentiation media. Replacing 2,4-D with PAA (phenyl acetic acid) in the induction medium did not influence callus induction but significantly improved the shoot differentiation from callus, particularly in the indica cultivar Teqing and the anther derived callus of all genotypes regenerate shoots directly on the callus induction medium containing PAA (Zhuo *et al.*, 1996).

2.2.7. Effect of carbon sources

Shahnewaz and Bari (2004) studied on effect of concentration of sucrose on the frequency of callus induction and plant regeneration in anther culture of rice (*Oryza sativa* L.). Effect of various concentrations of sucrose in culture media on the frequency of callus induction and regeneration of green plantlets from anther culture of rice are investigated. Cold pretreated anthers of BRRI dhan29 at 6^0C for seven days are cultured on N_6 medium containing sucrose at concentrations of 0, 1, 2, 3, 4, 5, 6%; 2.0 mg/l 2,4-D; 0.5 mg/l Kin and 2.5 mg/l NAA. Results reveals that 4% sucrose was suitable for inducing high frequency (7.5%) callus induction and high green plant regeneration (65%). Highest concentration of sucrose (6% and above) in the culture medium not only result in an increase in the percentage of callus formation but also prompt the regeneration of albino plants.

Yi *et al.* (2003) conducted an experiment to study the effect of maltose on callus formation and plant regeneration in anther culture of these recalcitrant varieties. Maltose increases anther response not only in Tongil and indica rice but also in japonica varieties. Interestingly, the percent callus induction was higher in Tongil and indica than in japonica varieties. Callus induction in indica was 2- to 14-fold higher in maltose (40–60 g/l) supplemented medium than in sucrose plus glucose supplementation medium.

Anderson and Al-Khayri (1996) showed that calli generally grew best on media containing sucrose alone, although a combination of sucrose and sorbitol enhanced regeneration of some cultivars. In other observations, all the anthers cultured in presence of compound carbon source (2% sorbitol + 4% sucrose) produced more callus (22.1%) in compare to culture in presence of the single source (6% sucrose) (11.5%). Callus emergence appeared by 7-10 days earlier on compound source medium compared to single source medium (Chu *et al.*, 1998).

Xie *et al.* (1994) noticed a high frequency of callus induction when microspores were cultured on media with maltose instead of sucrose was used as the carbon source. Pande and Bhojwani (1999) experienced that anther culture of indica rice cv. IR 43with maltose was four times more effective than with sucrose for callus induction and partial substitution of maltose by mannitol increased regeneration of green plantlets.

Ling *et al.* (1991), Miah *et al.* (1986) and Chen (1978) reported that a combination of 6% sucrose in the callus formation medium and 3% in the plant regeneration medium gave highest frequency of callus and green plant formation. Chaleff and Stolarz (1981) obtained high callus formation using sucrose concentrations of 4- 5% and suggested that the increased osmotic pressure of the culture medium may be partly responsible for accelerated callusing.

Other researchers recommend sucrose concentrations from 6 - 8% for dedifferentiation media and 4-5% for dedifferentiation media in order to maximize callus induction and plant regeneration (Croughan *et al.*, 1986 and 1989; Chu *et al.*, 1984, 1985, 1986ab; Zhang, 1982; Res. Group of Rice, 1976).

Sucrose, glucose or fructose were typically used as carbon sources (Miah *et al.*, 1986; Fadia and Metha, 1973) and sometimes as osmotic agents (Bhojwani and Razdan, 1983) in culture media.

High sucrose concentrations have been found to be beneficial for plant production from anther culture of many plant species, especially for the Gramineae (Oono and Larter, 1976; Ouyang *et al.*, 1973; Clapham, 1973). In rice opinions concerning the sucrose concentration vary.

2.2.8. Effect of organic substances

Mandal and Gupta (1997) showed that callusing, medium containing aqueous extract of potato favored differentiation of green plants in 15.8% of calluses on transfer to regeneration medium.

Mandal and Bandyopandhyay (1996) used 5% coconut water in MS medium and found higher callus induction. The potato media (Chuang *et al.*, 1978; Anonymous, 1976) have proved as very efficient for *in vitro* microspore development of many cereal crops including rice (Xu *et al.*, 1981; Huang and Sunderland, 1981).

Hirabayashi *et al.* (1992) reported that aspartic acid, glutamine, glutamic acid, tryptophan and casein hydrolysate (C:H) at 1-10 mg /l added to N_6 basal medium increased the frequency of callus formation and plant regeneration. They also observed that CH at 1000 mg /l was highly effective at improving plant regeneration and the combination of glutamine and tryptophan with CH further stimulated callusing.

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In contrast, Yoshida *et al.*(1983) reported that many rice varieties could develop good callus in the absence of yeast extract. Ginseng powder at 500-100 ppm reduced the rate of browning of rice anther culture and increased the callus forming ability and breeding efficiency (Chen and Tsay, 1984).

Various natural extracts, e.g., coconut water, yeast extract, potato extract, have been claimed to be beneficial for callus formation and / or plant regeneration in anther culture of rice (Liang, 1978; Oono, 1975; Wang *et al.*, 1974; Guha-Mukherjee, 1973; Guha *et al.*, 1970).

Nitsch (1974) emphasized the importance of inositol, serine and glutamine in the culture media. Zhou and Fan (1983) showed addition of DL-alanine (2-4 mg/L) to the medium enhanced the frequency of callus in Hsien rice. The addition of a mixture of amino acids to the medium promoted callus induction but not green plant regeneration, whereas proline at 17.4 mM induced green plant regeneration but not callus induction as observed by Aldemita and Zapata (1991). Medium containing 20 mM KNO_3 and 5 mM glutamine gave the highest number of calli which was 2- fold higher than the control medium

containing 40 mM KNO_3 and 2.5 mM $(NH_4)_2 SO_4$. Medium containing 20 mM $KN0_3$ and 5 mM alanine resulted in the highest number of regenerating calli and green plantlets (Ogawa *et al.*, 1995).

2.2.9. Effect of p^H

Ogawa *et al.* (2000) observed that in rice, callus induction frequency was highest at pH 5.7 and decreased with decreasing pH.

Owen *et al.* (1991) examined media pH as influenced by the inorganic salts, carbohydrate source, gelling agent, activated charcoal and medium storage method.

The p^H of plant tissue culture media is generally adjusted to p^H 5.5 to 6. Below 5.5, the agar will not gel properly and above 6.0, the gel may be too firm (Murashige, 1973). Media pH generally drops by 0.6 to 1.3 units after autoclaving (Sarma *et al.*, 1990).

2.2.10. Effect of solidifying agent

Feng and Li (1999) showed that callus induction was increased when the agar concentration increased from 0.6 to 0.8%, but that the callus formed with 1.0% agar was shriveled and slow growing, and green plantlet regeneration was best with 0.6 - 0.8% agar in the induction medium and 1.0% in the regeneration medium.

Aldemita and Zapata (1991) observed that addition of agarose in the culture media is beneficial for callus induction and plant regeneration of all genotypes (Taipei 309, IR43 and Pokkali).

Solidified medium is widely used because such cultures are very convenient to maintain, gives stationary support and good for oxygen supply, although, anther culture on liquid media perform better than those on agar solidified media (Tian and Chen, 1983; Wernick and Kohlenbach, 1976). Poor anther performance on solid medium has also been attributed to the presence of inhibitory compounds in the agar itself (Grifis *et al.*,1991;

Debergh, 1983; Halquist *et al.*, 1983; Wernick and Kohlenbach, 1976), as it has been showed that dialysis of the agar with activated charcoal (AC) is necessary before use (Kohlenbach and Wernick, 1978) or addition of activated charcoal directly to the culture medium (Anagnostakis, 1974; Nakamura and Itgaki, 1973) significantly increase anther response. But response of rice anthers has been found to be very poor on medium with AC (Chen, 1977).

Hu *et al.* (1978 b) showed that the optimum concentration of sucrose in agar medium was 4 - 6%, while a lower one (2 -3%) was better in liquid medium.

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Chapter III

Materials and Methods

CHAPTER III

MATERIALS AND METHODS

The experiment was conducted at the Biotechnology Laboratory, Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 during the period of July 2011 to December 2012. The experiment was conducted to fulfill the objectives of the present study. The materials and methods used to conduct the present study have been presented in this chapter.

3.1. Experimental Materials

3.1.1. Plant Materials

The following two crosses of indica rice hybrid were used as experimental materials:

1. BR 21 X BRRI dhan28 and
2. BR 26 X BRRI dhan29.

3.1.2. Source of Materials

The materials used in the experiment were obtained from Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University, Dhaka.

3.2. Plan of work

3.2.1. Rice seedling raising

- The seeds of different hybrid of rice were collected from my supervisor's own stock.
- Then seeds were washed with liquid detergent for cleaning purpose.
- Then washed them several times with running tap water to remove detergent.
- After that seeds were washed with distilled water.
- Then seeds were sown in the top in net house.

- Seeds were germinated and grown in the net house. The different boots were selected and closed flower buds (boots) were collected from the donor plants having the late uninucleated microspores. The boots were wrapped with aluminum foil and kept at 4^0C chamber for different time duration.

3.3. Explant Used

The anthers or pollens of hybrid obtained from BR 21 X BRRI dhan28 and BR 26 X BRRI dhan29 were used as explants for the study.

3.4. Methods

The culture media and the methods, which were used in the experiment, were as follows-

3.4.1. Culture media

Success of any experiment depends on the culture media, hormone combination, tissue and employing cell. Murashige and Skoog (1962) medium was used with different hormonal supplements as culture medium for callus induction and plantlet regeneration. The composition of MS medium has been presented in Appendix 1. Hormones were added to MS media as per treatment of the experiment. For the preparation of media, stock solutions were prepared at the beginning and stored in the refrigerator at 4 ± 1^0C. The respective media were prepared from stock solutions.

3.5. Preparation of stock solutions

The first step in the preparation of the medium was the preparation of stock solutions of various constituents of the MS medium. As different ingredients were required in different concentrations, separate stock solutions for macronutrients, micronutrients, vitamins and growth hormones were prepared separately for ready use.

3.5.1. Stock solution A (Macronutrients)

Stock solution of macronutrients was prepared up to 10 times the concentration of the final strength of the medium in 1000 ml of distilled water (DW) accurately. Dissolved all the macronutrient one by one except $CaCl_2$ was prepared separately in order to avoid precipitation. In this way, dissolved all the salts thoroughly by using a magnetic stirrer in about 750 ml of distilled water and then final volume was made up to 1000 ml by further addition of DW. To make the solution free from all sorts of solid contaminants, it was filtered through Whatman no. 1 filter paper. Then it was poured into a clean sterilized glass container, labeled with marker and stored in a refrigerator at 4^0C for later use.

3.5.2. Stock solution B (Micronutrients)

The stock solution of all the micronutrients was made up to 100 times the final strength of necessary constituents of the medium in 1000 ml of distilled water (DW) as described for the stock solution of macronutrients. The stock solution was filtered, labeled and stored in the refrigerator at 4^0C.

3.5.3. Stock solution C (Iron sources)

Iron-EDTA was added fresh and it was made 100 times the final strength of the medium in one litre DW. Here, two constituents, $FeSO_4 .7H_2O$ and Na_2EDTA were dissolved in 750 ml of DW in a conical flask and chelated by heating on a heater cum magnetic stirrer until the salts dissolved completely and final volume was made up to the 1000 ml by further addition of DW. Finally the stock solution was filtered, labeled and stored in an amber color bottle or a bottle covered with an aluminum foil in the refrigerator at 4^0C.

3.5.4. Stock solution D (Vitamins)

The following vitamins were used in the present study for the preparation of MS medium.

Myo-inositol (Inositol),

Thiamine HCl (Vitamin B_1),

Nicotinic acid (Vitamin B_3),

Pyridoxine HCl (Vitamin B_6) and

Glycine.

