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Improvement of Abiotic Stress Tolerant Rice (*Oryza Sativa* L.) Through Biotechnological Techniques

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**IMPROVEMENT OF ABIOTIC STRESS
TOLERANT RICE (*ORYZA SATIVA* L.) THROUGH
BIOTECHNOLOGICAL TECHNIQUES**



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

**BY
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MARCH 2016

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**Dedicated
to
the departed souls of my
father**

Shafi Ahmed
and mother
Zamila Ahmed

DECLARATION

I hereby declare that the research work embodied in this thesis entitled **“Improvement of Abiotic Stress Tolerant Rice (*Oryza sativa* L.) Through Biotechnological Techniques”** has been carried out by me for the degree of **Doctor of Philosophy** under the supervision of Dr. S. M. Shahinul Islam, Institute of Biological Sciences, University of Rajshahi, Bangladesh.

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CERTIFICATE

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The Author

Abstract

The present study was conducted to improve rice (*Oryza sativa* L.) cultivars considering the features related to tolerance in abiotic stresses, through biotechnological approaches. Seventeen Bangladeshi indica rice varieties were tested to evaluate the efficiency on somatic embryogenesis (SE), organogenesis, relative growth rate (RGR), tolerance index (TI), relative water content (RWC), test of viability of calli in salt stress, effect of partial air desiccation to enhance regeneration. Under this study regeneration has been enhanced by applying desiccation as stress pre-treatment factors. To develop transgenic plants tolerance to major abiotic stress (salt) *Agrobacterium*-mediated genetic transformation has been conducted.

For SE, callus induction (CI) and its subsequent regeneration were done to screen a suitable rice cultivar. For this purpose, effects of fifteen different concentration and combination (T₁-T₁₅) of plant growth regulators (PGRs) singly and/ or combined were tested for CI. Out of seventeen varieties, BRRI dhan29 performed the highest number of CI (78.67%) in T₅ (MS + 2.5 mg/l 2,4-D) and the lowest was for both BR11 and BRRI dhan27 (33.33%) in T₁ (MS + 0.5 mg/l 2,4-D). Effects of four basal media i.e. MS, N6, LS and SK1 were examined to CI where maximum callusing was recorded for BRRI dhan29 (77.33%) in MS; while the lowest was in SK1 for BR23 (36.00%).

Fifteen types of PGRs (H₁-H₁₅) individually and/ or combined were examined for regeneration; the maximum performance was found for BRRI dhan29 (70.67%) when 3 weeks old calli were transferred to MS that supplemented with 2.0 mg/l BAP + 1.0 mg/l Kin + 1.0 NAA (H₁₁); and BRRI dhan32 (22.67%) gave the lowest plant regeneration in H₁₅ (MS + 4.0 mg/l BAP + 0.5 mg/l Kin + 0.5 mg/l NAA). Considering 2.0 mg/l BAP + 1.0 mg/l Kin + 1.0 NAA (H₁₁) as constant, highest plant regeneration was recorded for BRRI dhan29 (69.33%) in MS, out of four basal media (MS, N6, LS and SK1) tested; and lowest in LS for BR10 (24.00%).

To observe the viability of calli in salt stress, four concentrations of NaCl (50, 100, 150 and 200 mM) were tested. The varieties BRRI dhan47, BR10 and BRRI dhan32 gave 53.33, 14.67 and 2.67% viable calli after one week cultured in 200 mM NaCl

levels respectively. When the calli of BRRI dhan32 were cultured for 4 weeks no calli were viable; while in case of BRRI dhan47 45.33% calli were alive at 200 mM salt level.

Three weeks old calli were cultured in 50, 100, 150 and 200 mM NaCl levels also for 3 weeks; and RGR (relative growth rate), TI (tolerance index) and RWC (relative water content) were determined. The recorded RGRs were 1.03, 0.23 and 0.11 at 200 mM for BRRI dhan47, BR10 and BRRI dhan32; while in control condition (without salt) RGRs were 5.12, 4.02 and 4.61 respectively. BRRI dhan47 carried the highest ability to grow in salt stress showing TI of 0.20 in 200 mM salt level. Comparatively lower TI values were recorded for BR10 (0.06) and BRRI dhan32 (0.02). The WRC were BRRI dhan47 (10.23%), BR10 (7.22%) and BRRI dhan32 (7.03%) in 200 mM salt. The growth pattern of BRRI dhan47 was determined in 0, 50, 150 and 200 mM salt stress. In this case RGR was increased up to two weeks at all NaCl levels. While after 2 weeks RGR was restricted at 100, 150 and 200 mM stress levels. In 50 mM, RGRs were gradually increased up to 4 weeks.

