EFFECTS OF DIFFERENT TYPES AND CONCENTRATION OF SUGAR ON CALLUS INDUCTION AND PLANT REGENERATION IN RICE (*Oryza sativa* L.) SEED CULTURE

BY

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This is to certify that thesis entitled, ***"Effects of Different Types and Concentration of Sugar on Callus Induction and Plant Regeneration in Rice (Oryza sativa* L.) *Seed Culture"*** *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* ***Mohammad Ataul Hoque, Roll No. 00428, Registration No. 26128/00428*** *under my supervision and my guidance. No part of the thesis has been submitted for any other degree or diploma.*

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

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Dedicated TO
MY
Beloved Parents

LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
°C	:	Degree Celsius
BRRI	:	Bangladesh Rice Research Institute
cm	:	Centimeter
Conc.	:	Concentration
Contd.	:	Continued
CRD	:	Completely Randomized Design
cv	:	cultivar
DMRT	:	Duncan's Multiple Range Test
dw	:	Distilled water
et al	:	Et alu = other people
Fig.	:	Figure
g/L	:	Gram per liter
$HgCl_2$	:	Mercuric Chloride
IRRI	:	International Rice Research Institute
KCl	:	Potassium Chloride
mg	:	Milligram
mg/L	:	Milligram per liter
mha	:	Million hectares
mL	:	Milliliter
MS	:	Murashige and Skoog
Na_2SO_4	:	Sodium Sulphate
NaCl	:	Sodium Chloride
NS	:	Not significant
var	:	variety
viz.	:	namely

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EFFECTS OF DIFFERENT TYPES AND CONCENTRATIONS OF SUGAR ON CALLUS INDUCTION AND PLANT REGENERATION IN RICE (*Oryza sativa* L.) SEED CULTURE

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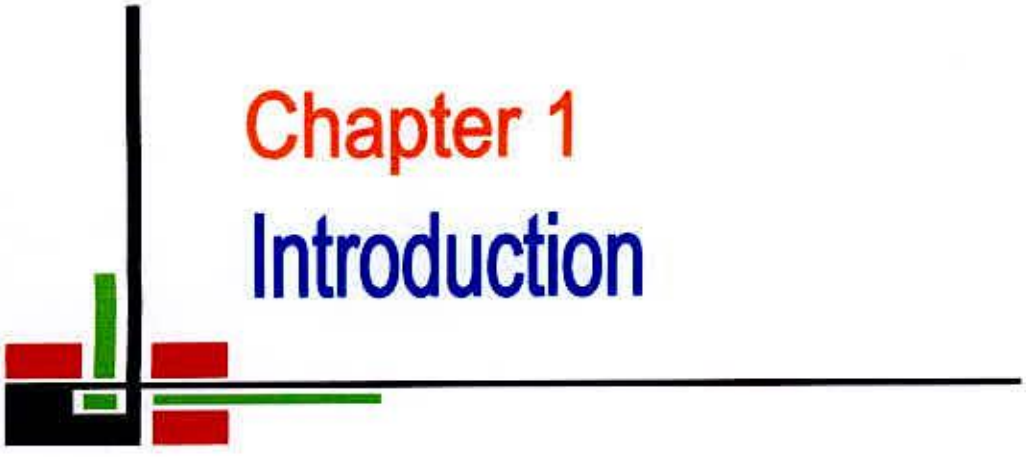
ABSTRACT

An experiment was conducted at Tissue Culture Laboratory, Biotechnology Division, Bangladesh Rice Research Institute (BRRI), Joydebpur, Gazipur during the period from January to May 2007 to investigate the effect of sugar on *in vitro* culture of six indica rice varieties, i.e. BR1, BR2, BR3, BR4, BR-5 and Taipei309 to find out the optimum concentration of sugar for germination, callus induction and plant regeneration. Murashige and Skoog (MS) medium supplemented with 2 mg/L 2, 4-D and three different types of sugar having the concentration of 2,3,and 4 g/L were used as treatment and mature seeds of rice varieties were used as explant. The highest germination (97.14%) was obtained from variety BR3 from sucrose treated explant at a concentration of 2 g/L and the lowest germination (35.71%) was recorded from BR4 treated with maltose at a concentration of 2 g/L. The highest callus induction (96.46%) was obtained from BR5 with sucrose treatment at a concentration of 2 g/L but the lowest callus induction (35.15%) was recorded from BR4 from plate treated with at a concentration of 2 g/L. The highest regeneration (54.55%) was recorded from BR5 treated with sucrose at a concentration of 2 g/L followed by Taipei309 (34.78%) at a concentration of 2 g/L. No regeneration was found in case of sucrose from BR2, BR3, BR4 at 2 g/L, BR1, BR2, BR3 at 3 g/L and BR2, BR3 at 4 g/L concentration, respectively. Again, Incase of maltose no regeneration was obtained from BR1, BR3, BR4 at 2 g/L, BR1, BR3 at 3 g/L and BR1, BR3, BR4, BR5 at 4 g/L, respectively. The same result recorded for BR2, BR3, BR4, Taipei309 at 2g/L and BR2, BR4, BR5 at 3g/L and BR2, BR5 and Taipei309 g/L with manitol, respectively. Sucrose exhibited its superiority in plant regeneration compared to maltose and manitol. Among the sugars, manitol performed poorly in *in vitro* condition whereas maltose gave intermediate effect. Among the varieties, BR5 appeared to be the best *in vitro* responder followed by Taipei309.



Chapter 1
Introduction

Rice (*Oryza sativa L.*) belongs to the genus *Oryza* and family Gramineae (Roy, 1985). It is the staple food of more than 50% of the world's population (Zheng *et al.*1995). About 40% of the world's population consumes rice as a major source of calorie (Banik, 1999). Livelihood patterns of our nation as well as farmers of the country are governed by the cultivation of rice. Particularly the poor farmers of the country feel that without production of rice there is no food security of their family household. The escalating population growth, rapid industrialization and urbanization during past few decades remarkably reduced the cultivable land in our country. The cultivable land was 0.97 million hectare in 1990 and it was tremendously reduced to 0.86 million hectare in 2005 (BBS, 2006).

The reduction of cultivable land per year is alarming. From the period of unknown decades, the farmers of our country have been cultivating their poor yielding land races of rice. After introduction of high yielding variety (HYV) like IR8 since 1966 (BRRI, 1985), a dramatic change occurred in rice production. The development of more than 50 HYV by conventional method yet could not meet up the food deficit rather gradual replacement of our local cultivars.

The populations of rice consuming countries including Bangladesh are rapidly increasing and that will be double the consumers within 30 years (IFPRI, 1997). Bangladesh is the fourth larger producer of rice with an annual production ranging from 17-18 million tons. It occupies 77% of the total cropped area. An empirical study has over thrown information that the demand of rice in Bangladesh will have increased by over 80% in the next twenty years (Zamman, 1996). At present rice alone covers about 92% of the total food grains

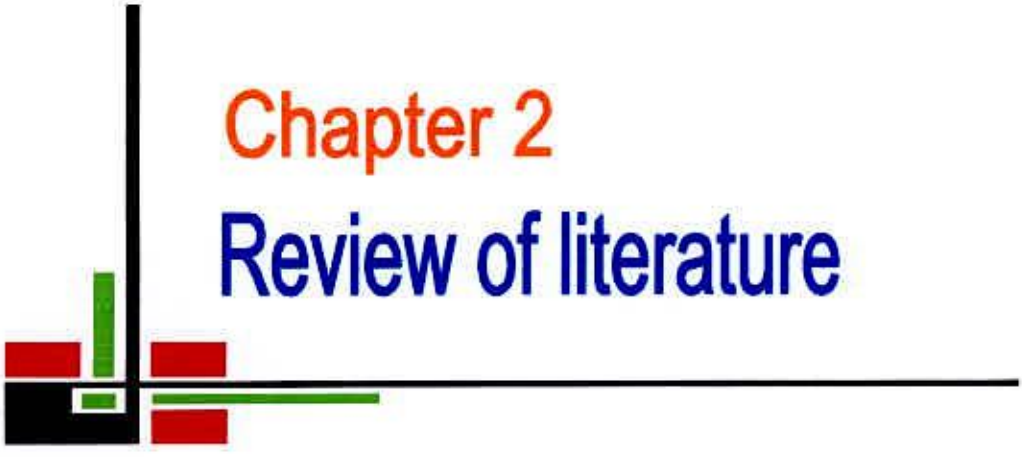
produced annually in the country and supplies 75%of the total calories and 55% of the proteins in over average daily diets (Bhuiyan, *et al.* 2002).

A considerable improvement has already been made through traditional rice breeding. Traditional rice breeding has resulted higher yield, improved quality, higher disease resistance and other important agronomic traits in the past and it will still play an important role in the future (Sun and Zheng, 1990). Now a days various tissue culture techniques are being applied for varietal development of cereal crops including rice in different countries (Dorosieve, 1996). Among the techniques, anther culture, protoplast fusion, leaf culture, root culture and mature embryo culture are important in rice tissue culture to somaclonal variation for creation of novel rice varieties (Ram and Singh, 1998). To overcome hybrid sterility, diploidization of agronomically important traits and insertion of diseased traits into the target varieties, micropropagation (tissue culture) techniques has been invented.

Rice seed culture is an important tool for crop improvement program, which is widely used in variety development programs. It offers many advantages to rice breeders because of their shortened breeding periods and high efficiency in selecting useful recessive agronomic traits. Cell and callus cultures are two systems in assessing the physiological effects of sucrose at the cellular level. Development of regeneration protocol is essential for incorporation of quality traits and developing new varieties by various biotechnological methods. Tissue culture technique has been carried out using sucrose as a medium component. Most plant tissue cultures are not highly autotrophic, that is, capable of fixing carbon through photosynthesis, due to limitations in culture of CO_2 availability, among other factors.Therefore, sugar is added to the medium as an energy source. Sucrose is the most common sugar added, although glucose, fructose, and sorbitol are also used in certain instances. Sucrose is the sugar form most commonly transported in plants; it is broken down

into glucose and fructose during metabolism. It is also partially hydrolyzed into glucose and fructose during autoclaving. Sugars also act as an osmoticum in the medium. Osmotic potential can have an important effect on *in vitro* response. Nutrient salts contribute from 20% to 50% of the osmotic potential of media, with sucrose making up the rest. Unfortunately, reports on the effect of sugar and its level on *in vitro* regeneration are not enough. Considering the above aspects the present investigation was under taken with the following objectives:

1. To observe the effect of different types of sugar on *in vitro* culture of five BRRI released rice varieties,
2. To find out the optimum concentration of sugar for germination, callus induction and plant regeneration,
3. To investigate callus induction and plant regeneration ability of the said released rice varieties.