Each of the desired ingredients except myo-inositol were taken at 10 folds (100x) of their final strength in a measuring cylinder and dissolved in 750 ml of distilled water. Then the final volume was made up to the 1000 ml by further addition of distilled water. The solution was dispensed into 10 ml aliquots, labeled and stored at -20^{0}C. Myo-inositol was used directly at the time of media preparation.

3.5.5. Hormone stock solutions

All the hormones are not soluble in water. Solubility of hormones are given in Table 1. Separate stock solution of hormones was prepared by dissolving the desired quantity of ingredients to the appropriate solvent and made the final volume with distilled water and stored in a refrigerator at 4^{0}C for later use.

The following growth regulators were used in the present investigation:

a. Auxins

2, 4-dichlorophenoxy acetic acid (2,4-D)

α –Naphthalene acetic acid (NAA)

Indole-3-acetic acid (IAA)

Indole-3-butyric acid (IBA)

b. Cytokinins

6- Benzyl amino purine (BAP)

6- Furfuryl amino purine (Kinetin)

c. Giberrelin

Giberrelic acid (GA_3)

Table 1. Molecular weight and solubility of some hormones

Hormones (Solute)	Molecular weight (gm)	Solvent
2,4-D	221.0	70% ethyl alcohol
NAA	186.2	1(N) NaOH
IAA	175.2	70% ethyl alcohol
IBA	203.2	70% ethyl alcohol
BAP	225.3	1(N) NaOH
KI	215.2	1(N) NaOH
GA_3	330.0	70% ethyl alcohol

To prepare the stock solution of hormones, 10 mg of solid hormone was placed on a clean beaker and then dissolved in 1 ml of particular solvent. The mixture was then washed off with distilled water and collected in a 100 ml measuring cylinder and was made up to 100 ml by the further addition of DW. Concentrations of compounds can be taken as mg/l or molarity. The solution was then poured and stored at 4^0C in a refrigerator for use up to four weeks.

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3.6. Preparation of other stock solutions

3.6.1. Preparation of 1N NaOH

a. 40gm NaOH pellets were weighed.
b. Then those pellets were put in dry 1 L volumetric flask.
c. 900ml distilled water was added slowly and stirred until dissolved.
d. The flask in a thermostat at 20^0C and maintained for 1 hour.
e. Distilled water was added up to the 1 L-mark and mixed the closed bottle.

3.6.2. Preparation of 70% Ethanol

a. In a 100ml measuring cylinder 70ml 99.9% ethanol was poured.
b. Double DW was poured up to the level of 100ml.

c. The solution was stored in a sterilized glass bottle.

d. This solution was made fresh each time before use.

3.7. Preparation of MS media

To prepare one litre of MS media, following steps were followed:

- The required volume of each stock solution (100 ml of stock solution "A", 10 ml of stock solution "B", 10 ml of stock solution "C" and 10 ml of stock solution "D") were pipetted into a 2 litre Erlenmeyer flask on a heater cum magnetic stirrer.
- 500 ml of double distilled water was added in the flask to dissolve all the ingredients.
- Myo-inositol and sucrose were added directly to the solution as per requirement and dissolved well with the help of magnetic stirrer.
- Different concentrations of hormonal supplements were added to the solution either in single or in different combination as required and mixed well throughly.
- Later different combination of three hormones NAA, 2,4-D and Kin, respectively were used viz. 1.5 mg/l NAA, 2.0 mg/l NAA + 0.5 mg/l Kin, 2.0 mg/l NAA + 2.0 mg/l 2,4-D and 2.0 mg/l NAA + 2.0 mg/l 2,4-D + 0.5 mg/l Kin for callus induction and callus maintenance.
- Later different combination of four hormones IAA, BAP, IBA and GA_3, respectively were used viz. 1.0 mg/l BAP + 1.0 mg/l IAA, 1.0 mg/l IBA + 1.0 mg/l BAP + 2.0 mg/l GA_3 and 2.0 mg/l IBA + 2.0 mg/l BAP + 2.0 mg/l GA_3 for rooting.
- Later different combination of three hormones BAP, IBA and GA_3, respectively were used viz. 1.0 mg/l IBA + 1.0 mg/l BAP + 2.0 mg/l GA_3, 2.0 mg/l IBA + 1.0 mg/l BAP + 2.0 mg/l GA_3 and 2.0 mg/l IBA + 2.0 mg/l BAP + 2.0 mg/l GA_3 for shooting.
- The whole mixture was then made up to a 1 litre with further addition of distilled water.

3.8. p^H of the medium

The p^H of the medium was adjusted to 5.8 with a digital p^H meter with help of 0.1 (N) HCl or 0.1 (N) NaOH as necessary.

3.9. Agar

After adjusting the p^H, 8 gm agar was added to solidified the medium. The whole mixture was then gently heated on microwave oven at 25^0C temperature for 8- 10 minutes.

3.10. Sterilization

In *in vitro* techniques, aseptic condition is a prerequisite. So, all instruments, glasswares and culture media were sterilized.

3.10.1 Sterilization of culture medium

Fixed volume of medium was dispensed into test tube. The test tubes were plugged with non-absorbent cotton. After dispensing the test tubes were covered with aluminum foil paper and marked with different codes with the help of a permanent glass marker to indicate specific hormonal supplement. Then the test tubes containing the medium were autoclaved with 1.16 kg/cm^2 or 15 psi of pressure at 121^0C for 20 minutes. After autoclaving the test tubes containing the medium were then transferred into the culture room and cooled at 24^0C temperature before used. Marking was also necessary.

3.10.2. Sterilization of glasswares and instruments

Beakers, culture vessels, glasswares, test tubes, petridishes, conical flasks, pipettes, plastic caps, metal instruments viz. forceps, scissors, scalpels, needles, spatulas, surgical blades, instrument stand and aluminum foils, brush and cotton were sterilized in a autoclave at a temperature of 121^0C for 30 minutes at 1.16 kg/cm^2 (15 Psi) of pressure. Before this, all types of glassware instrument was washed properly by liquid detergent, cleaned with running tap water and finally washed with DW.

3.10.3. Sterilization of culture room and transfer area

In the beginning, the culture room was spray with formaldehyde and then the room was kept closed for one day. Then the culture room was cleaned by gently washing all floors and walls with a detergent followed by wiping with 70% ethyl alcohol. The process of sterilization was repeated at regular intervals.

Generally, switching on the cabinet with UV light for 30 minutes before working in empty condition and for 20 minutes all the instruments. The working surface was wiping with 70% ethyl alcohol sterilized laminar airflow cabinet before starting the transfer work.

3.10.4. Sterilization of explant

Sterilization of the panicles was practiced by sterilized distilled water then 70% (v/v) ethanol for 20 seconds, again washed by sterilized distilled water. The spikelets were removed and they were surface sterilized with 30% (v/v) commercial Clorox solution for 20 minutes or transfer them to 0.1% mercuric chloride ($HgCl_2$) solution for one minute and then rinsed thoroughly with double distilled water five to six times to remove the chemicals in the laminar air flow. Different phytohormones were sterilized by using filter (Sieve size 0.45µm). Growth medium and required glasswares and small instruments were sterilized with autoclave (at 121^0C, 15 psi and for 20 minutes).

3.10.5. Precaution of aseptic conditions

All inoculation and aseptic manipulations were carried out under laminar air flow cabinet. The cabinet was usually switched on with ultra violet light half an hour before use and wiped with 70% ethanol to reduce the chances of contamination. The instruments like forceps, scissors, scalpels, needles, spatulas, surgical blades, pipettes, plastic caps, brush, cotton etc. were pre-sterilized by autoclaving and subsequent sterilization were done by dipping in 70% ethanol followed by flaming and cooling method inside the laminar air flow cabinet. While not in use, the instruments were kept inside the laminar air flow cabinet into the instrument stand. Hands were also sterilized by 70% ethanol and wearing of hand gloves. It is also necessary to wear apron and mask to avoid

contamination rate. Other required materials like DW, culture vessels, beakers, glass plates, petri dishes etc. were sterilized in an autoclave following method of media sterilization. The neck of test tubes were flamed before open and also dipping with ethanol with the help of soaked cotton before closing it with the aluminum foil paper. Aseptic conditions were followed during each and every operation to avoid the contamination of cultures.

3.11. Culture methods

The following culture methods were in work in the present investigation.

1. Explant culture
2. Subculture or transfer

3.11.1. Preparation of explants

The spikelets were cut from the panicle and washed thoroughly with double distilled water (DDW) into the laminar air flow cabinet. For surface sterilization, spikelets were sterilized with 70% (v/v) ethanol for one minute. After sterilization, spikelets were then rinsed and washed with brush for three times with sterilize DW into the petridish to remove the trace of alcohol. Afterwards the spikelets were transferred into another petridish and were again surface sterilized by immersing in 0.1% $HgCl_2$ solution supplement with three drops of Tween-20 mixtures and then finally rinsed and washed four times with sterilized DW. The surface sterilized disinfected spikelets were then cut with sterilized scissor and anthers were kept into the sterilized petridishes to make the anthers alive. Now the explants were ready for inoculation.

3.11.2. Placement of the anther or pollen

The sterilized anthers were transferred aseptically to the pre-sterilized petridishes. Pollen grains were released from the anthers by pressing them gently, and the remaining somatic tissues were removed using a nylon mesh with a pore size of 40μm. The pollen suspension (2-3 ml) was poured directly into a petridish containing callus induction medium. Each and every petridish was labeled with a code that indicates hormonal dose, replication number and date of culture. Then the petridishes were incubated at 25±2^{0}C in

dark under 24 hours dark periods into the growth chamber for 5-7 days or until callus induction. After initiation of callus, it was kept in light where it was 16/8 hours photoperiod. The observation of cultures was started from the 5^{th} day of inoculation and continued up to 45^{th} days.

3.11.3. Inoculation of culture

The explants were prepared carefully under aseptic condition inside the laminar airflow cabinet. One explants was directly inoculated to each petridish containing 10 ml of MS medium supplemented with different hormone concentrations as per treatments. There were three replications for each treatment. The petridishes were covered with lid and sealed with parafilm. The total operation was in the laminar airflow cabinet on the clean bench in sterilize condition.

3.11.4. Maintenance of calli

Callus initiated after 5-7 days of explants inoculation in the medium. The developed calli were also kept under 16 hours photoperiod at 25 ± 2^{0}C. The petridishes were checked daily to note the response and the development of contamination.

3.11.5. Culture incubation conditions

The prepared cultures were kept in a growth room on the shelves. All the cultures were kept at 25 ± 2^{0}C illuminated with 1.83 m florescent tubes (4.83 ft C84 TDFL/Philips). Those tubes broad spectrum of light, especially in the red wave length. The was illuminated 12 hours daily with a light intensity of 2000-3000 lux and monitored by using luxmeter. After five weeks of culture, callus tissues those does not produce any shoot or root, were sub cultured at 28-35 days interval and finally transferred into the new medium. Plantlets developed from callus in this medium after 21-35 days.

3.12. Subculture

3.12.1. Subculture of the callus for shoot regeneration

Five weeks after incubation of explants, the calli attained convenient size. Those which did not produce any shoot or root, were then removed in sterilized petridishes and callus

was cut into small pieces with the help of sharp sterilized surgical blade and placed again on freshly prepared sterilized MS medium in test tubes inside the laminar airflow cabinet for shoot and root initiation. The subcultured test tubes were then incubated at 25±2^{0}C with 12 hours photoperiod. After shoot initiation, more light intensity was given for shoot elongation. The test tubes showing sign on contamination were discarded from the laboratory to reduce the contamination. Repeated subculture was attended at regular of 28-35 days while incubated under the same temperature. The observations and data collections were noted regularly. Rice root tips of regenerated plants were prepared for determination of haploidy chromosome. Chromosome number was determined by chromosome counting of root tips.