Applying air desiccation pretreatment (15, 30, 45 and 60 hrs) to the calli of four age groups (3, 4, 5 and 6 w), around 2 folds enhanced regeneration was gained for BRRI dhan32 when the calli age of 4 weeks was desiccated by 45 hrs. Desiccated calli at optimal level (45 hrs) gave enhanced plant regeneration in salt stress. In this case, BRRI dhan47 gave 1.98 folds higher regeneration (26.98%) than undedicated (control) calli (14.29%) at 200 mM NaCl. In the same stress level (200 mM), BR10 and BRRI dhan32 could not be regenerated from undedicated calli; whereas, after desiccation pretreatment they were been capable to regenerate plants with frequency of 11.11% and 4.76%, respectively.

The experiments on organogenesis were done for investigating regeneration efficiency of nine explants *viz.* mature seeds (zygotic embryos, ZE), radicle, adventitious root (AT), adventitious root tips (ART), mesocotyl nodal segments (MNOS), mesocotyl internodal segments (MINS), coleoptile, primary leaf (PL) and secondary leaf (SL) of BRRI dhan29. The highest callusing was found in ZE (70.63%) and lowest in coleoptile (12.50%) in MS + 2.5 mg/l 2, 4-D + 1.0 mg/l kin +

300 mg/l L-proline + 400 mg/l. It was observed that maximum primary calli of ZE couldn't be proliferated for producing secondary callus, while almost all the primary calli of MNOS and MINS were proliferated and produced secondary callus. The calli of four age groups (3, 4, 5 and 6 weeks) induced from different explants were separately transferred to regeneration medium (MS + 2.0 mg/l BAP + 0.5 mg/l NAA + 1.0 mg/l Kin) for plant regeneration. Among nine explants, 6 weeks old calli of MNOS gave the highest regeneration (90.63%), and lowest in AR (32.50%) for 3 weeks old calli. ZE gave maximum 55.00% regeneration by 4 weeks old calli which was lower than MNOS (90.63%), MINS (89.06%), PL (75.00%), SL (72.92%) and coleoptile (71.88%).

Agrobacterium-mediated transformation was conducted using BRRI dhan29 by targeted gene of *PDH47* (pea DNA helicase 47). The co-cultured calli derived from ZE and MNOS produced 23.33 and 18.33% putatively transgenic plants respectively after infection of *Agrobacterium tumefaciens* strain of LBA4404. Out of eleven putatively transgenic plants, four showed PCR positive which obtained from ZE derived calli, analyzed through genomic DNA isolation. Further studied have been done and all four showed positive results that confirmed gene integration by southern hybridization analysis. It was observed that the potentiality of explants to regenerate transgenic plants were varied 6.67% for ZE and 3.33% for MNOS. In the experiment of leaf disk senescence (LDS), the transgenics were able to hold chlorophyll contents in their mesophyll tissues and remaining healthy with green in color.

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LIST OF ABBREVIATIONS

The following abbreviations have been used through the text.

0.1N	:	0.1 Normal solution
2,4-D	:	2,4-dichloro phenoxy acetic acid
%	:	Percent
µg	:	Micro gram
µl	:	Micro liter
µM	:	Micro mole
e.g.	:	exempli gratia = for example
BAP	:	6-Benzyl amino purine
bp	:	Base pair
°C	:	Celsius
CH	:	Casein hydrolysate
cm	:	Centimetre
DMRT	:	Duncan's Multiple Range Test
dS/m	:	deciSiemens per meter
et al.	:	Et alia = and others
etc.	:	et cetra = and other
GFP	:	Green fluorescent protein
GUS	:	β-glucuronidase
g/l	:	Gram per litre
HCl	:	Hydrochloric acid
HgCl ₂	:	Mercuric chloride
IAA	:	Indole- 3-acetic acid
IBA	:	Indole-3- butyric acid
i.e	:	That is (to say)
kb	:	Kilobase
Kin.	:	Kinetin
KOH	:	Potassium hydroxide