Chapter 2

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 (Carlson, 1975) and emphasized wide attention of modern scientists (Skirvin, 1978). Rice is one of the most important field crops in the world. Developed countries of the world are now applying biotechnological tools for the improvement of rice. But that a very few studies on the related to growth medium and concentration of sugar carried out in our country. Nevertheless, some of the important and informative works and research findings related to the different concentration of sugar on callus induction and plant generation in rice variety so far been done at home and abroad on these issues have been reviewed in this chapter under the following headings:

2.1 Types of sugar in Culture Media

A laboratory experiment was conducted Deepinder *et al.* (2005) on indica rice cultivars Pusa Basmati 1, Basmati 370 and 386, to assess the promotive effect of maltose, sucrose, proline, cefotaxime and activated charcoal on the frequency of regeneration and embryogenesis. Factors affecting somatic embryogenesis and subsequent plant regeneration in vitro in Basmati rice cultures were highly genotype specific. Supplementation of the control medium independently with maltose (30 and 60 g/litre), proline (560 mg/litre), elevated sucrose (60 g/litre), activated charcoal (2 g/litre) and cefotaxime (100 mg/litre) considerably enhanced somatic embryogenesis. The nodular embryogenic calluses were further confirmed through histological analysis and their plant regeneration ability. Higher plant regeneration was achieved on medium containing maltose (30 and 60 g/litre).

Embryogenic calluses were initiated by Forkan *et al.* (2005) from mature seed scutellum (MSS) of salt tolerant indica rice cultivars BRRI Dhan-40, BRRI Dhan-41 and Binnatoa on LS 2.5 and MS 2.5 culture media supplemented with 2.5 mg litre-1 2,4-D. Among the cultivars used, high frequency callusing from mature seed scutellum was observed in BRRI Dhan-40 (74%) on MS 2.5 medium and BRRI Dhan-41 (79%) on LS 2.5 medium. In terms of plant regeneration, although all cultivars responded well on the both RM-1 and RM-2 media, the response of cultivars varied from medium to medium. The highest frequency (69%) of plant regeneration was observed in Binnatoa on RM-1 medium. In contrast, BRRI Dhan-41 produced the highest frequency (79%) of plant regeneration, while plant regeneration frequency was 75% in Binnatoa on RM-2 regeneration medium.

Yao *et al.* (2004) reported a mutant, in which the short-root phenotype at the seedling stage could be partially rescued by exogenous abscisic acid (ABA) exogenously applied sucrose (Suc), glucose (Glu) or fructose (Fru), but not mannitol (Mtl) or glucose analogs, can also rescue root growth of the mutant to extents greater than ABA. Collectively, these results suggest that energy deficiency is the cause of the phenotype, and ABA regulates root elongation of the mutant in a sugar-mediated way. The possible mechanism for ABA to promote root growth by enhancing the level of sugar in roots of is discussed.

Callus induction rate (CIR) and plantlet regeneration rate (PRR) of mature embryos (as explants) of 2 indica rice (Zhenshan 97B and Minghui 63) and 2 japonica rice (HI493 and Zhonghua 11) varieties were studied by Zhang *et al.* (2002). The callus induction medium was N_6 medium supplemented with 2,4-D (0.1, 2.0, 3.0 and 5.0 ml/litre), 4.5% sucrose and 0.75% agar. The callus differentiation medium was MS medium supplemented with KT [kinetin] (2 ml/litre), NAA (0.5 ml/litre), 3% sucrose and 0.75% agar. The highest CIR was recorded in N_6 medium supplemented with 2 ml 2,4-D/litre.

An efficient method was established by Sahrawat and Suresh (2001) for high-frequency embryogenic callus induction and plant regeneration from 3, 4, 5 and 7-day-old coleoptile segments of Indica rice (*O. sativa* cv. Kasturi). Compact and friable callus developed from the cut ends and also on the entire length of the coleoptile segments cultured on MS basal medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 4.50-18.0 micro M), kinetin (2.32 micro M) and sucrose (3%, w/v). Plant regeneration was achieved by transferring clumps of embryogenic callus to MS medium containing 2.85 micro M indole-3-acetic acid (IAA), 17.77 micro M 6-benzylaminopurine [benzyladenine] (BA) and 3% (w/v) sucrose. Histological observations of embryogenic calluses revealed the presence of somatic embryos and also plant regeneration via multiple shoot bud formation.

Terashima *et al.* (2000) conducted an experiment to recombinant human alpha 1-antitrypsin (rAAT) by genetically engineered rice cells using an inducible promoter has been studied by batchwise and continuous production. When the fresh medium containing 5 mM glucose was supplied to the continuous bioreactor, induction time was long and the productivity was low, indicating that the glucose concentration in the cells was high enough to repress the promoter. When the glucose concentration in the fresh medium was reduced to 0.5 mM, the total amount of rAAT produced in 70 h cultivation reached 6.7-7.6 mg/g-dry cell, which was two times larger than the control medium without glucose.

The germination and biochemical changes in rice cv. EMBRAPA 7 TAIM seeds treated with increasing rates (0, 0.1, 0.5, 1.0, 10.0 and 20.0 micro M) of salicylic acid (SA) for 24 h were evaluated by Silveira *et al.* (2000). Increase in the rate of SA, decreased seed germination percentage, first germination count, while higher rates of SA (10.0 and 20.0 μM) had strong inhibitory effects. The soluble sugar content decreased while the protein and starch content increased as SA rates increased.

Dode *et al.* (2000) carried out an experiment to find out the effects of growth regulators (2,4-D, kinetin, BAP and NAA on callus induction and proliferation from mature seeds of 10 rice cultivars were studied. Seeds incubated in MS media containing 2,4-D showed the highest callus induction frequency. The media containing 2,4-D + kinetin or 2,4-D + kinetin + BAP + NAA enhanced the proliferation of callus. Responses varied depending on the cultivar, plant growth regulator content of the media and possibly the physiological condition of the seed.

Bishnoi *et al.* (2000) improved procedure has been developed for high frequency androgenesis in indica x Basmati rice hybrids using a liquid culture medium. Anthers from fourteen genotypes comprising of indica x Basmati rice F_1 hybrids, F_2 plants and the parental rice cultivars, were floated in liquid RZM, N6M, and Heh5M media. Anther culture frequencies (percentage of anthers forming calluses) in most of the genotypes were significantly higher in RZM medium (16-75%) compared to those obtained in N_6M (7-29%). Agarose (1.0% w/v)-solidified MSR1 medium containing 3.0% (w/v) maltose, 1 mg kinetin, 1 mg benzyladenine (BA) and 0.5 mg NAA/litre induced green shoot regeneration at high frequencies compared to the medium (MSR2) lacking BA. High plant regeneration frequencies (up to 270 green plants/1000 anthers) were obtained from microspore-derived calluses of some of the F1 hybrids.

Sharma *et al.* (1999) evaluated callus initiation and plant regeneration was studied from immature embryos of three indica rice varieties (Heera, Pankaj and Basmati-370). MS medium supplemented with 100 mg adenine sulfate, 2 mg 2,4-D and 0.5 mg benzyladenine/litre was suitable for callus initiation. Callus proliferation and shoot bud regeneration was achieved on MS medium with 100 mg adenine sulfate, 2.0 mg benzyladenine and 1.0 mg NAA/litre. For root initiation, differentiated calluses were

transferred to MS medium containing 1.0 mg kinetin and 5.0 mg NAA/litre. Twelve-day-old embryos of the dwarf cultivar Heera and the semi-tall Pankaj and 18-day-old embryos of the late variety Basmati 370 were most responsive to in vitro culture. Regeneration was 20% for Heera and Pankaj and 6.6% for Basmati-370. Response to in vitro culture condition was varietal specific.

Saikia *et al.* (1999) evaluate plant regeneration from protoplasts of an high-yielding, cold-tolerant indica rice was standardized using anther-derived embryogenic cell suspensions. Sustained protoplast divisions were observed without the use of nurse cells on AA, N_6 and MS media supplemented with 12% sucrose, 1 g proline, 2 mg 2,4-D and 0.5 mg kinetin (Kn)/litre. The use of AA medium coupled with alginate bead technique gave the greatest plating efficiency (3.1%). Plant regeneration (25%) was observed on MS regeneration medium supplemented with 1 mg NAA, 2 mg BAP and 0.5 mg Kn/litre and 3% sucrose.

Anthers of F_1 generation plants of crosses between local and exotic rice cultivars were cultured on 2 callus induction media (C_1M_1 containing 30 g sucrose and C_1M_2 containing 50 g maltose) by Draz (1994). Resulting calluses were transferred to regeneration media (MSG_1, MSG_2 and MSG_3; MS salts + Gamborg's micronutrients and vitamins). The highest average regeneration was observed for the crosses GZ3766-38 x GZ4255-6-3 and GZ3766-38 x Kaohsuing Yu 1484 (both 22.2%).

Lenka and Reddy (1994) carried out an experiment to find out the influence of cold pretreatment, culture media, plant growth regulators and sucrose on callus formation from anthers of cultivars CR666-34-3, SR26-B, Basmati Sd-10 and Ptb.33 and plantlet regeneration were evaluated. The highest frequency of callus induction (42%) occurred when anthers of Ptb.33 were cultured on N6 medium supplemented with 2 mg NAA + 0.5 mg kinetin/litre. Culture of Ptb.33 calluses on MS medium supplemented with 6% sucrose +

1 mg IAA and NAA resulted in the highest regeneration frequency, with 45 green and 30 albino plantlets being produced..

2.2 Types of concentration in culture media

Al-Khayri and Al-Bahrany (2002) investigated the influence of osmotic stress, induced by sorbitol and sucrose combinations, on growth and proline accumulation in callus cultures of rice (*Oryza sativa* L.). Dehusked mature seeds, cv. Hassawi, were induced to callus on MS medium supplemented with 4.52 micro M 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.32 micro M 6-furfurylaminopurine (kinetin). The medium also contained 29.2, 58.4, 87.6, and 116.8 mM sucrose combined with 0, 54.9, 109.8, and 164.7 mM sorbitol. Callus formation was observed in about 35% of the cultured seeds irrespective of the sugar treatment. An increase in callus mass was observed as sucrose concentration increased reaching a maximum growth at 87.6 mM. Callus growth was enhanced in response to 54.9 mM sorbitol but at higher concentration it was inhibitory.