3.13. Transplantation

The regenerated plantlets with sufficient root system were ready to transfer in soil. The plantlets with sufficient roots were then taken out from the culture vessels and thoroughly washed in running tap water to remove all culture medium. The plantlets were then transplanted to small pots containing garden soil, sand and cow dung in the ratio 1:2:1. After transplantation, the pots with the plantlets were covered immediately with polythene bag to prevent desiccation. The pots were kept in the controlled environment of glass house for 1-2 days to reduce shock and transferred to normal environment. After 4 days the polythene bags were gradually perforated to expose the plantlets to natural environment and completely removed after 7 days.

3.14. Experimental factors

The experiment consisted of two factors.

I. Genotype
II. Different concentrations of 2, 4-D, NAA, IAA, IBA, BAP, KI and GA_3.

3.14.1. Treatments

In vitro techniques were applied for callus induction and rice plantlet regeneration. Anther and pollen were used as explant. MS (Murashige and Skoog, 1962) medium supplemented with 2, 4-D, NAA, IAA, IBA, BAP, KI and GA_3 was used as treatment combination.

Table 2. Treatments for callus induction in rice

Treatment	Medium/ Hormones
T_1	MS+1.5 mg/l NAA
T_2	MS+2.0 mg/l NAA+0.5 mg/l KI
T_3	MS+2.0 mg/l NAA+2.0 mg/l2,4-D
T_4	MS+2.0 mg/l NAA+2.0 mg/l2,4-D+ 0.5 mg/l KI

Table 3. Treatments for plantlet regeneration in rice

Treatment	Medium/ Hormones
T_1	MS+1.0 mg/l BAP+1.0 mg/l IAA
T_2	MS+1.0 mg/l IBA+1.0 mg/l BAP+2.0 mg/l GA_3
T_3	MS+2.0 mg/l IBA+1.0 mg/l BAP+2.0 mg/l GA_3
T_4	MS+2.0 mg/l IBA+2.0 mg/l BAP+2.0 mg/l GA_3

3.15. Collection of data

To investigate the effect of different treatments of the experiment, data were collected on the following parameters:

3.15.1. *In vitro* callus induction

3.15.1.1. No. of calli induction: After inoculating of anthers or pollens in different test tubes, starting date of inoculation was recorded for each F_1. As soon as any callus was initiated, data on the number of days required to callus induction were recorded.

3.15.1.2. Days to callus induction: Generally callus induction started after few days of explants incubation. Days to callus induction was recorded until callus was not induced from explants. The mean value of the data provided the days to callus induction.

3.15.1.3. Callus color: The color of the callus was taken whether it is white and creamy by visual observation. The color of the callus varies with the light intensity. It was white in color up to 5-7 days when it was kept in dark condition.

3.15.1.4. Texture of the callus: Texture of the callus was measured by either it is friable or non-friable/ compact for their physical characteristics.

3.15.1.5. Callus induction frequency (%): Percentage of callus induction was noted after 7-10 days of inoculation by using the following formula:

$$\text{Callus induction frequency} = \frac{\text{Number of explants induced calli}}{\text{Number of explants inoculated}} \times 100$$

3.15.1.6. Size of callus: It was measured with a plastic set under test tube from base to apex of callus. Size of the callus was recorded at 14, 21, 28 and 35 days after inoculation (DAI) of callus. Callus length was measured horizontally and breadth was measured vertically with the help of digital Slide Calipers. The formula used for estimation the size of callus is given below:

$$\text{Size of callus} = \frac{\text{Breadth} + \text{length}}{2}$$

3.15.1.7. Fresh weight of callus (mg): Callus weights were recorded at 21 and 35 days in grams after inoculation (DAI) of explants with the help of electrical digital balance inside the laminar airflow cabinet with proper precaution. After that the callus was place in its previous place.

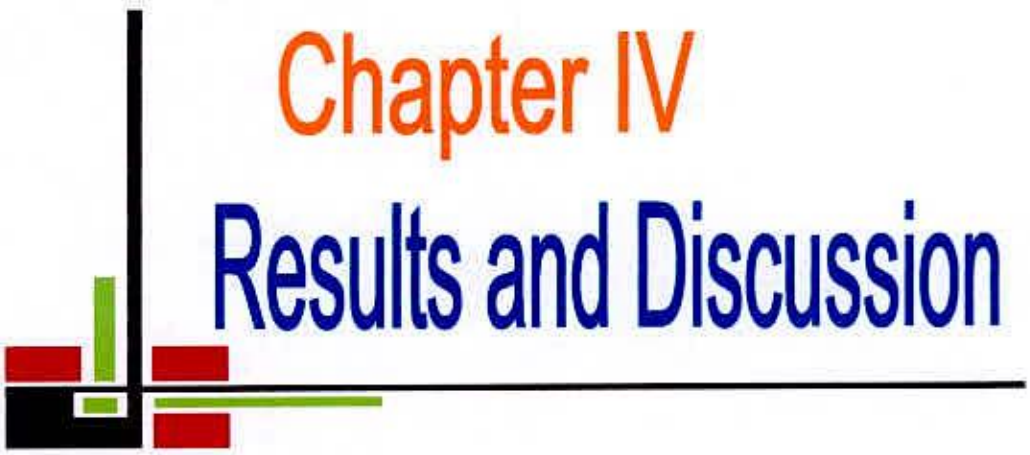
3.15.2. Days to plant regeneration: Shoot initiation started after 21-35 days of incubation of explants. The mean value of the data provided the days required for shoot initiation.

3.15.3. Plantlet regeneration frequency (%): Percentage of plantlet regeneration was noted after 21-35 days of inoculation by using the following formula:

$$\text{Plantlet regeneration frequency} = \frac{\text{Number of calli with plantlets}}{\text{Number of inoculated calli}} \times 100$$

3.16. Statistical analysis of data

The data for the characters under the present study were statistically analyzed wherever applicable. Data were analyzed using MSTAT-C statistical package programe. The experiment was conducted in growth room and arranged in Completely Randomized Design (CRD). The analyses of variance for the different test characters were performed and differences among means were compared by the Least Significant Different Test (lsd) at 5% level of significance.



Chapter IV

Results and Discussion

CHAPTER IV

RESULTS AND DISCUSSION

Two genotypes were used for callus induction and plant regeneration in the present study. The results obtained in different steps from this study have been presented and discussed with tables, figures and plates under the following headings. The analysis of variance has been presented in appendices.

4.1. Days to callus induction

Callus induction was studied on MS media supplemented with different concentration and combination of NAA, 2,4-D and KI and the data was presented in Table 4-5 and Plate 1.

The anther or pollen of two F_1 hybrids of BR 21 x BRRI dhan28 and BR 26 x BRRI dhan29 were used as explants. The effect of hybrids revealed that there was significant difference on days to callus induction. The maximum 24.00, 30.75 and 38.83 days were required at 25, 35 and 45 DAI, respectively to callus induction and the minimum days 23.33, 32.33 and 37.41were needed at 25, 35 and 45 DAI, respectively in both F_1 hybrids (Table 4).

The combined effect of F_1 hybrids and hormone showed significant variation on days to callus induction. The maximum 25.00, 34.67 and 41.67 days were needed to callus induction in the treatment T_1 (MS + 1.5 mg/l NAA) in F_1 hybrid of BR 21 x BRRI dhan28. The minimum 21.33, 28.67 and 36.33 days to callus induction were found in F_1 hybrid of BR 26 x BRRI dhan29 with T_4 (MS + 2.0 mg/l NAA + 2.0 mg/l 2, 4-D + 0.5 mg/l KI) (Table 5). Shikder *et al.* (2006) cultured the explants of aromatic rice (*Oryza sativa* L.) variety Chiniguri on MS media with 6 different concentrations of 2, 4-D and found that MS medium supplemented with 2.5 mg/l 2, 4-D produced the most desired calli. Pandey *et al.* (1994) cultured the explants of 10 rice genotypes on MS medium with 5 different concentrations of 2, 4-D and found that MS medium supplemented with 2.0 mg/l 2, 4-D produced the most desired calli. Azria and Bhalla (2000) also obtained

Table 4. Effect of F_1 hybrids on days to callus induction

F_1 hybrids	No. of anther/ pollen inoculation	No. of calli formed	Days to callus initiation		
			25 DAI	35 DAI	45 DAI
BR 21 x BRRI dhan28	256.66	6.25	23.33	32.33	37.41
BR 26 x BRRI dhan29	279.66	7.41	24.00	30.75	38.83

Table 5. Combined effect of F_1 hybrids and different hormones on days to callus induction

F_1 hybrids	Hormones (mg/l)	No. of anther/ pollen inoculation	No. of calli formed	Days to callus initiation		
				25 DAI	35 DAI	45 DAI
BR 21 x BRRI dhan28	T_1= MS + 1.50 NAA	120b	2.33d	25.00a	34.6a	41.67bc
	T_2= MS + 2.0 NAA + 0.5 KI	115b	2.66d	24.00c	34.0a	38.00cd
	T_3= MS + 2.0 NAA + 2.0 2,4-D	130a	8.66c	22.00d	31.3c	36.33 e
	T_4= MS + 2.0 NAA + 2.0 2, 4-D + 0.5 KI	120b	11.33b	21.33d	29.3d	36.67de
BR 26 x BRRI dhan29	T_1= MS + 1.50 NAA	120b	1.33ef	24.33ab	33.3ab	38.67a
	T_2= MS + 2.0 NAA + 0.5 KI	100c	1.66e	23.33bc	32.0bc	38.00b
	T_3= MS + 2.0 NAA + 2.0 2, 4-D	110c	10.33b	22.33d	29.0d	37.33cd
	T_4= MS + 2.0 NAA + 2.0 2, 4-D + 0.5 KI	120a	16.33a	21.33d	28.67d	36.33e
$Lsd_{(0.05)}$		16.18	0.99	1.32	1.58	1.499
% CV		3.48	8.45	3.27	2.89	2.27

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plantlets from callus, induced from embryos of mature seeds of 4 Australian varieties (Amaroo, Millin, Pelde and Langi) of rice. They found that MS medium supplemented with 0.5- 2.0 mg/l of 2, 4-D is suitable for callus formation.