LS	:	Linsmaier and Shoog (1965) medium
LSD	:	Least Significance Difference
mg/l	:	Milligram per litre
mM	:	Millimole
MS	:	Murashige and Skoog (1962) medium
N6	:	Chu et al. (1975) medium
NAA	:	α -naphthalene acetic acid
NaOH	:	Sodium hydroxide
NptII	:	Neomycin phosphotransferase gene
OD	:	Optical density
PGRs	:	Plant growth regulators
Sig.	:	Significance
SK1	:	Basal medium Raina et al. (1989)
w/v	:	Weight by volume
v/v	:	Volume by volume
<i>viz.</i>	:	Videlicet (L.); namely
ANOVA	:	Analysis of variance
EDTA	:	Ethylenedinitrilo tetra acetic acid
LSD	:	Least significant difference
p ^H	:	Negative logarithm of hydrogen ion (H ⁺) concentration
SE	:	Standard error/ Somatic embryogenesis

Chapter I

General Introduction



1. General Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops belongs to the family of Poaceae. It was cultivated first in South-east Asia, India and China between 8000 and 15000 years ago (OECD 1999, Normile 2004). It contains well developed classic genetics having a simple diploid genome ($2n=2X=AA=24$) which relatively small in size (4.2×10^8 bp) for each haploid (Ayres and Park 1994).

Rice is the food crop for nearly half of the world's population and the staple food in Bangladesh (Sellamuthu et al. 2011). The food and energy source of the world's population entirely depends on cereal crops. Among the cereals, rice currently provides 20% of the world's dietary energy, while wheat supplies 19% and maize 5% (FAO 2004). In another report it has been mentioned that rice, wheat, maize and soybean provide nearly 43% of global dietary energy and 40% of daily protein (Ray et al. 2013). More than 95% of population consumes rice and it alone provides 76% of calorie requirement of daily food intake (Bhuiyan et al. 2002). It provides approximately 30% and 27% of the dietary energy in India and China, respectively (Ray et al. 2013). In Bangladesh rice supplies 75.6% of dietary energy per capita. Above two billion people worldwide totally depend upon rice and in Asia rice is the basic food, where it provides 40 - 70% of the total food calories consumed (Datta 2004). About 95% of the rice is consumed by human as unprocessed food and remaining 5% as the processed foods like industrial products and alcoholic beverages. Rice provides fully 60% of the food intake in South-east Asia and about 35% in East Asia and South Asia. The highest level of consumption per capita is 130 - 180 kg yearly and 55 - 80% of total caloric source takes place in Bangladesh, Cambodia, Indonesia, Laos, Myanmar, Thailand and Vietnam.

Rice contains low fat and protein compared with other cereal grains. Brown rice has a greater food value than white, since the outer brown coating contains the proteins and minerals. It is also a source of magnesium (Mg), thiamine, niacin, phosphorus (P), copper (Cu), zinc (Zn) and vitamin-B6. Some varieties have iron, potassium and folic acid. White rice is one of the poorest cereals in proteins; some improved varieties

however provide 14% of proteins. It is low in riboflavin and thiamine content. Golden rice, a genetically modified food contains β -carotene which is the source of vitamin A. According to Helen Keller International (HKI) 190 million of pre-school children and 19 million pregnant women are currently deficient from vitamin A (Golden rice project, <http://www.goldenrice.org/>). Each year, an estimated 670,000 children die from vitamin A deficiency (VAD), and 350,000 are going to blind. In Bangladesh, one in every five of pre-school children is estimated to have VAD. In 2012 the World Health Organization (WHO) reported that about 250 million pre-school children were affected by VAD.

In recent rice represents 30% of global cereal production that has been doubled over the past 30 years. Worldwide, rice is cultivated on an area of 154 million hectares with an annual production of 700 million tons. China is the 1st largest rice producer in the world and the 2nd is India; while Bangladesh ranked as the 4th, producing 34.6 million metric tons of rice (USDA 2015). Considering the cultivable area, India ranked the 1st; yet 2nd in production, producing 131 million of tons. FAO has maintained its positive outlook in Bangladesh at production of 52.5 million tons (FAO 2014).