Ito *et al.* (2000) conducted an efficient anther culture system in rice cv. Koshihikari was developed using KSP medium and the double-layer technique. Callus induction frequency was higher in the double-layer technique with KSP medium than in the liquid KSP medium consisting of a three-step culture. Green plantlet differentiation frequency per anther was higher in the double-layer technique than in the three-step culture. Callus induction frequency was best on the medium containing one-fourth of the KNO_3 concentration of the N_6 medium, which is the same as the N concentration in KSP medium. Using the double-layer technique, with callus dessication prior to the transfer to regeneration medium, the green plantlet differentiation frequency per anther was 25-38%.

Anthers of the F_1 hybrid from the cross Khao Dawk Mali 105 x Skybonnet were cultured on various media by Yoshida and Oosato (1998). The best for callus induction was N_6 medium

containing 2 mg 2,4-D, 1 mg kinetin, 500 mg casein hydrolysate (CH) and 50 g sucrose/litre. Plantlets were produced from callus on MS medium supplemented with 2 mg benzyladenine (BA), 1 mg kinetin, 0.2 mg NAA, 300 mg CH and 20 g sucrose/litre and 15% coconut water. After transfer of plantlets onto MS medium containing 1 g yeast extract and 3 mg BA/litre, plus 15% coconut water and 0.01% colchicine for 5 days, doubled haploid plants were recovered at a frequency of about 60%.

Young ears and mature embryos of wild rice species were used as explants in culture on N_6 induction medium supplemented with 2 mg 2,4-D/litre and 4.5% sucrose, at pH 5.8 by Vijayalaxmi and Reddy (1997). Differentiation medium was MS supplemented with 2 mg kinetin/litre, 0.5 mg NAA/litre and 3% sucrose, at pH 5.8. After 15-20 days of culture on induction medium, callus was produced which became embryogenic. Induction rates differed between the different species, ranging from 94.7% for *O. rufipogon*.

In pollen culture during 1984-89, the basal medium for callus induction was evaluate by Hu, *et al.* (1992) added 2,4-D and kinetin (2 and 0.5 mg/litre, respectively) and a sucrose concentration of 80 g/litre. Mean percentages of callus formation and green shoot induction in 28 cross combinations with restorer lines were respectively 22.2% and 9.89%, while in 23 combinations without restorer lines they were respectively 5.2% and 2.44%.

2.3 Effect of genotype in culture media

Two F_4 lines derived from Hs-3 × 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.

In vitro regeneration through anther culture was studied by Lannes *et al.* (2004) in 2 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.

Hoque and Mansfield (2004) undertake an attempt to establish an efficient plant regeneration system in vitro from 3, 5, 7 and 9-days-old root segments of four Indica (Bangladeshi) rice genotypes. 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 in callus formation 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.

An experiment was conducted in India by Pushpam and Rangasamy (2004) to study the effect of salinity on calcium, sodium and potassium content of different rice cultivars (IR 50,Co 43, Dasal, SR 26 B and Pokkali) was studied by subjecting them to different levels of salt stress (0, 0.2, 0.4, 0.6, 0.8 and 1.0% NaCl). The results indicated that the *in vitro* and *in vivo* behaviour of genotypes was similar in terms of sodium, which increased as the level of stress increased. In contrast, the potassium content increased only in *in vitro* condition and decreased in seedling. The Na : K ratio behaved similarly in *in vitro* and *in vivo* conditions.

Shah *et al.* (2002) reported that cell lines of *Oryza sativa* (cv. Taipei-309) were adapted to 30 mM LiCI and 150 mM NaCI. Both adapted lines were considerably more tolerant than non-adapted line when grown on 200, 250 and 300 mM NaCI and 30 mM LiCI stresses. The tolerance of LiCI-adapted line to NaCI (150 to 300 mM) and the tolerance of NaCI-adapted cells line to LiCI (30 mM) indicated that there was a cross-adaptation towards alkali metals (Na^+ and Li^+) not the Cl^-, Na^+ and K^+ contents . Proline content of all lines increased with the increase in NaCI- stress but the magnitude of increase was much higher in the LiCI-adapted than other lines. The differential response of adapted lines to NaCI Stress in accumulation praline and maintaining the ionic contents reveals that adapted lines have evolved different features of adaptation to cope with NaCI stress.

In vitro screening at the cellular level was performed by Joyeeta *et al.* (2002) with mature seed-derived callus from five rice cultivars, viz. IR 18351-229-3, IR 3185-6-3-3-2, SR 26-B, Nona Bokra and C 14-8, of diverse geographical origin and with differential drought resistance at the in planta level. Callus was induced from mature seeds on Murashige and Skoog medium supplemented with 2.0 mg 2,4-dichlorophenoxyacetic acid (2,4-D) l-1 (9 micro M) and 5.0, 10.0 and 15.0 g high molecular weight polyethylene glycol (PEG, 6000) l-1 as stressing agent to create chemical drought. Seed germination was almost unaffected in SR 26-B and C 14-8, unlike in other cultivars where germination was seriously affected.

Callus induction and plant regeneration from mature embryo was studied by Deepti *et al.* (2001) in six indica rice cultivars (Pusa Basmati 1, Basmati 370, Type 3, CSR10, Pant Dhan 4 and Pokkali). Maximum callus initiation was observed on MS medium supplemented with 2 mg 2,4-D/litre in all the cultivars. Wide variation in callus growth was observed among the cultivars tested. MS medium without 2,4-D but supplemented with different

concentrations of BAP and IAA was used to evaluate the regeneration efficiency. BAP at 0.5 mg/litre was found to be optimum for the regeneration of plantlets in all cultivars.

Hossain (2000) observed callus induction followed by selection of salt tolerant cell lines in four varieties of *indica* rice. High frequency of callusing from mature embryo was observed in Binnatoa (70%) followed by Pokkali (60%) BR 16(52%) and BR 26(34%) when cultured in MS medium. The calli were then transferred to MS regeneration medium supplemented with different doses of NaCI(0, 0.1, 0.3 and 0.5%) to select salt tolerant cell lines. Both callus growth and plant regenerations were decreased with the increased level of NaCI in the media. The highest frequency of plant regeneration (33%) was observed in the variety Pokkali with 0.1% NaCI followed by Binnatoa (30%). BR 26(14%) and BR 16(12%) respectively. Pokkali and Binnatoa exhibited 12% and 7% regeneration respectively at 0.5% NaCI whereas no regeneration was observed in BR 16 and BR 26 at the same concentration.

Tang *et al.* (2000) developed a simple and efficient method has been developed to improve plant regeneration from protoplasts isolated from more 1-yr-old cell-suspension culture of indica rice (*Oryza sativa*) cv. Pusa Basmati 1. A two-step regeneration procedure, involving the transfer of calluses to shoot-regeneration medium containing 1% (w/v) agarose prior to culture on a medium containing 0.4% (w/v) agarose, was found to improve plant regeneration. High concentrations of kinetin in the regeneration medium were also found to be beneficial. By this method, even though protoplasts were isolated from over 1-yr-old cell-suspension cultures, protoplast-derived plant regeneration frequency reached 16.1%, compared with <4% regeneration frequency without such treatment.

Abbasi *et al.* (2000) Plant regeneration was investigated from seed derived callus-cultures in three cultivars of rice *Oryza sativa* viz. Basmati-370, Basmati-385 and KS-282. Seeds were cultured on Murashige and Skoog medium supplemented with 2 mg/litre 2,4-

dichlorophenoxy acetic acid (2,4-D). KS-282 exhibited a high embryogenic callus induction efficiency (31.25%) followed by Basmati-385 (17.60%) and Basmati-370 (6.50%). Calluses were maintained for 90 days on the same medium by regular subculturing after every 15 days. Thereafter, they were transferred to Linsmaier and Skoog supplemented with various concentrations of 1-napthaleneacetic acid (NAA) and benzyl-amino purine (BAP). Plant regeneration frequencies were the highest when NAA and BAP were used at 0.4-0.5 and 0.8-1.0 mg/litre respectively.

The effect of brassinosteroid analogues DAA-6, DI-31 and ME was evaluated by Prede *et al.* (2000) for more efficient callus induction media, as an initial phase to rice in vitro culture establishment of mature seeds of cv. Jucarito 104 (J-104) and Pokkali (Pok). Seeds were dehusked and disinfected before culturing in 4 culture media. Media were combinations of Murashige and Skoog salts (MS), hormonal supplement of 2 mg 2,4-D/L, 1 mg kinetin (KIN)/L and the corresponding brassinosteroids (BRs), at 10-5 mg/L. The best callus induction response was obtained after one month of culture, with the combination MS + 2,4-D + BRs at 10-5 mg/L for all brassinosteroids tested. The addition of 2,4-D was an essential requirement for callus induction.

Varietal specificity with respect to in vitro culture response involving callus induction and plantlet regeneration was investigated in indica rice by Biswas and Mandal (1999). Their performance was concomitantly compared with an in vitro culture responsive japonica cultivar Taipei 309. Maximum per cent plantlet regeneration and number of plantlets obtained from individual seed calli were found to be maximum in rice cv. Annada. Furthermore, the use of embryogenic induction medium (EIM) under in vitro culture was evaluated. That clearly elucidated the positive influence of EIM in facilitating more plantlet regeneration via induction of more somatic embryos.

Protoplasts were isolated from callus/suspension cultures of three Basmati varieties, viz., Pusa basmati 1, Basmati 370 and Basmati 386 by Deepinder *et al.* (1999). A fairly good yield of protoplasts (up to 1.2 x 106 protoplasts/ml) was obtained from cell suspension cultures after 4.5 h of incubation in enzyme mixture containing cellulase R-10 (1%) and pectolyase (0.1%). Protoplasts cultured on Millipore filter membrane placed on feeder cells of Pusa basmati 1 embedded in KPR medium exhibited best colony formation and plant regeneration (37.39%). Protoplast-derived plants exhibited normal growth and tillering in the soil. Protoplast to plant regeneration systems have been established in two commercial basmati varieties viz. Pusa basmati 1 and Basmati 370.