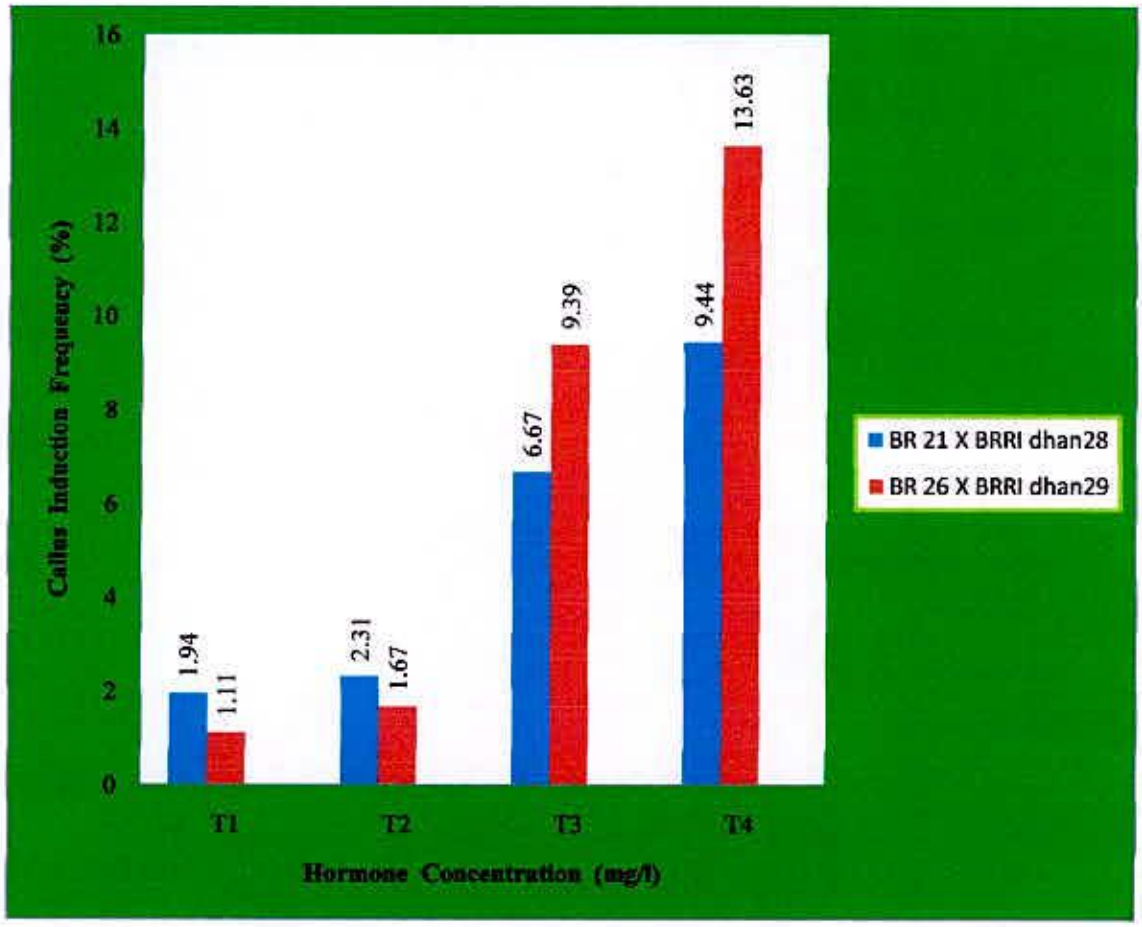
4.2. Callus color and callus texture

Color of calli were white from beginning of induction and changed into creamy white color. Most of the calli were friable in texture. There was variation in days to callus induction.

4.3 Callus induction frequency (%)

Callus induction frequency was varied in the present study. Maximum (9.44%) of callus was induced in treatment T_4 (MS + 2.0 mg/l NAA + 2.0 mg/l 2, 4-D + 0.5 mg/l KI) and minimum (1.94%) of callus was induced in T_1 (MS + 1.5 mg/l NAA) in BR 21 x BRRI dhan28 (Figure 1).

The F_1 hybrid BR 26 x BRRI dhan29 showed maximum (13.63 %) of callus induction in treatment T_4 (MS + 2.0 mg/l NAA + 2.0 mg/l 2, 4-D + 0.5 mg/l KI) and minimum (1.11%) of callus induction in T_1 (MS + 1.5 mg/l NAA). Similar results have been reported by Thuan *et al.* (2001), Yan *et al.* (1996) and Miah *et al.* (1985).



* T_1= 1.5 mg/l NAA, T_2= 2 mg/l NAA + 0.5 mg/l KI, T_3= 2 mg/l NAA + 2 mg/l 2,4-D and T_4= 2.0 mg/l NAA + 2.0 mg/l 2, 4-D + 0.5 mg/l KI supplemented with MS media.

Figure 1. Effect of hormone concentration (mg/l) on callus induction frequency (%)

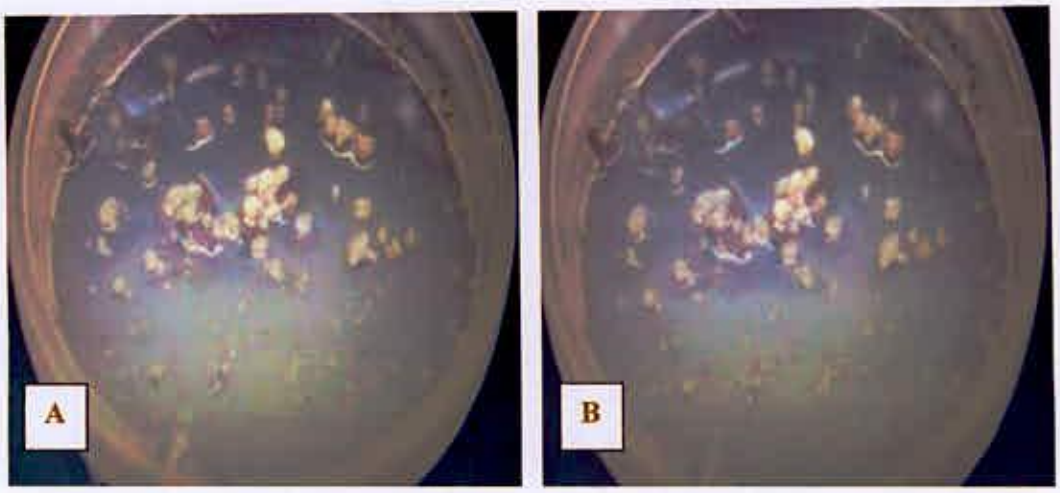


Plate 1. Callus induction on MS media supplemented with 2.0 mg/l NAA + 2.0 mg/l 2,4-D + 0.5 mg/l KI in F_1 hybrids (A) BR 21 x BRRI dhan28 and (B) BR 26 x BRRI dhan29

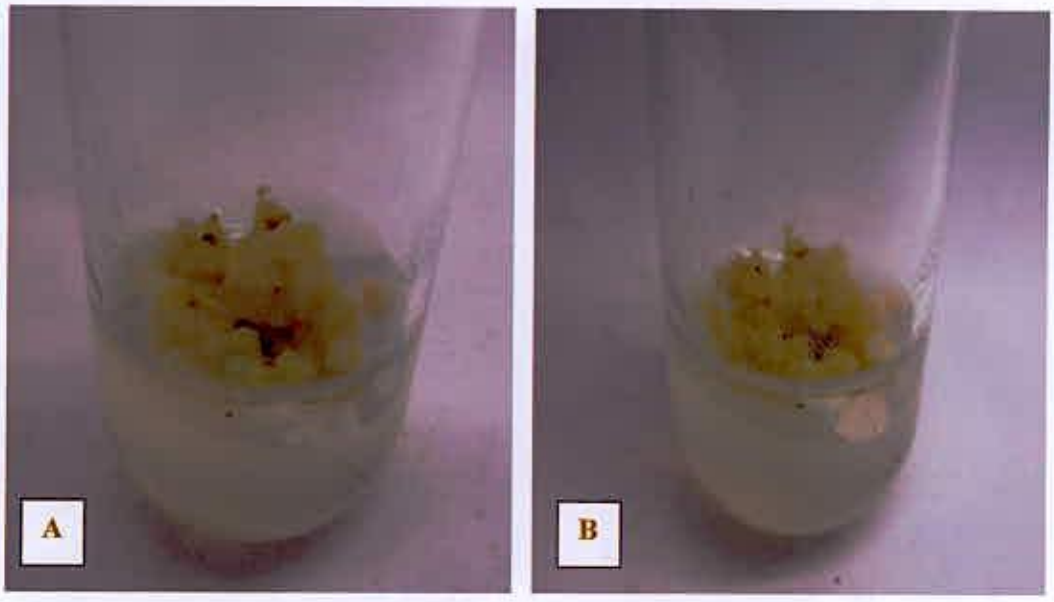


Plate 2. Maximum breadth of callus on MS medium supplemented with 2.0 mg/l NAA + 2.0 mg/l 2,4-D + 0.5 mg/l KI in F_1 hybrids (A) BR 21 x BRRI dhan28 and (B) BR 26 x BRRI dhan29

4.4 Breadth of callus

The breadth of callus was studied on MS media containing with different concentrations of NAA, 2,4-D and KI and the data was recorded in Table 6-7 and Plate 2.

The effect of F_1 hybrid showed significance variation on breadth of callus at different DAI. The highest breadth (1.95 mm) of callus was found in F_1 generation of BR 21 x BRRI dhan28. The breadth of callus gradually increased with the advancement of DAI in this genotype. The minimum breadth (1.52 mm) of callus was found in F_1 of BR 26 x BRRI dhan29 (Table 6).

The combined effect of genotypes and hormone showed significant variation on breadth of callus. The maximum breadth of callus (4.52 mm) was observed at treatment T_4 (MS + 2.0 mg/l NAA + 2.0 mg/l 2, 4-D + 0.5 mg/l KI) in F_1 generation of BR 21 x BRRI dhan28. The lowest breadth of callus (0.64 mm) was noticed at T_1 (MS + 1.5 mg/l NAA) hormonal concentration in same F_1 hybrid (Table 7).

4.5 Length of callus

The length of callus was studied on MS media containing with different concentrations of NAA, 2,4-D and KI and the data was recorded in Table 6-7 and Plate 3.

The effect of F_1 hybrid showed both significance variation on length of callus at different DAI. The maximum length (3.17 mm) of callus was observed in F_1 of BR 26 x BRRI dhan29 whereas the minimum length (2.72 mm) of callus was recorded in F_1 generation of BR 21 x BRRI dhan28 (Table 6).

The combined effect of F_1 hybrid and hormone showed significant variation on length of callus. The maximum length of callus (5.62 mm) was observed at T_4 (MS + 2.0 mg/l NAA + 2.0 mg/l 2, 4-D + 0.5 mg/l KI) in F_1 generation of BR 26 x BRRI dhan29 and the minimum length of callus (1.17 mm) also observed in same hybrid at T_2 (MS + 2.0 mg/l NAA + 0.5 mg/l KI) hormonal concentration (Table 7).

Table 6. Effect of F_1 hybrids on size and fresh weight of callus

F_1 hybrids	Size of callus			Fresh weight of callus (mg)
	Breadth (mm)	Length (mm)	Size (mm)	
BR 21 x BRRI dhan28	1.95	2.72	2.34	1.42
BR 26 x BRRI dhan29	1.52	3.17	2.35	1.80

Table 7. Combined effect of F_1 hybrids and different hormones size and fresh weight of callus

F_1 hybrids	Hormones (mg/l)	Size of callus			Fresh weight of callus (mg)
		Breadth (mm)	Length (mm)	Size (mm)	
BR 21 x BRRI dhan28	T_1= MS +1.50 NAA	0.64 f	2.56 d	1.85 c	0.36 e
	T_2= MS +2.00 NAA + 0.5 KI	0.91 e	2.41 e	1.21 e	0.36 e
	T_3= MS +2.00 NAA + 2.00 2, 4-D	1.23 d	2.23 f	1.73 c	0.67 d
	T_4= MS +2.0 NAA + 2.0 2, 4-D + 0.5 KI	4.52 a	4.93 b	4.73 a	3.84 b
BR 26 x BRRI dhan29	T_1= MS +1.50 NAA	1.13 d	1.91 g	1.70cd	0.35 e
	T_2= MS +2.00 NAA + 0.5 KI	1.53 c	1.17 h	1.97 c	0.53 d
	T_3= MS +2.00 NAA + 2.00 2, 4-D	1.03 de	2.72 c	1.87 c	1.32 c
	T_4= MS +2.0 NAA + 2.0 2, 4-D + 0.5 KI	2.35 b	5.62 a	3.98 b	5.02 a
$Lsd_{(0.05)}$		0.21	0.10	0.50	0.05
% CV		7.43	2.16	12.31	1.40

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4.6 Size of callus

The size of callus was studied on MS media containing with different concentrations of NAA, 2,4-D and KI and the data was recorded in Table 6-7 and Plate 4.

The effect of F_1 hybrid showed significance variation on size of callus at different DAI. The maximum size (2.35 mm) of callus was observed in F_1 generation of BR 26 x BRRI dhan29 whereas the minimum size (2.34 mm) of callus was recorded in F_1 generation of BR 21 x BRRI dhan28 (Table 6).