The total population of the world is exceeded 7 billion on 12 March, 2012. At recent it is estimated as 7.075 billion. World population is increasing day by day and it will reach 9.1 billion by the year 2050. United States Census Bureau (USCB) has developed software of 'Population Clock' which is counting the population of the world every moment automatically (<http://www.census.gov/popclock/>). The software showed that the total number of population of the world is 7302820001 at the moment of 02 February, 2016, 14:42:07 UTC and it is increasing by every moment. In another report USCB has been expressed that global population is increasing above 1.8% yearly; and world population may rise to 16 billion by 2100. However, according to USCB Bangladesh is the 8th largest country having population of 168,957,745 at 02 February, 2016. It is the 3rd densely populated country in the world with density of 1034 per sq. kilometer; and population growth rate is 1.58%. So that the world with rapidly increasing population is being gone to be faced the challenge of food security. In order to feed this population, food production needs to be

increased by 70% of the present production; and global agricultural production must be increased by 60 - 110% (FAO 2009, Tilman et al. 2011). Current rate of grain production are not sufficient to meet the increasing food demand. Nearly 2.4% increase in grain yield per year is needed to double the crop production by 2050. Another reports expressed that in order to avoid shortages, rice yields must increase by 30% over the next 20 years. But at current rates, approximately 60, 42, 38 and 55% increase in maize, rice, wheat and soybean production respectively is possible by that time (Ray et al. 2013). Increase in rice yield by 1.0% per year, may not result in any change in the per capita rice harvest by 2050. In Bangladesh, India, Afghanistan, Laos, Vietnam and Cambodia, rice production is doubling, but significant reduction of yield is found in some local parts of India like Uttar Pradesh, Maharashtra and Tamil Nadu and there is no significant change in per capita rice harvest in Pakistan, Nepal, Malaysia and South Korea (Ray et al. 2013). According to Bangladesh Bureau of Statistics (BBS) agriculture contributes about 22% of the gross domestic product (GDP) including 9.5% of rice contribution alone in 2008 (www.bbs.gov.bd/).

Bangladesh is an agro-based developing country where rice is grown in three distinct seasons, namely Boro (January to June), Aus (April to August), and Aman (August to December) covering almost 11.0 million hectares of land, producing nearly 95% of the total food requirements. Our cultivable land is very insufficient, so that it is not possible to ensure food security without development of high yielding rice varieties. On relation to increase rice consumer, its production has to be increased obviously. So it's the crying need to develop high yielding rice varieties to ensure the food security of the world. Using the methods of traditional breeding a lot of high yielding varieties have been developed by Bangladesh Rice Research Institute (1999) in Bangladesh. By crossing between the varieties BR6 and Purbachi, the hybrid BRRI dhan28 has been developed. Similarly BRRI dhan37 has also been developed from BR5 and Basmati. But this process is very lengthy and sometimes it takes several years.

The International Rice Research Institute (IRRI) is employing *in vitro* tissue culture techniques to develop rice varieties (Bajaj and Rajam 1996). Regeneration from callus was achieved long back in japonica varieties (Nishi et al. 1973). Strategies to improve plant regeneration frequency in cereals, including rice have been steadily evolving

during the last decade (Kyozyuka et al. 1988, Datta et al. 1992). The totipotent character of plant cells that retains its nucleus has the ability to regenerate entire new plant by somatic embryogenesis (SE) or organogenesis (Fortes and Pais 2000). Protocol of SE has been reported to regenerate plants *in vitro* for indica rice (Rance et al. 1994, Seraj et al. 1997, Islam et al. 2005, Makerly et al. 2012, Haque et al. 2013) and also for japonica rice (Lee et al. 2002, Sah et al. 2014). Some author have experimented the potentiality of Bangladeshi indica rice for plant regeneration via SE (Hoque et al. 2007, Haque et al. 2013, Islam et al. 2013, Zinnah et al. 2013, Fook et al. 2015, Hossain et al. 2015, Din et al. 2016). In 2014 Siddique et al. regenerated Bangladeshi indica rice viz. BR10, BRRI dhan32 and BRRI dhan47 with high frequency through somatic embryogenesis. Furthermore, Bangladeshi indica rice has also been regenerated through anther and microspore culture (Khatun et al. 2010, Islam and Tuteja 2012a, Islam et al. 2013). Regeneration of *in vitro* plants has been done by organ culture (Organogenesis) in rice (Sahrawat and Chand 2001, Khatun et al. 2003, Ramesh et al. 2009, Ghobeishavi et al. 2014).