Cell suspension cultures were established from mature embryo calluses of indica rice cultivars viz. Tellahamsa, Rasi and Getu by Rani and Reddy (1996). Establishment of fine suspensions was possible with Tellahamsa on N6 medium supplemented with proline followed by Rasi and Getu. Potentiality of suspension cultures varied with respect to genotype and media. Growth parameters of the cell suspension cultures of Tellahamsa were maximum in N_6 medium. However, Getu and Rasi exhibited better growth with AA medium. The suspensions were composed of two distinct types of cells: large vacuolated and often elongated cells with sparse cytoplasm devoid of storage starch, most of which do not undergo many divisions; and small generally rounded cells, highly cytoplasmic with prominent nuclei and referred as embryogenic cells; these occurred in compact groups of cells. Globular somatic embryos were continuously produced in N_6 medium with 2.5 mg 2,4-D and 0.5 mg kinetin/litre, 3% maltose and 10 mM proline. These somatic embryos under went further developmental stages after transfer onto plating medium. Plant regeneration was obtained upon transfer to N_6 medium with 0.5 mg BA and 0.5 mg NAA/litre and 3% maltose.

Pongtongkam *et al.* (1995) conducted an experiment with mature seeds of 4 cultivars of rice (RBL-4, RBL-6, RBL-121 and Beziamah) were analysed for nutritional and antinutritional factors. The cultivars showed little variation in chemical composition. On a DM basis, the percentage of CP varied from 16.9 to 18; crude fat 0.46 to 0.52, crude fibre 6.3 to 7.5, total soluble sugar 4.9 to 5.6, starch 52.2 to 55 and ash 4.2 to 4.4. Callus induction rate varied from 35.1 to 83.4% with genotype and root induction rate was from 0 to 51.3%. Genotypes genetically related to Koshihikari tended to have a low induction rate.

The nutritional requirements for androgenic callus induction and green plant regeneration were studied by Mandal and Gupta (1995) in 8 rice genotypes, viz. *Oryza sativa* subsp. indica, *O. rufipogon* and doubled haploid lines of their intervarietal and interspecific hybrids. Culture response varied with genotype. Indica rice cultures were highly sensitive to the presence of NH_4+ and $MgSO_4$ in the medium. Sucrose at 3 to 5% was optimum for growth. High concentrations of sucrose were deleterious for callus induction and green plant regeneration. NAA or 2,4-D at 2 mg/litre were effective for callus induction. Calluses initiated in NAA containing media were more efficient for regenerating green plantlets than those induced in 2,4-D. Callusing medium containing aqueous extract of potato favoured differentiation of green plants in 15.8% of calluses on transfer to regeneration medium. Higher concentrations of auxins inhibited the regeneration of green plants.

Pandey *et al.* (1994) carried out an experiment with explants from 10 rice genotypes cultured on MS medium with 5 different concentrations of 2,4-D were compared for 8 characteristics related to callus formation and plant regeneration. Among the genotypes, Pusa Basmati 1 had the best overall callusing response, Pant Dhan 1 produced the most green spots and shoots, while Sarju 52 had the highest potential for long shoots and roots. MS medium supplemented with 2.0 mg 2,4-D/litre produced the most callus; 2.0 mg IAA

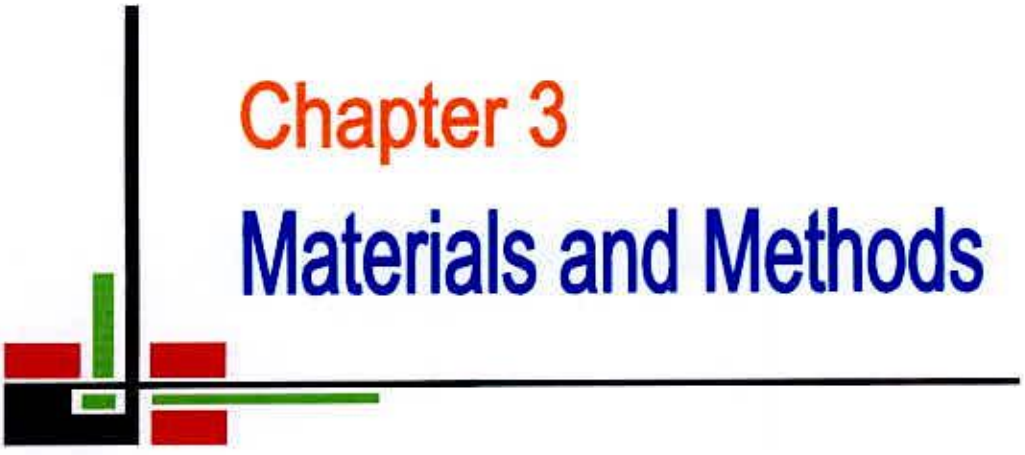
and 3.0 mg kinetin/litre produced the most shoots; and 3.0 mg IAA and 4.0 mg kinetin/litre produced the most roots.

Nilufer and Nabors (1994) reported that the callus forming ability of seed derived embryos of IR 36 and Calrose 76, the two rice varieties were examined in 0, 5, 10 and 15g/L NaCI. An increase in salt concentration resulted in decreased shoot and root formation. After two weeks of inculation, IR 36 and Calrose 76 callused differently indicating that Indica and Japonica cultivars require different combinations of nutrients and growth regulators. In all the media containing 3 levels of NaCI, embryogenic (E) callus of IR 36 was found to be higher than non-embryogenic callus (NE).

Heszky *et al.* (1987) reported that plant regeneration frequency from plumule meristem-derived callus ranged from 25 to 69% among 13 genotypes, the range for grain-derived callus being 0-27%. In a study of haploid production in 2 genotypes, callus derived from young inflorescences gave higher regeneration frequencies than that derived from anthers (90 vs. 25%), with no albino plants and only slight genotypic effects. Supplementation of the culture medium with NaCI (0.5 or 1.0%) appeared to prolong the retention of regeneration capacity in callus. Somaclonal variants were obtained, including lines with early heading and high lysine and methionine contents of the grain. Cytologically stable lines were obtained in the third generation after regeneration.

Li *et al.* (1987) studied that six genotypes were subjected to *in vitro* selection for tolerance of NaCI solution (0.5-2.0%) and artificial sea water (18-75%). Results were influenced by genotype, ploidy level of the explant, weight of callus inoculum and subculture state of the callus. The recommended method involves repeated *in* vitro selection at the callus induction, callus growth and plant regeneration stages. Of 24 lines derived from plants regenerated from tolerant callus, one was more tolerant than the control to 2.0% NaCI.

Heszky *et al.* (1986) observed that callus from 5 types of explant from 2 lines and 4 cultivars were cultured on a modified Murashige Skoog medium. The expression of totipotency during subculturing depended more on genotype than on explants. In cultures of callus from immature inflorescences of haploids, 90% of regenerated plants from the first subculture were haploid, but none were haploid after 2 subcultures. Addition of NaCl (0.5 or 1.0%) to the culture medium increased the percentage of plant regeneration., particularly in Unggi 9, and allowed plants to be regenerated more readily from later subcultures.



Chapter 3

Materials and Methods

An experiment was conducted at the Tissue culture Laboratory of Biotechnology Division, Bangladesh Rice Research Institute (BRRI), Joydebpur, Gazipur to find out the effect of different types and different concentrations of sugar on callus induction and plant regeneration in rice seed culture during the period January to May, 2007.

3.1 Experimental materials

The rice varieties BR1, BR2, BR3, BR4, BR5 and Taipei309 were used as experimental materials in the present investigation. Among them BR1, BR2, BR3, BR4, BR5 were a high yielding short duration indica variety developed by BRRI. Taipei309 a Japonica variety having high culturability under *in vitro* condition.

3.2 Source of experimental materials

Mature seeds of six indica rice varieties obtained through the courtesy of Biotechnology Division, BRRI, Gazipur were used for this experiment. .

3.3 Types of explants

The healthy and dehusked seeds of six rice varieties viz. BR1, BR2, BR3, BR4, BR5 and Taipei 309 were used as explants for the study.

3.4 Media used

MS medium with sugar for callus induction was used for culturing seeds of six rice varieties. The pieces explants at appropriate size were transferred to MS regeneration medium for differentiation.

A. For callus induction

The MS medium was used supplemented with 2 mg/L 2,4-D and three types sugars at different concentration for the study.

i. MS medium + Sucrose: Three levels of Sucrose

a. MS medium supplemented with 2 g/L Sucrose

b. MS medium supplemented with 3 g/L Sucrose

c. MS medium supplemented with 4 g/L Sucrose

ii. MS medium + Maltose: Three levels of Maltose

a. MS medium supplemented with 2 g/L Maltose

b. MS medium supplemented with 3 g/L Maltose

c. MS medium supplemented with 4 g/L Maltose

i. MS medium + Manitol: Three levels of Manitol

a. MS medium supplemented with 2 g/L Manitol

b. MS medium supplemented with 3 g/L Manitol

c. MS medium supplemented with 4 g/L Manitol

B. For regeneration

The MS (Murashige and Skoog, 1962) medium was supplemented with 1.0 mg/L NAA and 1.0 mg/L Kinetin.