The combined effect of F_1 hybrid and hormone showed significant variation on size of callus. The maximum size of callus (4.73 mm) was observed at treatment T_4 (MS + 2.0 mg/l NAA + 2.0 mg/l 2, 4-D + 0.5 mg/l KI) and the minimum size of callus (1.21 mm) at T_2 (MS + 2.0 mg/l NAA + 0.5 mg/l KI) with the F_1 generation of BR 21 x BRRI dhan28 (Table 7). The size of callus varied genotype to genotype. Rajbhog had maximum size (4-6mm) of callus but Chinigura produced poor size (1-3mm) of callus had been reported by Iqbal (2006).

4.7 Fresh weight of callus

The fresh weight of callus was studied on MS media containing with different concentrations of NAA, 2,4-D and KI and the data was recorded in Table 6-7 and Plate 5.

There was significant difference in weight of callus between F_1 hybrids. The maximum weight of callus was observed in F_1 generation of BR 26 x BRRI dhan29 (1.80 mg) whereas the minimum weight of callus was recorded (1.42 mg) in F_1 generation of BR 21 x BRRI dhan28 (Table 6).

There was significant interaction effect in combination the F_1 hybrid and hormone concentration on callus weight. The maximum weight of callus (5.02 mg) was observed in the T_4 (MS + 2.0 mg/l NAA + 2.0 mg/l 2, 4-D + 0.5 mg/l KI) and the minimum weight of callus (0.35 mg) at T_1 (MS + 1.5 mg/l NAA) in F_1 generation of BR 26 x BRRI dhan29 (Table 7).

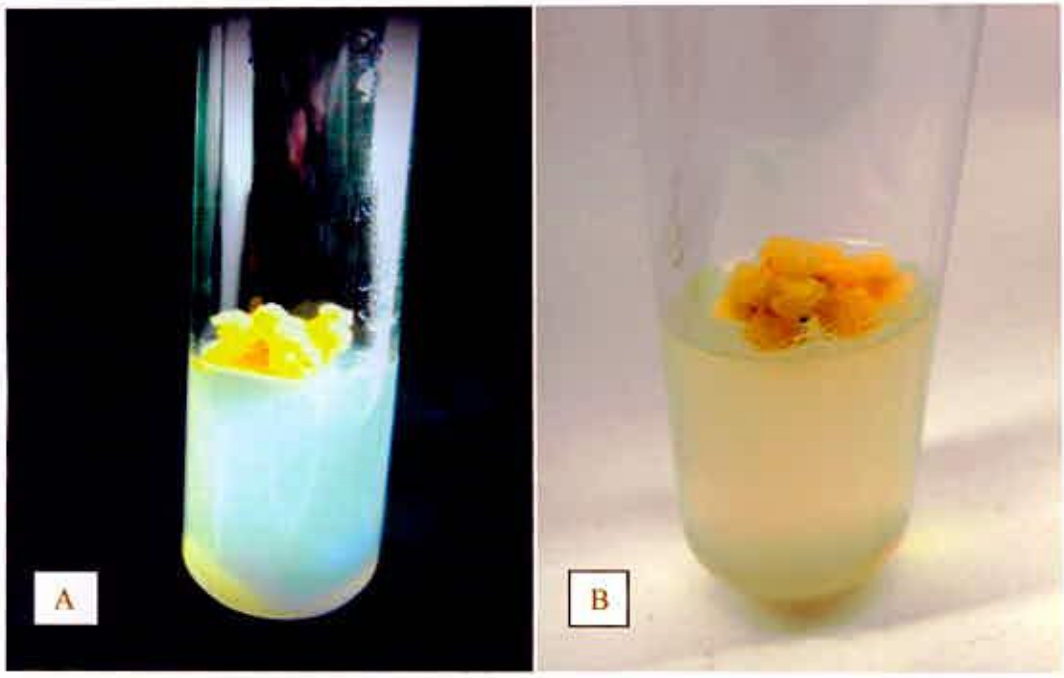

Plate 3. Maximum length of callus on MS medium supplemented with 2.0 mg/l NAA + 2.0 mg/l 2,4-D + 0.5 mg/l KI in F_1 hybrids (A) BR 21 x BRRI dhan28 and (B) BR 26 x BRRI dhan29

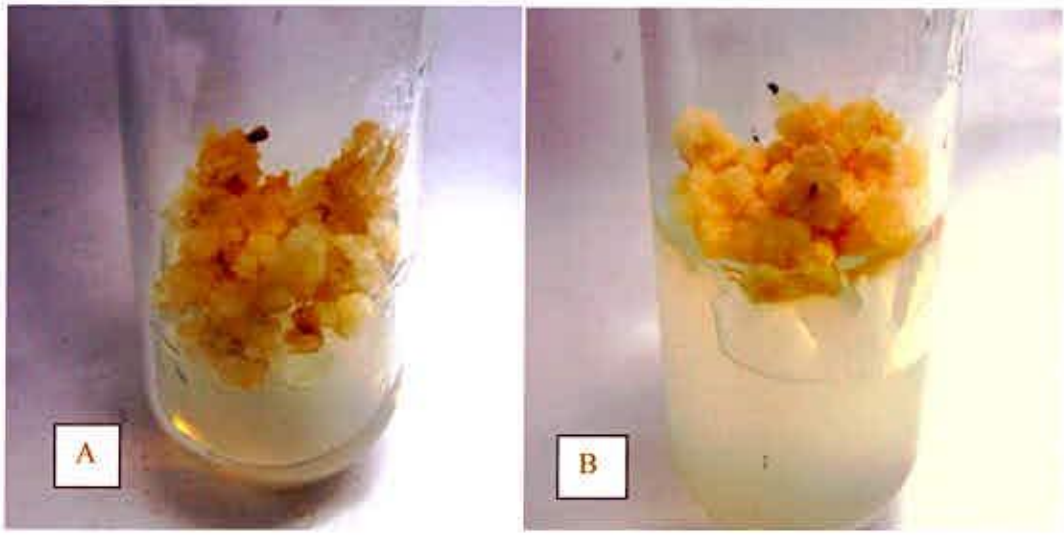

Plate 4. Maximum size of callus on MS medium supplemented with 2.0 mg/l NAA + 2.0 mg/l 2,4-D + 0.5 mg/l KI in F_1 hybrids (A) BR 21 x BRRI dhan28 and (B) BR 26 x BRRI dhan29

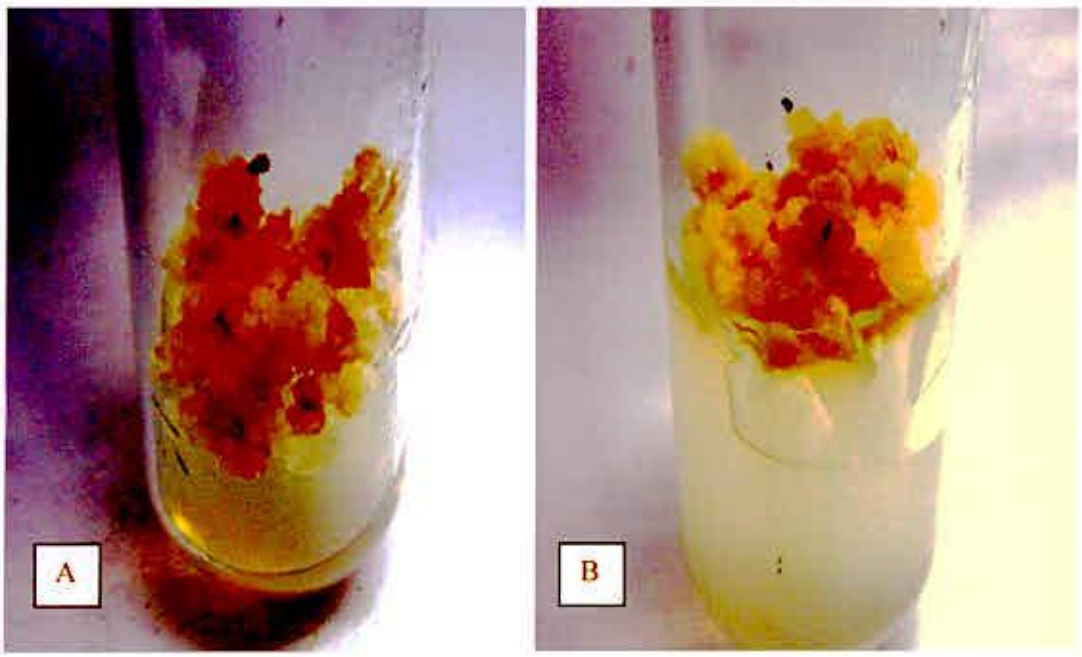

Plate 5. Maximum weight of callus on MS medium supplemented with 1.5 mg/l NAA + 2.5 mg/l 2,4-D + 0.5 mg/l KI in F_1 hybrids (A) BR 21 x BRRI dhan28 and (B) BR 26 x BRRI dhan29

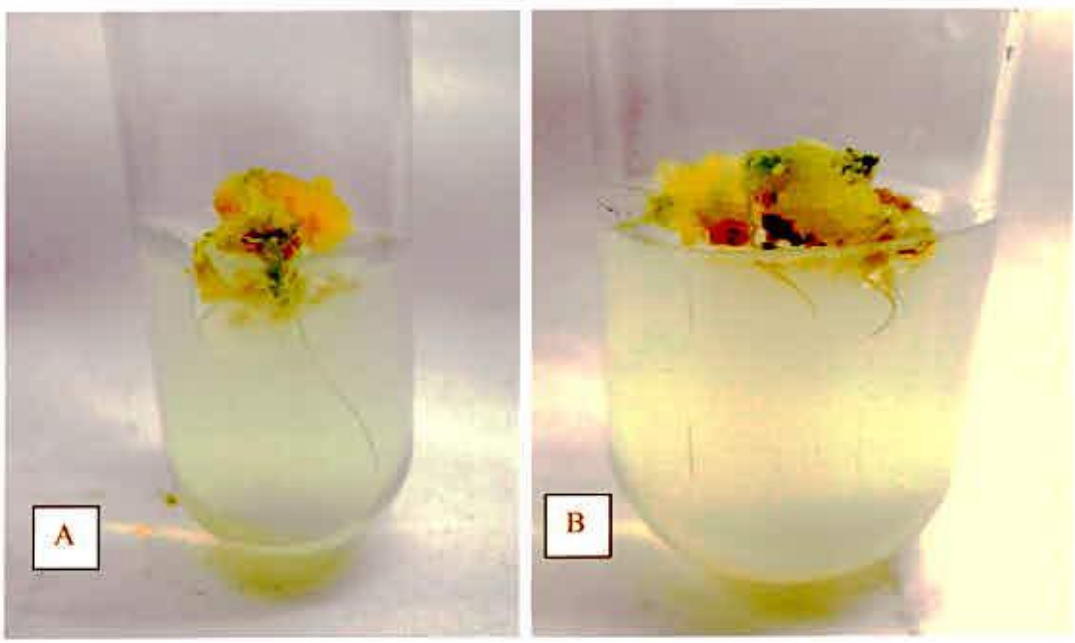

Plate 6. Initiated plantlet on MS media containing with 2.0 mg/l IBA + 2.0 mg/l BAP + 2.0 mg/l GA3 in F_1 hybrids (A) BR 21 x BRRI dhan28 and (B) BR 26 x BRRI dhan29

4.8. Days to plant regeneration

The data of days to plant regeneration was recorded in Table 8-9 and Plate 6.