Genetic factors are considered to be a major contributor to *in vitro* response of cultured tissues. Peng and Hodges (1989) analyzed genetically the phenomenon of regeneration. Differences in the production of embryogenic calli and the regenerated plantlets have been observed, depending on the genotype and source of the explants (O'Toole 1982, Rashid et al. 1996, Al-Bahrany 2002a, Wang et al. 2003, Lutts et al. 2004, Khalequzzaman et al. 2005, Mahajan and Tuteja 2005). It was reported that the factors like explants, media and genotype were characterized and optimized for reproducible and efficient *in vitro* tissue culture system for various rice genotypes (Nishi et al. 1968, Oinam and Kotharii 1995). A comprehensive study of 500 rice varieties showed that callus formation and plant regeneration both were highly genotype dependent (Peng and Hodges 1989). Regeneration efficiency is affected by a number of factors including the genotype, the type and physiological status of the explants, media composition, plant growth regulators (PGRs) and culture conditions (Rueb et al. 1994). Mannan et al. (2013) investigated the effect of various PGRs on callus induction and plant regeneration efficiency in two Bangladeshi rice varieties, Kalijira and Chinigura; and reported that MS medium supplemented with 2 mg/l of

2,4-D was the most effective PGR to induce calli for both cultivars. Abe and Futsuhara (1989) reported that selection and differentiation of embryogenic calli is necessary to get efficient regeneration. Efficient plant regeneration has been widely reported in japonica and indica rice by optimizing several factors influenced such as sugars (Geng et al. 2008, Feng et al. 2011), amino acids (Pons et al. 2000), micronutrients (Rueb et al. 1994) and hormones (Zuraida et al. 2011). Aananthi et al. (2010) tested two basal media MS (Murashige and Skoog 1962) and N6 (Chu et al. 1975) for induction in five indica rice (*Oryza sativa* L.) cultivars viz., ASD 16, White Ponni, Pusa Basmati 1, Pusa Sugandh 4 and Pusa Sugandh 5.

However, Abe and Futsuhara (1986) tested 66 rice varieties and reported large differences between indica and japonica *in vitro* manipulation. Ge et al. (2006) reported that among the rice sub-species, indica and javanica were recalcitrant to tissue culture manipulation. Many indica rice genotypes including Malaysian rice are recalcitrant to tissue culture manipulation due to poor callus induction, proliferation and regeneration (Zuraida et al. 2010). Moreover, it has been reported that the success for reproducible fertile plant regeneration in indica rice varieties limited (Kyozyuka et al. 1988). The japonica rice showed higher response in tissue culture as compared to indica varieties (Abe and Futsuhara 1984). Till now reports on *in vitro* plant regeneration as well as on advance biotechnological research in Bangladeshi indica rice is limited. However, due to various abiotic stresses like drought, submergence tolerance and salinity, production of rice is decreased; therefore, development of abiotic stress-tolerant rice cultivars is the main priority (Grover and Minhas 2000). Against the increasing population, production of rice at the rate of our expectation is hampered due to many threats around the environment, such as drought, salinity, heat, cold etc.

Drought is a critical and most important abiotic stress to influence rice productivity. It is a main problem for crop production specially rice worldwide, limiting the growth and productivity of many crop species particularly in rainfed agricultural areas (Chaves and Oliveira 2004). It is estimated to be one of the most serious yield reducing stresses in the agriculture. However, water deficit is subjected to a range of

abiotic stresses that affect growth and development; and it affects global crop yields (Hasegawa et al. 2000). It limits rice productivity in rainfed and upland ecosystems and worldwide, affects approximately 27 million hectares of rainfed (Bimpong et al. 2011). Seventy percent of the world's food-growing areas turn increasingly parched (IRRI 2009). Drought reduces yield by 15 - 50% depending on the stress intensity and crop growth period at which the stress occurs in rice (Srividhya et al. 2011). Root traits are the key component of rice plant adaptation to drought stress (Courtois et al. 2009). It is important for numerous functions including water and nutrient uptake (MacMillan et al. 2006).