Table 1. Composition of the media used

Components	MS (Induction) Conc. (mg/L)	MS (Regeneration media) Conc. (mg/L)
Macro nutrients		
KNO_3	1900	1900
NH_4NO_3	1650	1650
$MgSO_4.7H_2O$	370	370
$CaCl_2.2H_2O$	440	440
KH_2PO_4	170	170
Micro nutrients		
$MnSO_4.4H_2O$	22.3	22.3
H_3BO_3	6.2	6.2
$ZnSO_4.7H_2O$	8.6	8.6
$CuSO_4.5H_2O$	0.025	0.025
$Na_2MoO_4.2H_2O$	0.25	0.25
$CoCl_2.6H_2O$	0.025	0.025
KI	0.83	0.83
Iron Source		
$FeSO_2.7H_2O$	27.8	27.8
Na_2-EDTA	37.3	37.3
Organic nutrients		
Nicotinic acid	0.5	0.5
Pyridoxine HCl	0.5	0.5
Thaimine HCL	0.1	0.1
Glycine	2.0	2.0
Myo-inositol	100	100
Sucrose	40000	40000
Agar	9000	9000
Phytohormones		
2,4-D	2.0	--
NAA		1.0
Kinetin		1.0

3.4.1 Preparation of culture media

To prepare the culture medium the same procedure was followed everywhere unless otherwise stated. Different steps of media preparation are described below:

3.4.1.1 Preparation of stock solutions

As different ingredients were required in different concentrations, separate stock solutions for macronutrients, micronutrients, vitamins, growth hormones etc. were used.

i) Stock solution A (Macro nutrients)

Stock solution of macronutrients was prepared up to 10 times the concentration of the final medium in 1000 ml of distilled water (dw). Ten times the weight of the salts required per litre of the medium were weighed properly and dissolved by using a magnetic stirrer in about 750 ml of distilled water and then made up to 1000 ml by further addition of dw. To make the solution free from all sorts of solid contaminants, it was filtered though Whatman no.1 filter paper. Then it was poured into a plastic container, labeled with marker and stored in a refrigerator at 4^0C for later use.

ii) Stock solution B (Micro nutrients)

The stock solution of micro nutrients 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 macro nutrients. The stock solution was filtered, labeled and stored in the refrigerator at 4^0C.

iii) Stock solution C (Iron sources)

This was prepared at 10 times the final strength of $FeSO_4$ and Na_2 EDTA in 100 ml of dw and chelated by heating on a heater cum magnetic stirrer. Then the volume was made up to 1000 ml by further addition of distilled water. Finally the stock solution was filtered and stored in a refrigerator at 4^0C.

iv) Stock solution (Vitamins)

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

v) Hormone stock solutions

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:

Auxins

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

∞- naphthalene acetic acid (NAA)

Cytokinins

6-furfuryl amino purine (Kinetin)

These hormonal supplements were dissolved in proper solvent as shown against each of them.

Hormones (Solute)	**Solvent**
2,4-D	70% ethyl alcohol
NAA	0.1 (N) NaOH
Kn (Kinetin)	0.1(N) HCl

To prepare the stock solution of hormones, 10 mg of solid hormone was placed on a clean watch glass 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. The solution was then stored at 0^0C for use upto four weeks.

After the preparation of the stock solution the later step was the preparation of culture media. To prepare one litre of above-mentioned media following steps were followed.

i) The required volume of each stock solution (100 mL of stock solution " A'', 10 mL of stock solution "B'', 100 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.

ii) 500 mL of distilled water was added in the flask to dissolve all the ingredients.

iii) Myo-insitol and sucrose was added directly to the solution as per requirement and dissolved well.

iv) Different concentrations of hormonal supplements were added to the solution either in single or in combination as required and mixed well.

v) Other additives, such as sodium chloride (NaCl), sodium sulphate (Na_2SO_4), potassium chloride (KCl) were measured as per requirement and directly added to the medium.

vi) The mixture was then poured into a 1 litre measuring cylinder and made the volume up to 1000 ml with addition of distilled water and poured back to a 2 litre conical flask and mixed well.

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

viii) After adjusting the p^H, 9 g agar was added to solidified the medium. The mixture was then gently heated with continuous stirring till complete dissolution of agar.

ix) Required volume of hot medium was dispensed into culture vessels viz. test tubes or conical flasks. After dispensing the medium the culture vessels were plugged with Aluminum foil and marked with different codes with the help of a glass marker to indicate specific media.

3.4.2 Sterilization

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

3.4.2.1 Sterilization of culture medium

The culture vessels containing the medium were autoclaved with 1.16 kg/cm ^{2} of pressure at 121^{0}C for 20 minutes. After autoclaving the test tubes containing the medium were allowed to cool as slants or vertical position whichever necessary.

3.4.2.2 Sterilization of glassware and instruments

Beakers, test tubes, conical flasks, pipettes, metal instruments viz, forceps, scalpels, needles, spatulas and aluminum foils were sterilized in an autoclave at a temperature of 121^{0}C for 20 minutes at 1.16 kg/cm^2 pressure.

3.4.2.3 Sterilization of culture room and transfer area

The culture room was initially 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 and wiping the working surface with 70% ethyl alcohol sterilized laminar airflow cabinet.

3.4.3 Culture methods

3.4.3.1 Seed selection

Healthy 1000 seeds of six rice varieties (BR1, BR2, BR3, BR4, BR5 and Taipei309) were collected and dehusked.

3.4.3.2 Seed treatment

The dehusked seeds were taken in small beaker. Then the seeds were sterilized with the help of following procedure:

i) Distilled water was taken 1/3 of the beaker and two drops of Twin 20 was added and shake for 10 minutes. Then the water was discarded.

ii) Then the seeds were washed with 70% ethanol for 3 minutes.

iii) After discarding the ethanol, the seeds were washed with 0.1% Mercuric Chloride ($HgCl_2$) for 20 minutes and then rinsed in distilled water for 4 times to remove $HgCl_2$).

Then the seeds were air dried in the Laminar Air Flow cabinet and were transferred in callus induction media.

3.4.3.3 Inoculation

The culture was inoculated with the treated seeds at 25 ± 2^0C for callus induction. After 4-6 weeks of inoculation, seeds of the responsive varieties started to produce callus. Callus induction frequency was calculated on the basis of the number of seeds producing callus. Some seeds produced more than one callus but for the calculation all calli originating from one seed were considered as one.

3.4.3.4 Callus transfer (Incubation)

Calli with a size of least 2 mm were transferred to regeneration medium (MS basal semi-solid medium), and were incubated in a temperature controlled growth room at 25+ 2^0C under a 12 hr light photoperiod with a light intensity of 2000-3000 lux for plant regeneration. Day to day observations was carried out to note the response. Regenerated plants were counted on the basis of the number of callus producing plantlets.

3.5 Recording of data

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

$$\text{i. Percent seed germination} = \frac{\text{Number of seeds germinated}}{\text{Number of seeds incubated}} \times 100$$

$$\text{ii. Percent callus induction} = \frac{\text{Number of seeds induced calli}}{\text{Number of seeds incubated}} \times 100$$

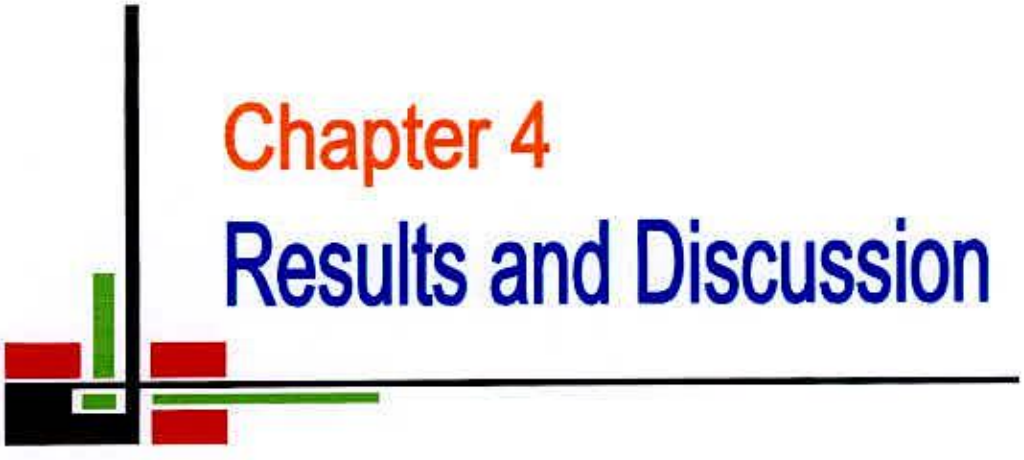
$$\text{iii. Percent plant regeneration} = \frac{\text{Number of calli with plantlets}}{\text{Number of incubated calli}} \times 100$$

3.6 Statistical analysis of data

The data for the characters under present study were statistically analyzed wherever applicable. The experiment were conducted in growth room and arranged in Completely Randomized Design (CRD) with 6 replications. The analyses of variance for different characters was performed and means were compared by the Duncan's Multiple Range Test (DMRT).

Chapter 4

Results and Discussion



High frequency of plant regeneration in sugar supplemented media canoffers a feasible propagation method in rice. In this experiment, the techniques for *in vitro* plantlet regeneration have been established very carefully using seed as explants of six varieties viz. BR1, BR2, BR3, BR4, BR5 and Taipei309. Taipei309 was used as a standard check for callus induction and regeneration which also used internationally for the same purpose. The effects of three different sugars(sucrose, maltose and manitol) on test parameters (germination, callus induction and plant regeneration) of the variety were also investigated. Under these major headings the results are elaborated based on the nature of the morphogenic response in different type of sugar, sugar concentration and variety of rice and their interaction. The analysis of variance of different parameters has been present in Appendices.

4.1 Effect of different types of sugar

Different types of sugars showed significant differences for germination, callus induction and regeneration (Appendix I). The results on effect of different sugars are presented in Table 2. The highest percent of (81.83%) germination was recorded from sucrose treatment and the lowest (77.37%) from maltose treatment. The highest (80.21%) callus induction was recorded form sucrose treatment and the lowest (75.64%) callus induction was recorded for maltose treatment which was closely (77.03%) followed by manitol treatment. In case of regeneration highest (9.62%) was recorded for sucrose, while the lowest (5.62%) was recorded from manitol treatment which was intermediate (6.66%) in case of maltose treatment.

On the basis of above results, all the characters such as germination, callus induction and regeneration were influenced due to presence of sugar. Considering germination response of the variety, sucrose was the best followed by manitol and maltose. In case of callus induction

response, the similar results was obtained. But for regeneration response of the variety, sucrose was the best, followed by maltose and than manitol.

Sucrose provide better result due to more soluble and available for tissue culture in the medium.The above findings agrees with that of Deepinder *et al.* (2005) who reported sucrose have more promotive effect than maltose and manitol.

4.2 Effect of sugar concentration

Germination, callus induction and regeneration showed significant differences for different concentration of sugars in the present study (Appendix I). Different levels of sugar concentration showed different responses (Table 3). The highest (82.27%) germination was recorded from sugar concentration at 2 g/L which was closely followed (79.30%) by 3 g/L and the lowest (76.85%) from 4 g/L. The highest (80.62%) callus induction was recorded for sugar at the concentration of 2 g/L which was closely (77.36%) followed by 3 g/L concentration and the lowest (74.90%) callus induction was recorded form 4 g/L. In case of regeneration highest (8.38%) was recorded for the sugar concentration of 3 g/L which was closely (7.81%) followed by sugar concentration 2 g/L. On the other hand the lowest (5.70%) was recorded from sugar concentration 4 g/L.