Rice F_1 hybrid was differed significantly on days to plant regeneration from callus. The effect of F_1 hybrid on days to plant regeneration have been presented in Table 8. The callus of two F_1 hybrids BR 21 x BRRI dhan28 and BR 26 x BRRI dhan29 were used as explants and cultured on MS medium supplemented with different concentrations of IBA, BAP, GA_3 and IAA. The effect of F_1 hybrid revealed that there was significant difference on days to plant regeneration. The maximum 33.83 days were required to plant regeneration and the minimum 31.00 days were needed in both F_1 hybrids.

The combined effect of F_1 hybrid and hormone showed significant variation on days to plant regeneration. The minimum (21.67 days) to plant regeneration was noticed at T_3 (MS + 2.0 mg/l IBA + 1.0 mg/l BAP + 2.0 mg/l GA_3) in F_1 generation of BR 26 x BRRI dhan29. The maximum (39.83 days) to plant regeneration was observed in F_1 generation of BR 21 x BRRI dhan28 with T_1 (MS + 1.0 mg/l BAP+1.0 mg/l IAA) (Table 9). Regeneration of green plants is greatly influenced by age and size of callus. Wang *et al.* (1977) and Chen *et al.* (1986 and 1978) reported that rice callus induced in early stage (around 30- 50 days after inoculation), offers high differentiation for green plants.

4.9. Green and albino plant regeneration from pollen derived calli

Green and albino plant regeneration was studied on MS media containing with different concentrations of IBA, BAP and IAA and the data was recorded in Table 8-9, Plate 7-8 and Figure 2.

The effect of F_1 hybrid revealed that there was significant difference on plant regeneration. The maximum number of green plants (0.94) and the minimum number of green plants (0.82) were noticed in F_1 generation of BR 21 x BRRI dhan28 and BR 26 x BRRI dhan29. The maximum number of albino plants (1.33) and the minimum number

Table 8. Effect of F_1 hybrids on no. of plantlet regeneration from callus and days to plantlet regeneration

F_1 hybrids	No. of calli cultured	No. of plantlet regeneration		Days to plantlet regeneration
		Green	Albino	
BR 21 x BRRI dhan28	13.00	0.82	1.09	31.0
BR 26 x BRRI dhan29	12.33	0.94	1.33	33.83

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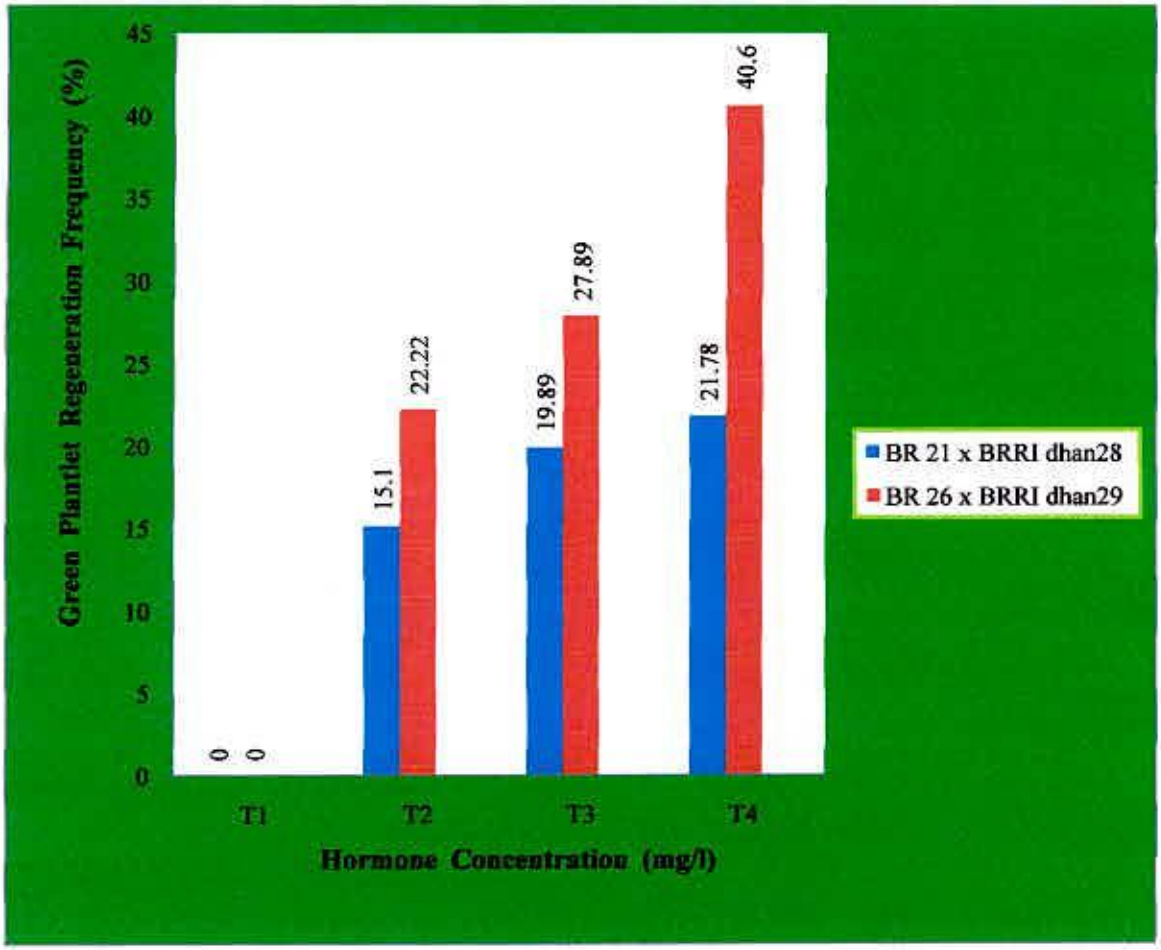
Table 9. Combined effect of F_1 hybrids and different hormones on no. of plantlet regeneration from callus and days to plantlet regeneration

F_1 hybrids	Hormones (mg/l)	No. of calli cultured	No. of plantlet regeneration		Days to plantlet regeneration
			Green	Albino	
BR 21 x BRRI dhan28	T_1= MS +1.0 BAP + 1.0 IAA	10.67 a	0 d	1.23bc	39.83a
	T_2= MS +1.0 IBA + 1.0 BAP + 2.0 GA_3	10.00 a	1.51 c	1.30b	31.83c
	T_3= MS +2.0 IBA + 1.0 BAP + 2.0 GA_3	9.50 b	1.89 c	0.90c	32.50c
	T_4= MS +2.0 IBA + 2.0 BAP + 2.0 GA_3	9.00 c	1.96 c	0.95de	38.00a
BR 26 x BRRI dhan29	T_1= MS +1.0 BAP + 1.0 IAA	9.16 c	0 d	1.15bc	25.67d
	T_2= MS +1.0 IBA + 1.0 BAP + 2.0 GA_3	9.00 c	2.00 b	1.22bc	34.83b
	T_3= MS +2.0 IBA + 1.0 BAP + 2.0 GA_3	9.50 b	2.65 b	1.10cd	21.67e
	T_4= MS +2.0 IBA + 2.0 BAP + 2.0 GA_3	10.00 a	4.06 a	1.87 a	35.00b
Lsd $_{(0.05)}$		1.75	0.11	0.17	0.98
% CV		10.87	10.90	12.12	2.61

of albino plants (1.09) were noticed in F_1 generation of BR 21 x BRRI dhan28 and BR 26 x BRRI dhan29. The highest normal plantlet regeneration (40.60%) was found in the F_1generation of BR 26 x BRRI dhan29 at the T_4 (MS + 2.0 mg/l IBA + 2.0 mg/l BAP + 2.0 mg/l GA_3) but no plantlet regeneration was found from both F_1 hybrids with T_1 (MS + 1.0 BAP + 1.0 IAA) (Figure 2). Plantlet regeneration was observed in MS media with different combination and concentration of BAP, kinetin and NAA by Jubair *et al.* (2008). Roy and Mandal (2011) clearly indicated that higher concentration of BAP had inhibitory effects on microtillering of androgenic plantlets of rice variety IR 72.

The combined effect of F_1 hybrid and hormone showed significant variation on green and albino plant regeneration. The maximum number of green plants (4.06) and albino plants (1.87) regeneration were noticed at T_4 (MS + 2.0 mg/l IBA + 2.0 mg/l BAP + 2.0 mg/l GA_3) in F_1 of BR 26 x BRRI dhan29. There was no regeneration was observed in both F_1 hybrids with T_1 (MS + 1.0 mg/l BAP+1.0 mg/l IAA). Regeneration of albino plants has been reported as a major problem in rice anther culture especially in indica rice (Asaduzzaman, *et al.*, 2003; Chen *et al.*, 1991; Shi-Wei and Zhi-Hong, 1991). Oono (1975) reported the differentiation of primarily green plant from micro spore derived callus of the japonica variety Minehikari. Occurrence of albino pollen plants seems to be a common phenomenon in pollen plants of *Gramineae.* The recover of primarily albino plants from micro spore derived calli has been a formidable obstacle to the utilization of rice anther culture.



* T_1= 1 mg/l BAP + 1 mg/l IAA, T_2= 1 mg/l IBA + 1 mg/l BAP + 2 mg/l GA_3, T_3= 2 mg/l IBA + 1 mg/l BAP + 2 mg/l GA_3 and T_4= 2 mg/l IBA + 2 mg/l BAP + 2 mg/l GA_3 supplemented with MS medium

Figure 2. Effect of hormone concentration (mg/l) on green plantlet regeneration frequency (%)

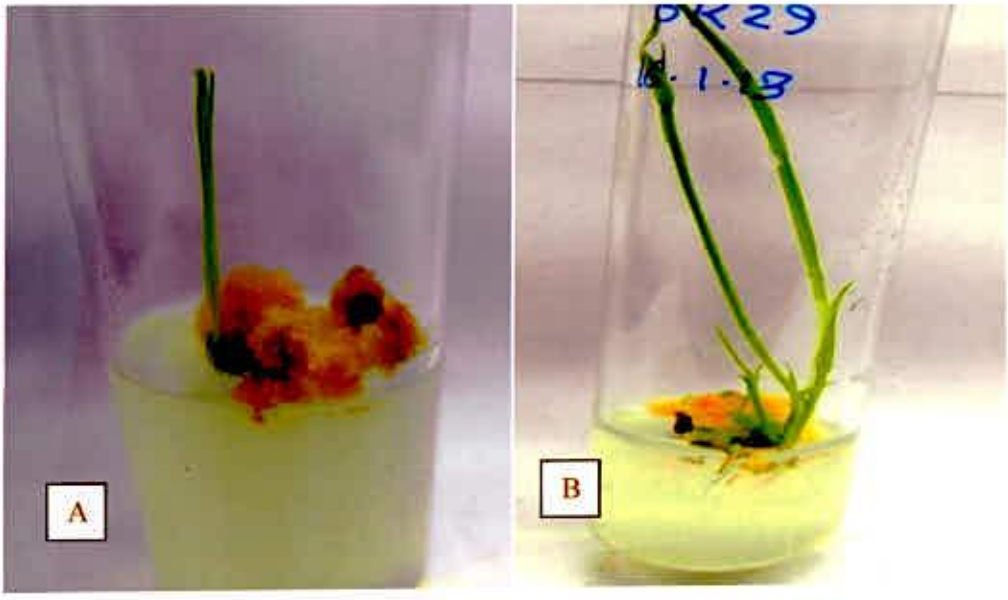


Plate 7. Regenerated green plantlets on MS media containing with 2.0 mg/l IBA + 2.0 mg/l BAP + 2.0 mg/l GA_3 in F_1 hybrids (A) BR 21 x BRRI dhan28 and (B) BR 26 x BRRI dhan29