Plant response to drought stress is one of the most complex biological processes, and it involves numerous changes at the physiological, cellular and molecular levels. Many genes have been identified involving in response to drought stress in plants (Zhang et al. 2012). Usually plants respond and adapt themselves to dehydration by altering their cellular metabolism and activating defense mechanisms. Characterization of physio-morphological changes in the transgenic material is very important to unearth mechanism of drought tolerance (Wang et al. 2012). The effect of drought on rice plants considerably varies with genotypes, developmental stages, and degree and duration of drought stress (Wang et al. 2011, Siddique et al. 2014). Biochemical changes i.e. proline, glycine betaine, soluble sugars, photosynthetic pigments and defensive proteins and physiological as well as water use efficiency, osmotic adjustment, chlorophyll a fluorescence, and net-photosynthetic rate in plants growing under salt or water-deficit conditions have been broadly investigated in rice (Ashraf and Harris 2004, Castillo et al. 2007, Ashraf 2010). Thus different physiological processes in plants are more or less affected by drought. Drought stress responses involving physiological processes are operational in certain plants (Passioura 2007). Haque et al. (2013) reported that there was reduction in callus induction and plant regeneration efficiency with increasing levels of mannitol stress. They mentioned that mannitol can be used as drought stress creating agent under *in vitro* conditions and rice variety BRRI dhan29 was relatively tolerant to drought stress as compared to BRRI dhan28.

Salinity is another major abiotic stress which is one of the main problems to produce rice with high rate. In Bangladesh, greater than 1 million hectares of coastal area are affected by varying degrees of soil salinity. Rice is sensitive above a soil conductivity of 3 dS/m. During salinity stress, several classes of Na⁺ transporters have been shown to play central roles in Na⁺ homeostasis. High salinity stress causes an imbalance in sodium ion (Na⁺) homeostasis, which is normally maintained by the coordinated action of various pumps, ions, constitutive overexpression of the plasma membrane Na⁺/H⁺ antiporter gene OsSOS1 in rice, cloned downstream of the CaMV35S promoter was overexpressed in the high yielding variety BRRI dhan28 (Razzaque et al. 2014). A gene ‘Saltol’ on salt tolerance in rice located on chromosome No. 1 was reported by Causse et al. (1994).

Partial desiccation, a physical stress factor influenced positively to produce rice plants *in vitro* with high frequency (Saharan et al. 2004, Haq et al. 2009). This factor has also been reported to be beneficial for regeneration of indica rice varieties (Jain et al. 1996, Diah and Bhalla 2000, Chand and Sahrawat 2001, Makerly et al. 2012) and in japonica rice (Tsukahara and Hirose, 1992). In Bangladeshi indica rice varieties viz. BR10, BRRI dhan32 and BRRI dhan47, beneficial effect of partial air desiccation has been reported by Siddique et al. (2014). They also investigated that partially desiccated calli were more able to be regenerated in salt induced stress condition than undesiccated calli. Desiccation improves the efficiency of plant regeneration in date palm (Ibrahim et al. 2012). Such beneficial effect was reported in several plants viz. sugarcane (Kaur and Gosal 2009), citrus (Singh and Sengar 2014) and wheat (Stipešević and Kladić 2005, Scotti-Campos et al. 2014).

Tolerant lines could be screened via *in vitro* culture. *In vitro* selection of salt tolerant cell lines has been reported for several species (Dracup 1991, Tal 1994). Research has been conducted on *in vitro* selection for salt tolerance in wheat (Karadimova and Djambova 1993). Salinity is the main abiotic stress that has been addressed by *in vitro* selection, and applications of heat and drought have also been reported (Lutts et al. 1996).

Plant response to abiotic stress is a complex phenomenon, which could be approached efficiently through tissue culture. Genetic transformation is a technique by which functional foreign genes are inserted into genome of the crop plants from diverse biological system. In 2005 the mapping of entire genetic code (genome) for rice was announced by an international team of scientists and mentioned that rice contains 37544 genes. More than 20 genes for resistance to various plant diseases have been isolated from a wild range of plant species including monocotyledonous and dicotyledonous viz. tomato, rice, tobacco and maize. Transgenic technology can significantly strengthen rice-breeding programs, enabling breeders to achieve results more quickly and efficiently than conventional techniques. Genetic engineering is more advanced for rice than any other crop plants cultivated in agriculture (Swaminathan 1982). Many insect resistance transgenic cotton (Awan et al. 2015) and rice (Chen et al. 2011) plants have been developed through gene transformation.

Chan et al. (1992) reported that *Agrobacterium*-mediated transformation was highly genotype dependent. Cereals lack wound response and leads towards the cell death but researchers had reported *Agrobacterium*-mediated transformation in rice (Li et al. 1992). This type of genetic transformation of different japonica cultivars were also achieved using *Agrobacterium* (Hiei et al. 1994). The size of introduced DNA also affected the stable transformation and integration of DNA into rice genome (Uchimiya et al. 1986). In Bangladesh there have tremendous scopes for developing its agricultural crops through transgenic approaches, in particular, to develop the high yielding, disease resistant, abiotic stress tolerant rice.