It was reveal that, lower level of sugars (2 g/L) showed higher germination and callus induction whereas moderate level of sucrose gave high regeneration. Considering germination response of the varieties, 2 g/L concentration was the best followed by 3 g/L and than 4 g/L. Callus induction response gave the same result. But for regeneration response, 3 g/L concentration was best, then comes 2 g/L and 4 g/L.

The above findings agrees with that of Deepfinder *at el.*(2005) that higher plant regeneration was achieved on medium concentration of sugar. The above findings alse agree with that of Mandal and Gupta(1995) that high concentration of sucrose were deleterious for callus induction and green plant regeneration.

Table 2. Main effect of different sugars on germination, callus induction and regeneration

Sugar	Germination (%)	Callus induction (%)	Regeneration (%)
Sucrose	81.83 a	80.21 a	9.62 a
Maltose	77.34 c	75.64 c	6.66 b
Manitol	79.24 b	77.03 b	5.62 c

In a column, having similar letter(s) are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05 level of probability

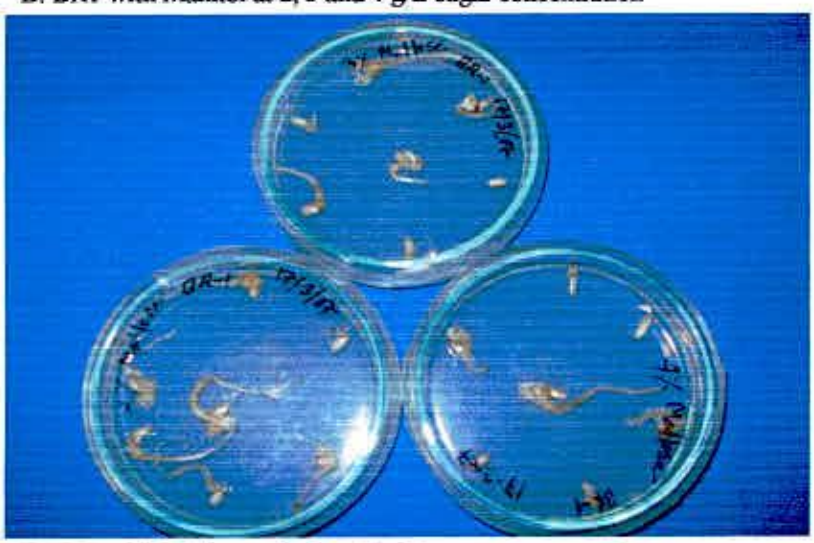
Table 3. Main effect of different sugar concentrations on germination, callus induction and regeneration

Sugar concentration (g/L)	Germination (%)	Callus induction (%)	Regeneration (%)
2	82.27 a	80.62 a	7.81 b
3	79.30 b	77.36 b	8.38 a
4	76.85 c	74.90 c	5.70 c

In a column, having similar letter(s) are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05 level of probability

A. BR1 with Sucrose at 2, 3 and 4 g/L sugar concentration

B. BR1 with Manitol at 2, 3 and 4 g/L sugar concentration



C. BR1 with Maltose at 2, 3 and 4 g/L sugar concentration

Plate 1. Germination and callus induction of variety BR1 in different sugars and conc.

A. BR2 with Manitol at 2, 3 and 4 g/L sugar concentration

B. BR2 with Maltose at 2, 3 and 4 g/L sugar concentration

C. BR2 with Sucrose at 2, 3 and 4 g/L sugar concentration

Plate 2. Germination and callus induction of variety BR2 in different sugars

A. BR3 with Sucrose at 2, 3 and 4 g/L sugar concentration

B. BR3 with Maltose at 2, 3 and 4 g/L sugar concentration

Plate 3. Germination and callus induction of variety BR3 in different sugars

A. BR4 with Sucrose at 2, 3 and 4 g/L sugar concentration

B. BR4 with Maltose at 2, 3 and 4 g/L sugar concentration

C. BR4 with Manitol at 2, 3 and 4 g/L sugar concentration

Plate 4. Germination and callus induction of variety BR4 in different sugars

A. BR5 with Maltose at 2, 3 and 4 g/L sugar concentration

B. BR5 with Sucrose at 2, 3 and 4 g/L sugar concentration

C. BR5 with Manitol at 2, 3 and 4 g/L sugar concentration

Plate 5. Germination and callus induction of variety BR5 in different sugars

A.Taipei309 with Sucrose at 2 ,3 and 4 g/L sugar concentration

B. Taipei309 with Manitol at 2 ,3 and 4 g/L sugar concentration

C. Taipei309 with Maltose at 2 ,3 and 4 g/L sugar concentration

Plate 6. Germination and callus induction of variety Taipei 309 in different sugars

4.3 Effect of genotype

Different rice varieties showed significant variations for germination, callus induction and regeneration (Appendix I). The results were presented in Table 4. The highest (95.11%) germination was recorded from BR3 which was closely (90.71%) followed by BR5 and the lowest (64.37%) from BR4 which was closely followed (67.25%) by BR1. The highest (93.21%) callus induction was recorded form BR3 which was closely (88.94%) by BR5 and the lowest (62.89%) callus induction was recorded for BR4 which was closely (65.52%) followed by BR1. In case of regeneration highest (16.90%) was recorded form BR5 which was closely (11.62%) followed by Taipei309, while the lowest (1.01%) was recorded from BR3 which was closely (4.07%) followed by BR2.

In case of germination performance, the varieties can be arranged BR3, BR5, Taipei309, BR2, BR1 and BR4 according to descending order. For callus induction ability the varieties were at BR3, BR5, Taipei309, BR2, BR1 and BR4 according to same order. For regeneration ability the highest performance showed BR5 followed by Taipei309, BR1, BR4, BR2, BR3, respectively.

Thus it may be concluded that BR5 is the best variety in terms of regeneration ability which is a very desirable attribute in *in vitro* culture.This above finding agrees with that of Deepinder *et al.*(2005) who repoted that the factors affecting plant regeneration in *in vitro* in rice cultivers were highly genotype specific.

Table 4. Main effect of different variety on germination, callus induction and regeneration

Variety	Germination (%)	Callus induction (%)	Regeneration (%)
BR1	67.25 e	65.52 e	5.68 c
BR2	78.09 d	75.87 d	4.07 e
BR3	95.11 a	93.21 a	1.01 f
BR4	64.37 f	62.89 f	4.50 d
BR5	90.71 b	88.94 b	16.90 a
Taipei309	81.31 c	79.32 c	11.62 b

In a column, having similar letter(s) are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05 level of probability

4.4 Combined effect of different types of sugar and sugar concentration

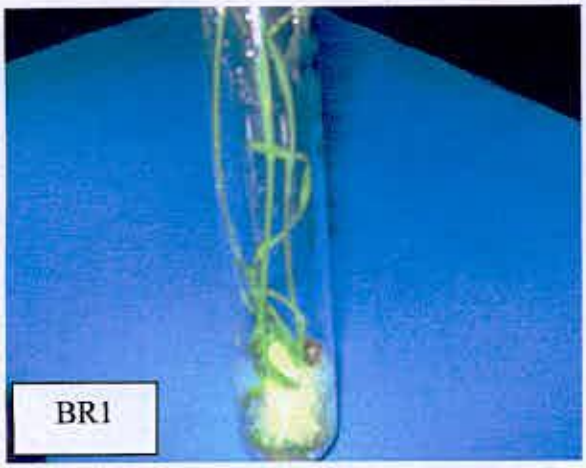
Germination, callus induction and regeneration showed significant differences for combined effect of sugar and different sugar concentrations in the present Study (Appendix I). Different combination of sugar and levels of sugar concentration showed different values that were presented in Table 5. The highest (86.06%) germination was recorded from sucrose at the concentration of 3 g/L which was statistically identical (85.23%) with sucrose at concentration of 2 g/L and the lowest (74.20%) was recorded from sucrose at 4 g/L which was closely (75.07%) followed by maltose at concentration of 3 g/L . The highest (84.22%) callus induction was recorded from sucrose at the concentration of 3 g/L which was statistically similar (84.01%) with sucrose at concentration of 2 g/L and the lowest (72.39%) was recorded from sucrose at 4 g/L which was closely (73.51%) followed by maltose at concentration of 3 g/L. The highest (12.05%) regeneration was recorded from sucrose at the concentration of 2 g/L which was closely followed (11.55%) with sucrose at concentration of 4 g/L and the lowest (1.67%) was recorded from manitol at 4 g/L which was closely (3.89%) followed by maltose at concentration of 4 g/L .

It was reveal that, sucrose showed its superiority in plant regeneration (12.05% and 11.55%) at both high and lower level of concentrations, which to be ambiguous and need to be verified. Both maltose and manitol showed good plant regeneration (10.73% and 9.15%, respectively) at 3 g/L although it is higher than that of sucrose at the same level. It can be concluded that sucrose favors plant regeneration compared to maltose and manitol. Manitol showed to be poor performance in case of plant regeneration.

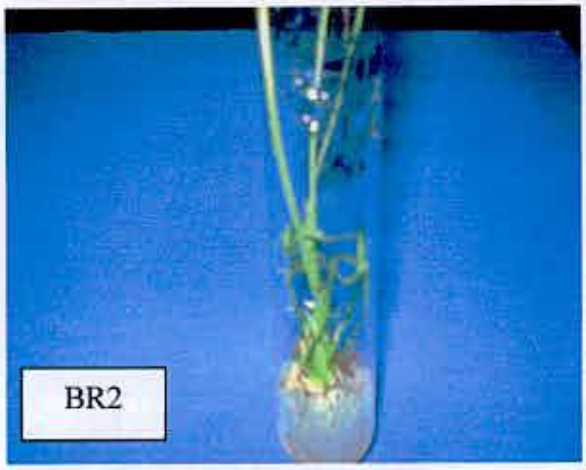
Table 5. Combined effect of different types of sugar and sugar concentration on germination, callus induction and regeneration

Treatment		Germination (%)	Callus induction (%)	Plant regeneration (%)
Sugar	Sugar conc. (g/L)			
Sucrose	2	85.23 a	84.01 a	12.05 a
	3	86.06 a	84.22 a	5.26 f
	4	74.20 e	72.39 d	11.55 b
Maltose	2	80.55 b	78.85 b	5.35 f
	3	75.07 de	73.51 cd	10.73 c
	4	76.41 cd	74.56 c	3.89 g
Manitol	2	81.02 b	78.99 b	6.04 e
	3	76.76 c	74.36 c	9.15 d
	4	79.95 b	77.74 b	1.67 h