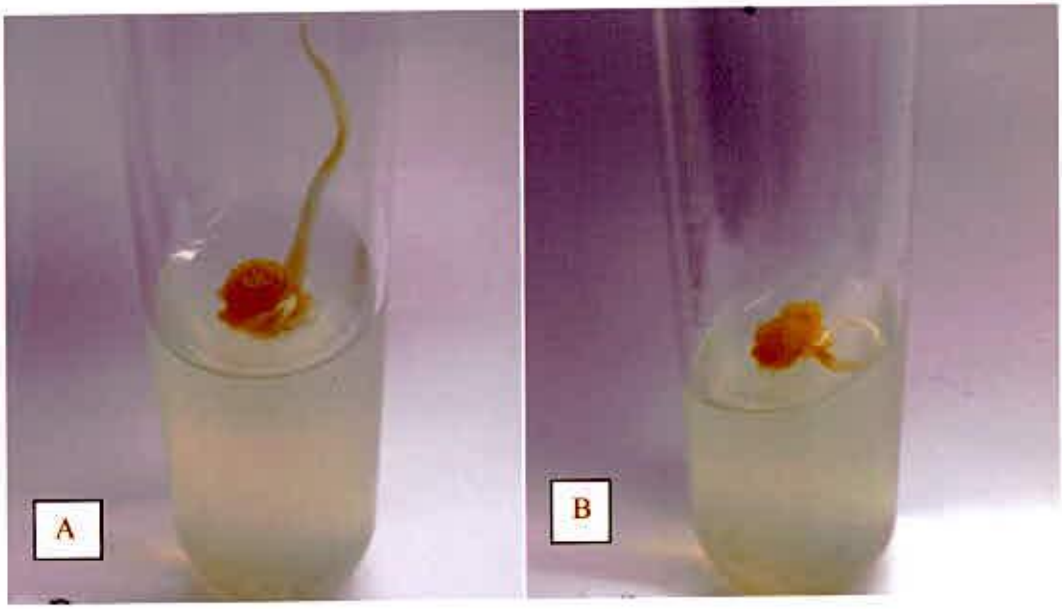


Plate 8. Regenerated albino plantlet on MS media containing with 2.0 mg/l IBA + 2.0 mg/l BAP + 2.0 mg/l GA_3 in F_1 hybrids (A) BR 21 x BRRI dhan28 and (B) BR 26 x BRRI dhan29

4.10. Number of leaf and root per plantlet

The data of number of leaf and root per plantlet was recorded in Table 10-11 and Plate 9.

The effect of F_1 hybrid revealed that there was significant difference on number of leaf and number of root per plantlet. The highest number of leaves (4.85 leaves/plantlet) and the lowest (3.20 leaves/plantlet) were produced in F_1 generation of BR 26 x BRRI dhan29 and BR 21 x BRRI dhan28. The highest number of roots (15.16 roots/plantlet) and the lowest (12.45 roots/plantlet) were found in F_1 generation of BR 26 x BRRI dhan29 and BR 21 x BRRI dhan28 (Table 10).

The combined effect of F_1 hybrid of rice and hormone showed significant variation on number of leaf and number of root per plantlet. The highest number of leaves (5.00 leaves/plantlet) was noticed at T_4 (MS + 2.0 mg/l IBA + 2.0 mg/l BAP + 2.0 mg/l GA_3) in F_1 generation of BR 26 x BRRI dhan29. The highest number of roots (21.00 roots/plantlet) were produced at T_3 (MS + 2.0 mg/l IBA + 1.0 mg/l BAP + 2.0 mg/l GA_3) in F_1 generation of BR 26 x BRRI dhan29 (Table 11). Average number of roots, leaves, root length and shoot length was better in LS (agar) medium in both the cultivars (Super Basmati and IRRI-6) had been reported by Afrasiab and Jafar (2011).

4.11. Transplanting and growing of anther derived plants

Garden soil, sand and cow dung in the ratio 1:2:1 was mixed properly and autoclaved one hour in 121^{0}C for 20 minutes at 1.16 kg/cm^2. The soil mixture was taken into 10 cm pots after cooling for growing of plantlets in natural condition. When the plantlets became 5-8 cm in height with sufficient shoot and root system, they were taken out from the vials without damaging roots. Medium attached with root gently washed out in running tap water to prevent further microbial infection. The plantlets were then transplanted to pot containing sterilized potting mixture. Immediately after transplantation the plants along with the pots were covered with moist polythene bags to prevent desiccation. To reduce sudden shock the pots were kept in a growth room for 7-15 days under controlled environments (Plate 10).

Table 10. Effect of F_1 hybrids on number of leaf and root from one plantlet

F_1 hybrids	Number of leaf/plantlet	Number of root/plantlet
BR 21 x BRRI dhan28	3.20	12.45
BR 26 x BRRI dhan29	4.85	15.16

Table 11. Combined effect of F_1 hybrids and different hormones on number of leaf and root from one plantlet

F_1 hybrids	Hormones (mg/l)	Number of leaf	Number of root
BR 21 x BRRI dhan28	T_1= MS +1.0 BAP + 1.0 IAA	1.66 e	5.83 f
	T_2= MS +1.0 IBA + 1.0 BAP + 2.0 GA_3	3.33 c	8.00 e
	T_3= MS +2.0 IBA + 1.0 BAP + 2.0 GA_3	3.30 c	15.83 b
	T_4= MS +2.0 IBA + 2.0 BAP + 2.0 GA_3	4.00 b	20.17 a
BR 26 x BRRI dhan29	T_1= MS +1.0 BAP + 1.0 IAA	1.50 e	13.00 bc
	T_2= MS +1.0 IBA + 1.0 BAP + 2.0 GA_3	2.33 d	11.00 d
	T_3= MS +2.0 IBA + 1.0 BAP + 2.0 GA_3	3.36 c	21.00 a
	T_4= MS +2.0 IBA + 2.0 BAP + 2.0 GA_3	5.00 a	15.67 b
$Lsd_{(0.05)}$		1.92	1.13
% CV		13.17	7.04

Table 12. Survival rate of regenerated plantlets of rice

F_1 hybrids	Number of transplanted plants	Number of plants survives	Survival rate (%)
BR 21 x BRRI dhan28	20	8	40
BR 26 x BRRI dhan29	18	10	55.55

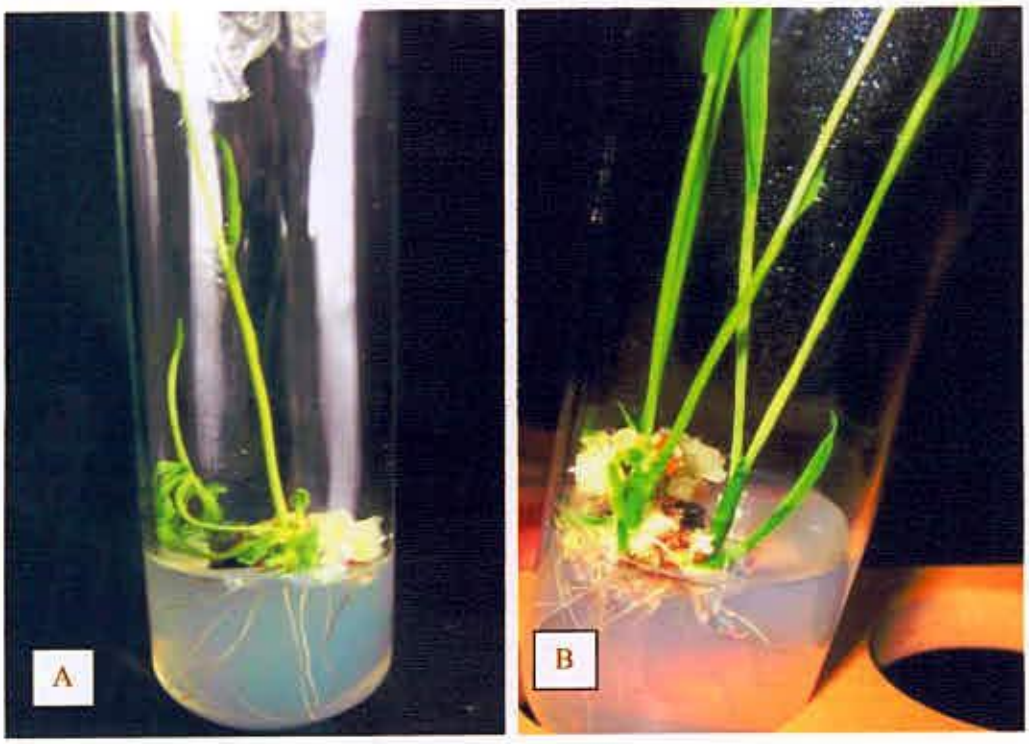


Plate 9. Maximum number of leaves and roots initiated on MS media containing with 2.0 mg/l IBA + 2.0 mg/l BAP + 2.0 mg/l GA_3 in F_1 hybrids (A) BR 21 x BRRI dhan28 and (B) BR 26 x BRRI dhan29

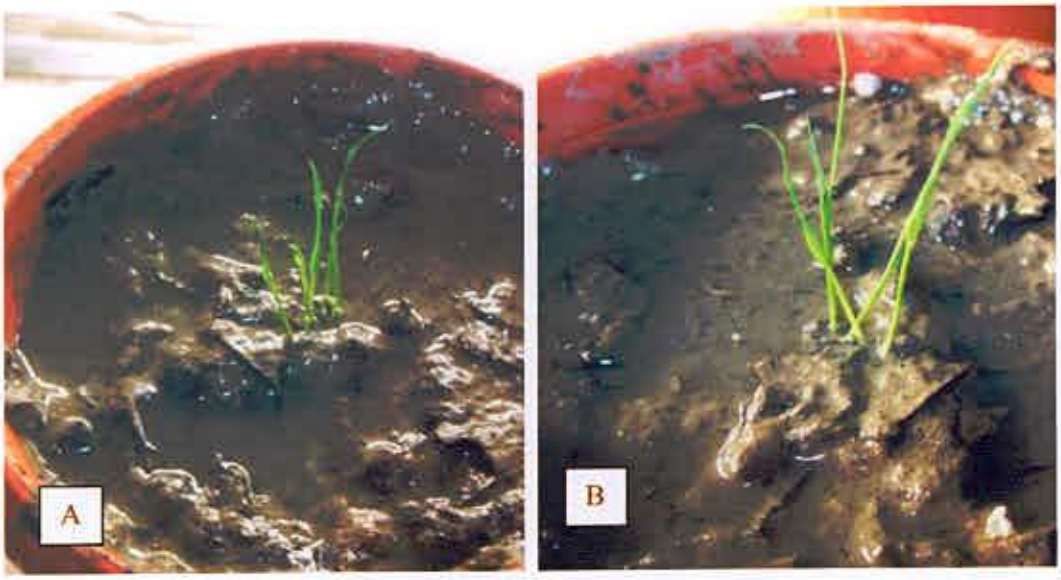


Plate 10. *In vivo* acclimatization of regenerated plantlets in growth chamber (A) BR 21 x BRRI dhan28 and (B) BR 26 x BRRI dhan29

Plate 11. *In vivo* establishment of regenerated plantlets in open atmosphere (A) BR 21 x BRRI dhan28 and (B) BR 26 x BRRI dhan29