Rice varieties having high yielding and highly adaptive features have to be developed through facing various stress i.e. salinity, drought. However, to do this development, genetic engineering and other molecular techniques are essential and effective. Hence, Biotechnological techniques can be used for *in vitro* micropropagation, callus induction, plant regeneration and genetic transformation for this purpose. To develop transgenic plant, *Agrobacterim*-mediated genetic transformation has been used in Bangladeshi indica rice (Amin et al. 2012, Yasmin et al. 2015). Progress towards the transformation of useful genes to indica rice has been slow and manipulation of genetic engineering has been hampered due to limitation of indica rice regeneration.

Efficient regeneration and genetic transformation in Bangladeshi indica rice is still poses a major problem for genetic manipulation through innovative approaches. Therefore, the present study was undertaken considering the following objectives.

- ⊙ Screening of suitable rice cultivars for advance biotechnological research.
- ⊙ Standardization of media, culture conditions, plant growth regulators (PGRs) and various combinations on callus induction and regeneration.
- ⊙ To evaluate the effect of partial air desiccation for enhancing *in vitro* plant regeneration.
- ⊙ Effect of salt stress to cell viability, cell growth and development, and proliferation of callus.
- ⊙ Enhancement of capability of cell existence saline condition applying physical stress factors.
- ⊙ To increase efficiency of plant regeneration in salt induced *in vitro* stress.
- ⊙ Organogenesis of rice using various types of explants of vegetative organs.
- ⊙ *In silico* analysis of targeted gene and cloning to related vectors for transformation. Confirmation of cloning by PCR, restriction enzyme (RE) analysis.
- ⊙ To determine the efficiency on response to *Agrobacterium*-mediated genetic transformation of Bangladeshi indica rice.
- ⊙ Analysis of transgenics for different abiotic stress specially salinity.

Chapter II

Review of Literature



2. Review of Literature

Rice is an annual plant and vital cereal crop entire the population of the world. Rice plant produces the grain is known as rice, a staple food which constitute the dominant part of the diet and supply a major proportion of energy and nutrient needs for a large part of the world's population. The vegetative body of rice plant composes of four main parts such as, root, stem, leaf and panicle. It is a monocotyledonous angiospermic plant.

2.1 Taxonomy and classification of rice

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

Taxon	Name of taxon
Kingdom	: Plantae
Subkingdom	: Viridiplantae
Infrakingdom	: Streptophyta
Superdivision	: Embryophyta
Division	: Tracheophyta
Subdivision	: Spermatophytina
Class	: Magnoliopsida
Superorder	: Lilianae
Order	: Poales
Family	: Poaceae
Genus	: <i>Oryza</i> L.
Species	: <i>Oryza sativa</i> L.

Oryza sativa L. Taxonomic Serial No. (TSN): 41976

http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=41976

According to Plants Database ITIS there are seven accepted species of rice:

- Species:*Oryza barthii* - Barth's rice
- Species:*Oryza glaberrima* - African rice
- Species:*Oryza latifolia* - Broadleaf rice
- Species:*Oryza longistaminata* - Longstamen rice
- Species:*Oryza punctata* - Red rice
- Species:*Oryza rufipogon* - Brownbeard rice
- Species:*Oryza sativa*–Rice

2.2 Origin, history and distribution

The genus *Oryza* includes approximately 22 species of which 20 are wild and only 2 species are cultivated. Two cultivated species are *O. sativa* L. and *O. glaberrima* Steud. *O. sativa* L. is common Asian rice found in most producing countries which originated in the Far East at the foot of the Himalayas. *O. sativa japonica* grew on the Chinese side of the mountains and *O. sativa indica* on the Indian side. The majority of the cultivated varieties belong to this species, which is characterized by its plasticity and taste qualities. *O. glaberrima* is annual species originating in West Africa, covering a large region extending from the central Delta of the Niger River to Senegal. African rice (*O. glaberrima* Steud.) is confined to West Africa, whereas common or Asian rice (*O. sativa* L.) is now commercially grown in 112 countries, covering all continents. The wild species are widely distributed in the humid tropics and subtropics of Africa, Asia, Central and South America and Australia (Chang 2003). In the beginning rice grew wild, but today most countries cultivate rice varieties belonging to the genus *Oryza*. Rice is grown in spanning an area from 53° latitude north to 35° south. It is widely grown both in upland and in water in tropical and subtropical region.