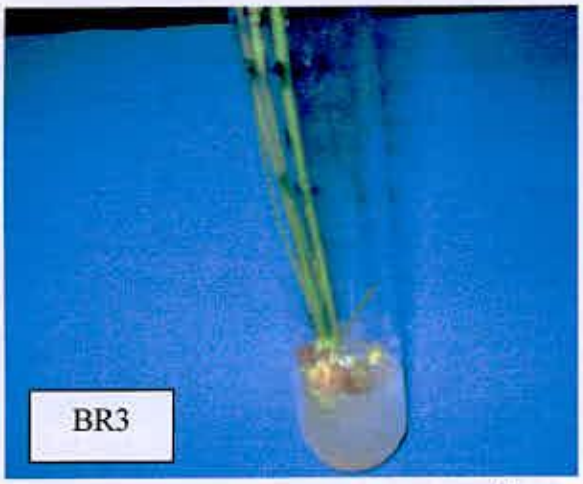
In a column, having similar letter(s) are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05 level of probability



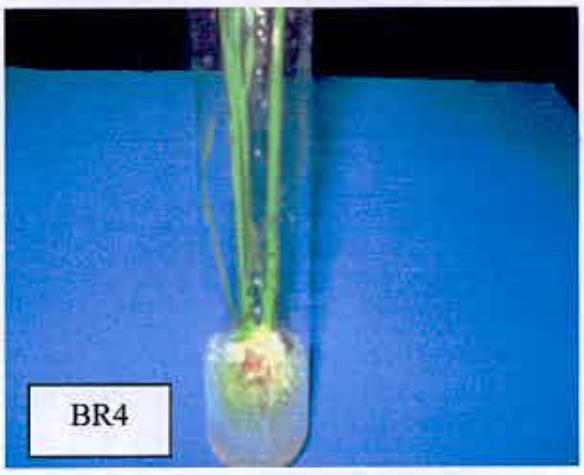
A Regeneration of BR1 with Sucrose at 2 g/L



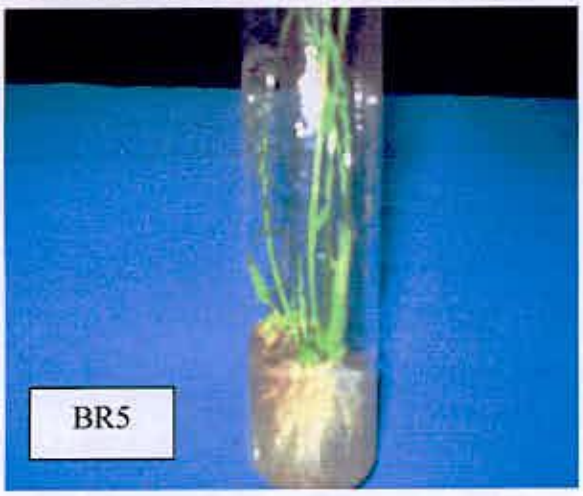
B. Regeneration of BR2 with Maltose at 2 g/L



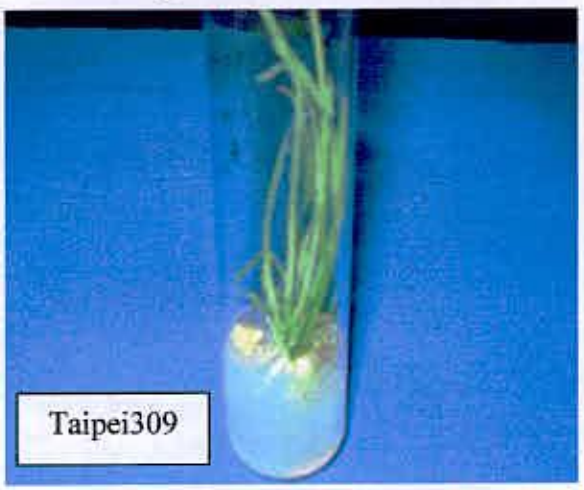
C. Regeneration of BR3 with Sucrose at 2 g/L



D. Regeneration of BR4 with Sucrose



E. Regeneration of BR5 with Sucrose



F. Regeneration of Taipei309 with sucrose

Plate 7. Regeneration of different rice genotypes

4.5 Combined effect of sugar and variety

Germination, callus induction and regeneration showed significant differences for combined effect of sugar and different rice variety in the present study(Appendix I). Different combination of sugar and variety performed different values that were presented in Table 6. The highest (95.47%) germination was recorded from manitol with BR3 which was statistically identical (95.08%, 94.77%, 94.76%) with maltose with BR3, sucrose with BR3 and maltose with BR5, respectively and the lowest (51.26%) was recorded from maltose with BR4 which was closely followed (61.88%) with manitol with BR1. The highest (93.51%) callus induction was recorded from manitol with BR3 which was statistically identical (93.15%, 92.98% and 92.90%) with maltose with BR3, sucrose with BR3 and maltose with BR5, respectively and the lowest (49.33%) was recorded from maltose with BR4 which was closely followed (59.82%) with manitol with BR1 . The highest (30.49%) regeneration was recorded from sucrose with BR5 which was closely (18.44%) with sucrose with Taipei309 and the no generation was recorded from sucrose with BR2, sucrose with BR3, maltose with BR1, maltose with BR3, manitol with BR2 .

Thus it may be concluded that the variety BR5 is a sound performer interaction between the variety and sugar, even high germination and callus induction was recorded from BR

4.6 Combined effect of sugar concentration and variety

Germination, callus induction and regeneration showed significant differences for combined effect of sugar concentration and different rice variety in the present investigation(Appendix I). Different combination of sugar concentration and variety showed different values that were presented in Table 7. The highest (95.72%) germination was recorded fro sugar concentration of 2 g/L with BR3 which was statistically identical (95.08%, 94.92%, 94.52%) with sugar concentration at 4 g/L with BR3, sugar concentration 2 g/L with BR5 and sugar concentration 4 g/L with BR3, respectively and the lowest (55.19%) was recorded from sugar concentration at 4 g/L with BR1 which was closely followed (61.37%) with sugar concentration at 3 g/L with BR4.

Table 6. Combined effect of different types of sugar and varieties on germination, callus induction and regeneration

Treatment		Germination (%)	Callus induction (%)	Plant regeneration (%)
Sugar	Variety			
Sucrose	BR1	76.73 f	75.13 e	3.37 i
	BR2	79.20 de	77.15 de	0.00 j
	BR3	94.77 a	92.98 a	0.00 j
	BR4	70.61 h	69.71 g	5.41 g
	BR5	91.90 b	90.26 b	30.49 a
	Taipei309	77.76 ef	76.01 de	18.44 b
Maltose	BR1	63.13 i	61.60 h	0.00 j
	BR2	74.12 g	72.30 f	12.22 d
	BR3	95.08 a	93.15 a	0.00 j
	BR4	51.26 j	49.99 i	4.76 gh
	BR5	94.76 a	92.90 a	10.69 e
	Taipei309	85.71 c	83.93 c	12.26 d
Manitol	BR1	61.88 i	59.82 h	13.67 c
	BR2	80.94 d	78.16 d	0.00 j
	BR3	95.47 a	93.51 a	3.03 i
	BR4	71.23 h	68.99 g	3.33 i
	BR5	85.47 c	83.66 c	9.52 f
	Taipei309	80.47 d	78.03 d	4.17 h

In a column,having similar letter(s) are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05 level of probability

Table 7. Combined effect of different sugar concentration and variety on germination, callus induction and regeneration

Treatment		Germination (%)	Callus induction (%)	Plant regeneration (%)
Sugar conc. (g/L)	variety			
2	BR1	81.73 de	79.81 de	3.71 h
	BR2	74.91 f	72.48 f	3.33 h
	BR3	95.72 a	93.89 a	0.00 j
	BR4	64.28 h	63.30 h	0.00 j
	BR5	94.92 a	93.61 a	33.96 a
	Taipei309	82.05 de	80.63 de	5.87 fg
3	BR1	64.83 h	63.55 gh	11.11 d
	BR2	83.96 d	81.72 d	5.56 g
	BR3	94.52 a	92.84 a	3.03 h
	BR4	61.37 i	59.58 i	7.14 e
	BR5	90.15 b	88.02 b	10.50 d
	Taipei309	80.94 e	78.47 e	12.96 c
4	BR1	55.19 j	53.20 j	2.22 i
	BR2	75.39 f	73.41 f	3.33 h
	BR3	95.08 a	92.90 a	0.00 j
	BR4	67.45 g	65.80 g	6.36 f
	BR5	87.06 c	85.20 c	6.25 f
	Taipei309	80.94 e	78.87 e	16.04 b

In a column, similar letter(s) are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05 level of probability

Table 7. Combined effect of different sugar concentration and variety on germination, callus induction and regeneration

Treatment		Germination (%)	Callus induction (%)	Plant regeneration (%)
Sugar conc. (g/L)	variety			
2	BR1	81.73 de	79.81 de	3.71 h
	BR2	74.91 f	72.48 f	3.33 h
	BR3	95.72 a	93.89 a	0.00 j
	BR4	64.28 h	63.30 h	0.00 j
	BR5	94.92 a	93.61 a	33.96 a
	Taipei309	82.05 de	80.63 de	5.87 fg
3	BR1	64.83 h	63.55 gh	11.11 d
	BR2	83.96 d	81.72 d	5.56 g
	BR3	94.52 a	92.84 a	3.03 h
	BR4	61.37 i	59.58 i	7.14 e
	BR5	90.15 b	88.02 b	10.50 d
	Taipei309	80.94 e	78.47 e	12.96 c
4	BR1	55.19 j	53.20 j	2.22 i
	BR2	75.39 f	73.41 f	3.33 h
	BR3	95.08 a	92.90 a	0.00 j
	BR4	67.45 g	65.80 g	6.36 f
	BR5	87.06 c	85.20 c	6.25 f
	Taipei309	80.94 e	78.87 e	16.04 b

In a column, similar letter(s) are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05 level of probability

The highest (93.89%) callus induction was recorded from sugar concentration of 2 g/L with BR3 which was statistically identical (93.61%, 92.90%, 92.84%) with sugar concentration 2 g/L with BR5, sugar concentration at 4 g/L with BR3, and sugar concentration 4 g/L with BR3, respectively and the lowest (53.20%) was recorded from sugar concentration at 4 g/L with BR1 which was closely followed (61.37%) with sugar concentration at 3 g/L with BR4 . The highest (33.96%) regeneration was recorded from sugar concentration at 2 g/L with BR5 which was closely followed (16.04%) with sugar concentration at 4 g/L with Taipei309 and no generation was recorded from sugar concentration at 2 g/L with BR3, BR4, sugar concentration with BR3.