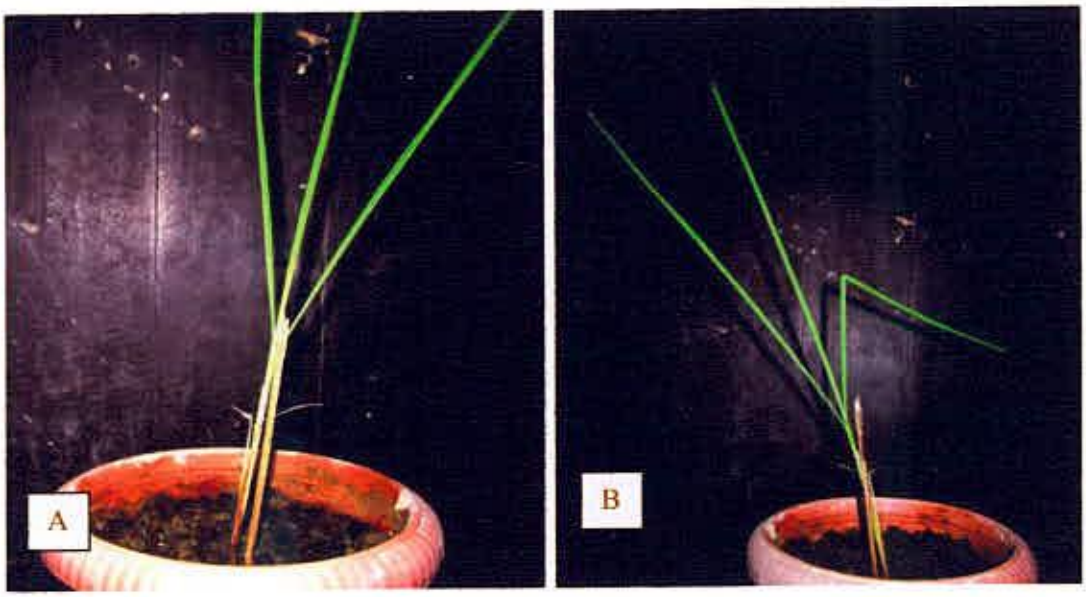


Plate 12. Hardening of plantlets in pot (A) BR 21 x BRRI dhan28 and (B) BR 26 x BRRI dhan29

The interior of the polythene bags was sprayed with distilled water at every 24 hours to maintain high humidity around the plantlets. After 2-3 days, the polythene bags were gradually perforated to expose the plants to natural environment. The polythene bags were completely removed after 10-15 days when the plantlets, appeared to be self-sustainable. At this stage, the plantlets were placed in natural environment for 3-10 hours daily (Plate 11).

Finally, after 15-20 days, they were transferred to the net house for hardening and after proper hardening the plantlets were transplanted to the soil (Plate 12).

As soon as new leaves started to initiate, plants were watered with ordinary tap water. Gradually the plantlets were adapted to the soil.

From Table 12, among 20 transplanted plants of F_1 hybrid BR 21 x BRRI dhan28 only 8 plants were survived. Ten (10) plants were survived among 18 transplanted plants of F_1 hybrid BR 26 x BRRI dhan29. 55.55% survival rate was obtained in F_1 hybrid BR 26 x BRRI dhan29. Similar result had been reported by Herath *et al.* (2007).

From the above investigation it can be revealed that BR 26 x BRRI dhan29 is a good F_1 hybrid for further studies.

Chapter V

Summary and Conclusion

CHAPTER V

SUMMARY AND CONCLUSION

The present experiment was conducted at the Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Sher-e Bangla Nagar, Dhaka-1207. The experiment was set up July, 2011 to December, 2012 from anther or pollen of two F_1 hybrid viz. BR 21 x BRRI dhan28 and BR 26 x BRRI dhan29 to obtain *in vitro* callus induction and haploid plant regeneration of rice by using different concentrations and combinations of 2,4-D, NAA, IBA, BAP, IAA, GA_3 and KI supplemented with MS media. The experiment was conducted at Completely Randomized Design (CRD) with three replications. The concentrations of NAA were 1.5, and 2.0 mg/l, 2,4-D were 2.0 mg/l, IAA were 1.0 mg/l, BAP were 1.0 and 2.0 mg/l , IBA were1.0 and 2.0 mg/l, GA_3 were 2.0 mg/l and KI were 0.5 mg/l. The combined effect of both hormones was also studied. The parameters were recorded on days to callus induction, callus color and texture, percentage of callus induction, breadth, length and size of callus, fresh weight of callus, days to plant regeneration, number of leaves/plantlet and number of roots/plantlet.

It revealed that days to callus induction, breadth, length, size and weight of callus were significantly influenced by two F_1s and different concentrations of 2,4-D, NAA and KI. The minimum 23.33, 32.33 and 37.41days were needed at 25, 35 and 45 DAI, respectively in F_1 hybrid BR 21 x BRRI dhan28. The minimum 21.33, 28.67 and 36.33 days were required to callus induction at T_4 (MS + 2.0mg/l NAA + 2.0mg/l 2, 4-D + 0.5 mg/l KI) in F_1 BR 26 x BRRI dhan29. Maximum (13.63 %) of callus was obtained at T_4 (MS + 2.0 mg/l NAA + 2.0 mg/l 2, 4-D + 0.5 mg/l KI) in F_1 hybrid BR 26 x BRRI dhan29 but the minimum (1.11%) of callus was induced in same genotype. The maximum size (4.73 mm) and the minimum size (1.21 mm) of callus were observed in F_1 generation of BR 21 x BRRI dhan28. Maximum length (5.62 mm) and the minimum (1.17mm) of callus were noticed in F_1 generation of BR 26 x BRRI dhan29. The maximum breadth (4.52 mm) and the minimum (0.64mm) were observed in BR 21 x

BRRI dhan28.The maximum weight (5.02 mg) of callus was observed in BR 26 x BRRI dhan29 at T_4 (MS + 2.0 mg/l NAA + 2.0 mg/l 2, 4-D + 0.5 mg/l KI).

In the case of days to plantlet initiation, it was noticed that the genotype BR 26 x BRRI dhan29 showed minimum (21.67 days) for green plantlet regeneration at T_3 (MS + 2.0mg/l IBA + 1.0mg/l BAP + 2.0mg/l GA_3). The maximum number of green plants (4.06) and albino plants (1.87) regeneration were noticed at T_4 (MS + 2.0mg/l IBA + 2.0mg/l BAP + 2.0mg/l GA_3) in F_1 generation of BR 26 x BRRI dhan29. Maximum (40.60%) green plant was regenerated in the F_1 generation of BR 26 x BRRI dhan29 at the T_4 (MS + 2.0 mg/l IBA + 2.0 mg/l BAP + 2.0 mg/l GA_3). The highest number of leaves (5.00 leaves/plantlet) was noticed in F_1 hybrid BR 26 x BRRI dhan29 at T_4 (MS + 2.0mg/l IBA + 2.0mg/l BAP + 2.0mg/l GA_3). The highest number of roots (21.00 roots/plantlet) were produced in F_1 generation of BR 26 x BRRI dhan29 at T_3 (MS + 2.0mg/l IBA + 1.0mg/l BAP + 2.0mg/l GA_3).

For acclimatization, plantlets were transplanted from culture medium to soil in pot, where percentage of survival of rice plantlet was the highest 55.55 in F_1 generation of BR 26 x BRRI dhan29.

There were two F_1 hybrids and four hormonal treatments were used to generate haploid of rice plants through pollen culture. 2.0mg/l NAA + 2.0mg/l 2, 4-D + 0.5mg/l KI hormonal treatment supplemented with MS medium showed the best result in response to days to callus induction and callus induction frequency. Considering the finding of the present study MS medium supplemented with 2.0mg/l IBA + 2.0mg/l BAP + 2.0 mg/l GA_3 was found to be the best for plantlet regeneration. Present study suggested that BR 26 x BRRI dhan29 was a good F_1 hybrid for further studies.

The protocol developed from the present study may be useful tool for breeder to develop homozygous haploid plants for rice. This protocol could be used for *in vitro* breeding programme.

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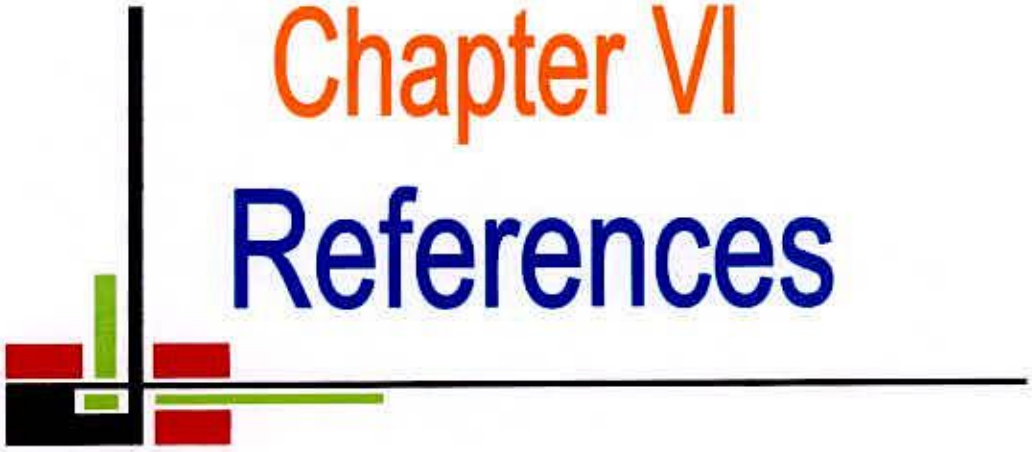

Chapter VI
References

CHAPTER VI

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Chapter VII
Appendices

CHAPTER VII

APPENDICES

Appendix I: Composition and stock solution of MS (Murashige and Skoog, 1962) medium

	Major salts (10x)	mg/l	g/l
STOCK A	KNO_3	1900	19.00
	NH_4NO_3	1650	16.50
	$MgSO_4.7H_2O$	370	3.370
	$CaCl_2.2H_2O$	440	4.40
	KH_2PO_4	170	1.70
STOCK B	**Minor salts (100x)**		
	KI	0.83	83
	H_3BO_3	6.2	620
	$MnSO_4.4H_2O$	22.3	2230
	ZnSO4.7H2O	8.6	860
	$Na_2MoO_4.2H_2O$	0.25	25
	$CuSO_4.5H_2O$	0.025	2.5
	$CoCl_2.6H_2O$	0.025	2.5
STOCK C	**Iron EDTA Soln. (100x)**		
	$FeSO_4.7H_2O$	27.8	2.78
	$Na_2EDTA.2H_2O$	37.3	37.3
STOCK D	**Organics (100x)**		
	Myo-inositol	100	10000
	Nicotinic acid	0.5	50
	Pyridoxin HCl	0.5	50
	Thamine Hcl	0.1	10
	Glycin	2.0	200

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Appendix II: Analysis of variance of days to callus induction and size of callus

Source of Variation	Degrees of freedom (df)	Callus induced frequency	Size of callus (mm)	Days to callus initiation		
				25 DAI	35 DAI	45 DAI
F_1 hybrid (A)	1	0.89**	0.00	69.00**	15.04**	12.04**
Hormones (B)	3	30.06**	10.46**	39.42**	33.04**	18.48**
Interaction (A x B)	3	1.19**	0.58**	29.22**	0.81*	3.04**
*: Significant at 0.05 level of probability; **: Significant at 0.01 level of probability						

Appendix III: Analysis of variance of days to plantlet and no. of plantlet regeneration

Source of Variation	Degrees of freedom (df)	No. of plantlet regeneration		Green plantlet frequency	Days to plantlet regeneration
		Green	Albino		
F_1 hybrid (A)	1	0.19**	0.71**	422.03**	96.33**
Hormones (B)	3	4.27**	0.34**	641.38**	432.55**
Interaction (A x B)	3	0.06**	0.66**	207.84**	55.66**
*: Significant at 0.05 level of probability; **: Significant at 0.01 level of probability					

Appendix IV: Analysis of variance of number of leaf and root

Source of Variation	Degrees of freedom (df)	Number of leaf	Number of root
F_1 hybrid (A)	1	40.33**	88.02**
Hormones (B)	3	292.08**	303.85**
Interaction (A x B)	3	6.55	77.96**
*: Significant at 0.05 level of probability; **: Significant at 0.01 level of probability			

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