On the basis of the above result it can be more or less generalized that the percentage of germination and callus induction declined with increase of sugar concentration and BR5 demonstrated its superiority for the percentage of regeneration.

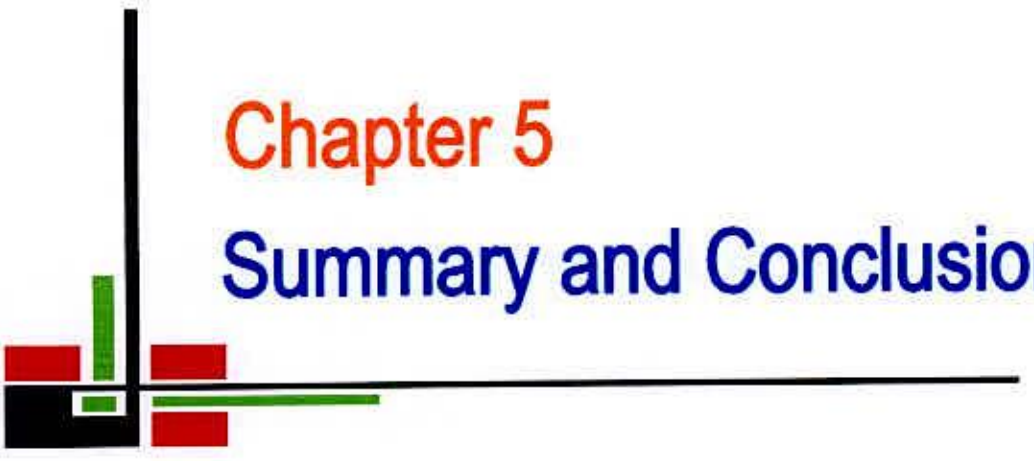
4.7 Combined effect of different types of sugar, sugar concentration and variety

Germination, callus induction and regeneration showed significant differences for combined effect of sugar, sugar concentration and different rice varieties in the present trial (Appendix I). Different combination of sugars, sugar concentration and variety showed different values that were presented in Table 8. The highest (97.17%) germination was recorded from sucrose at sugar concentration of 2 g/L with BR3 and the lowest (35.71%) was recorded from maltose at sugar concentration at 2 g/L with BR4. The highest (96.46%) germination was recorded from sucrose at sugar concentration of 2 g/L with BR5 and the lowest (35.15%) was recorded from maltose at sugar concentration at 2 g/L with BR4. The highest (54.55%) regeneration was recorded from sucrose at sugar concentration of 2 g/L with BR5 which was closely followed (34.78%) by maltose with sugar concentration at 2 g/L with Taipei309. From the above results it may be concluded that BR5 was the best performer followed by BR3 for the interaction of sugar, sugar concentration, it was better than the check variety Taipei309 in case of all test parameters.

Table 8. Combined effect of different sugars and sugar concentration and variety on germination, callus induction and regeneration

Treatment			Germination (%)	Callus induction (%)	Plant regeneration (%)
Sugar	Sugar Conc. (g/L)	Variety			
Sucrose	2	BR1	85.70 ef	83.82 fgh	3.44 m
		BR2	66.65 kl	64.17 qr	0.00 n
		BR3	97.17 a	95.43 ab	0.00 n
		BR4	88.56 cde	88.09 def	0.00 n
		BR5	97.14 a	96.46 a	54.55 a
		Taipei309	76.17 ij	76.00 lmnop	14.28 h
	3	BR1	80.24 ghi	79.83 hijkl	0.00 n
		BR2	97.14 a	94.93 ab	0.00 n
		BR3	94.29 ab	92.58 abcd	0.00 n
		BR4	73.29 j	71.58 p	7.14 kl
		BR5	90.47 bcd	88.45 de	18.18 f
		Taipei309	80.94 gh	77.92 klmn	6.25 l
	4	BR1	64.26 l	61.75 r	6.67 kl
		BR2	73.80 j	72.34 op	0.00 n
		BR3	92.86 abc	90.93 bcd	0.00 n
		BR4	49.99 n	49.46 t	9.09 j
		BR5	88.09 de	85.88 efg	18.75 f
		Taipei309	76.18 ij	74.01 mnop	34.78 b
Maltose	2	BR1	83.31 fg	81.44 ghijk	0.00 n
		BR2	77.14 hij	75.03 mnop	10.00 j
		BR3	97.14 a	94.49 ab	0.00 n
		BR4	35.71 o	35.15 u	0.00 n
		BR5	97.14 a	95.67 ab	18.75 f
		Taipei309	92.86 abc	91.34 bcd	3.33 m
	3	BR1	57.12 m	55.71 s	0.00 n
		BR2	78.56 hi	76.73 klmno	16.67 g
		BR3	92.86 abc	91.63 abcd	0.00 n
		BR4	51.41 n	50.11 t	14.28 h
		BR5	94.28 ab	92.13 abcd	13.33 hi
		Taipei309	76.18 ij	74.78 mnop	20.12 e
	4	BR1	48.96 n	47.64 t	0.00 n
		BR2	66.66 kl	65.15 qr	10.00 j
		BR3	95.23 a	93.32 abc	0.00 n
		BR4	66.66 kl	64.70 qr	0.00 n
		BR5	92.86 abc	90.90 bcd	0.00 n
		Taipei309	88.09 de	85.67 efg	13.33 hi
Manitol	2	BR1	76.17 ij	74.17 mnop	7.69 k
		BR2	80.94 gh	78.23 jklmn	0.00 n
		BR3	92.85 abc	91.75 abcd	0.00 n
		BR4	68.56 k	66.65 q	0.00 n
		BR5	90.47 bcd	88.69 cde	28.57 d
		Taipei309	77.13 hij	74.44 mnop	0.00 n
	3	BR1	57.12 m	55.10 s	33.33 c
		BR2	76.18 ij	73.51 nop	0.00 n
		BR3	96.42 a	94.32 ab	9.09 j
		BR4	59.42 m	57.05 s	0.00 n
		BR5	85.70 ef	83.46 ghi	0.00 n
		Taipei309	85.71 ef	82.72 ghij	12.50 i
	4	BR1	52.36 n	50.20 t	0.00 n
		BR2	85.71 ef	82.75 ghij	0.00 n
		BR3	97.14 a	94.46 ab	0.00 n
		BR4	85.71 ef	83.26 ghi	10.00 j
		BR5	80.24 ghi	78.82 ijklm	0.00 n
		Taipei309	78.56 hi	76.94 klmno	0.00 n

In a column, having similar letter(s) are statistically identical and those having dissimilar letter(s) differ significantly as per 0.05 level of probability



Chapter 5

Summary and Conclusion

Six rice varieties have been used in the present investigation for observing seed germination, callus induction and regeneration in three sugars supplemented media with three different concentrations. The study was carried out to standardize the different responses of rice for sugars in *in vitro* conditions. The experiments were conducted at the Tissue Culture Laboratory of Biotechnology Division, Bangladesh Rice Research Institute, Gazipur.

To conduct the experiment, dehusked seeds of six rice varieties such as BR1, BR2, BR3, BR4, BR5 and Taipei309 were placed in MS media supplemented with three sugars(Sucrose, Maltose, Manitol) at three different levels (2, 3 and 4 g/L).After germination followed by callus induction, the calli were transferred to MS media for regeneration. The experiment was conducted following Completely Randomized Design(CRD) with 6 replications.

Three sugars(Sucrose, Maltose, Manitol) were used to see their influence on callus induction and plant regeneration. Among three sugars, sucrose is easily available, readily assimilated and relatively stable and most commonly used carbon source. Sucrose is the sugar form most commonly transported in plants, it is broken down into glucose and fructose during metabolism. It is partially hydrolyzed into glucose and fructose during autoclaving. Sucrose contributes to the osmotic potential which have an important effect on *in vitro* response. Sucrose is more soluble and available in tissue culture media. Sucrose may influence secondary metabolism in cell culture. Low sucrose concentration in the medium increases the photosynthesis ability, thereby improving plantlet survival. Alleviated sucrose levels favored rooting and root quality. Not only biomass formation, but photosynthesis was positively affected by sucrose. High sucrose levels were stressful for the shoots which exhibit poor regeneration. Sucrose act as a regulator of xylem differentiation in cultured tissues.

Different sugar concentrations have paramount influence in germination and callus induction which regulate the regeneration capability. All the parameters were inhibited at high sugar level (4 g/L sugar) and magnitude of two parameters (germination and regeneration) were maximum at 2 g/L sugar concentration. However, all the parameters decreased with increased sugar level.

Out of six varieties, BR5 showed excellent performance for all the parameters against different sugar at different concentration levels. Taipei309 and BR1 also performed well against sugar. BR2, BR3, BR4 demonstrated their sensitivity at different sugar concentration levels.

Regeneration is the single most important character in *in vitro* culture. The test varieties showed their differential response in their parameters. From the interaction response of variety x sugar and variety x sugar x sugar concentration, BR5 demonstrated its superiority for percent germination and callus induction in most of cases at lower level of sugar (2 g/L). For regeneration in few cases no response was obtained, but statistically highly significant response was obtained from BR5 than those of other varieties. BR5 was most responsive to *in vitro* culture and showed high frequency of plantlet regeneration. I *In vitro* Response to culture condition was found genotype specific.

It appears from present study that the increased sugar level are not beneficial for the plant in tissue culture media. Considering the results of the present study, further research may be conducted with other varieties of rice to make a firm and general recommendation.

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Appendix

APPENDIX

Appendix 1. Analysis of variance of germination, callus induction and plant regeneration

Source of variation	Degrees of freedom	Mean square		
		Germination (%)	Callus induction (%)	Regeneration (%)
Variety (A)	2	547.851	591.582	464.841
Sugar (B)	2	793.861**	888.699**	215.473**
Genotypes (C)	4	628.268**	627.331**	638.193**
Interaction (A×B)	5	8138.748**	627.331**	1850.209**
Interaction (A×C)	10	784.890**	7998.304**	968.247**
Interaction (B×C)	10	676.447*	762.810**	1009.986**
Interaction (A×B×C)	20	590.281**	564.974**	318.678**
Error	270	11.342	12.939	0.984