It is difficult to cite the time for the botanist when the cultivation was first started in the world. It is believed that rice cultivation began simultaneously in many countries over 6500 years ago. The first crops were observed in China (Hemu Du region) around 5000 BC as well as in Thailand around 4500 BC. They later appeared in Cambodia, Vietnam and southern India. From there, derived species Japonica and Indica expanded to other Asian countries, such as Korea, Japan, Myanmar, Pakistan, Sri Lanka, Philippines and Indonesia. After the middle of the 15th century, rice spread throughout Italy and then France, later propagating to all the continents during the great age of European exploration. In 1694 rice arrived in the South Carolina, probably originating from Madagascar. The Spanish took it to South America at the beginning of the 18th century and soon became an important crop. Chief growing areas of the United States are in California, Mississippi, Texas, Arkansas and Louisiana. Between 1500 and 800 BC, the African species (*O. glaberrima*) propagated from its original center, the Delta of Niger River and extended to Senegal.

However, it never developed far from its original region. Rice has been cultivated in China since ancient times and was introduced to India before the time of the Greeks. Chinese records of rice cultivation go back 4,000 years.

Oryza sativa L. was first cultivated in south-east Asia, India and China between 8000 and 15000 years ago (Normile 1997). *O. glaberrima* has been cultivated since approximately 1000 BC (Ahn et al. 1992). Current cultivation for *O. sativa* is worldwide, extending from latitude 35°S (New South Wales and Argentina) to 50°N (Northern China), over 110 countries. Rice is also grown from sea level to 3000 m and in both temperate and tropical climates. Rice can grow in a wide range of soil types as well, including saline, alkaline and acid-sulfur soils (Ahn et al. 1992).

The International Rice Gene Bank holds approximately 100,000 different rice varieties, most of which are *O. sativa* (<http://www.knowledgebank.irri.org>). Cultivars can be divided into three ecological varieties, Indica (tropical and sub-tropical distribution), Javanica (grown in Indonesia) and Japonica (temperate distribution). Japonica cultivars are grown predominantly in temperate regions and can germinate and grow under lower temperatures (15 to 20°C) than the tropical and sub-tropical indica cultivars. Temperatures below 18°C at night during pollen formation result in sterile pollen in all cultivars (McDonald 1994).

2.3 Rice and Myths

Rice is an integral part of many cultures folklore. In Myanmar, the Kachins were sent forth from the center of the earth with rice seeds and were directed to a country where life would be perfect and rice would grow well. In Bali, Lord Vishnu caused the earth to give birth to rice and the God Indra taught people how to raise it. In China rice is the gift of animals. Legend says after a disastrous flooding all plants had been destroyed and no food was available. One day a dog ran through the fields to the people with rice seeds hanging from his tail. The people planted the seeds, rice grew and hunger disappeared. All of these stories and many others have rice as their foundation and for generations people have believed these lores of rice (<http://www-plb.ucdavis.edu/labs/rost/Rice/introduction/intro.html>).

2.4 Rice in Bangladesh

In Bangladesh rice is very popular and main food where a proverb ‘MASE VATE BANGALI’ is established. The economy and culture are closely related to rice in this country. Though Bangladesh is 4th on accordance of rice production, its yield is 4.32 ton/ha; whereas in China, Japan, and Korea it is 6-6.5 ton/ha. In 1968 the cultivation of high yielding rice variety IR8 of International Rice Research Institute (IRRI) was been initiated. This rice variety was known to the people of Bangladesh as ‘IRRI dhan’. However, in 1970 Bangladesh Rice Research Institute (BRRI) has been established and the institute released 53 high yielding and 4 hybrid rice varieties till 2012. According to BRRI, Bangladeshi rice varieties are divided into three groups, such as Sonatony dhan (local), Upshi dhan (high yielding) and Adhunik dhan is high yielding along with special features (BRRI 2011). Rice production in Bangladesh is shown in **Table 1**.

Table 1: Rice production in Bangladesh from 2006 to 2015 (Unit of measurement = 1000 MT)

Market year	Production	Growth rate	Market year	Production	Growth rate
2006	29000	0.84 %	2011	33700	6.31 %
2007	28800	-0.69 %	2012	33820	0.36 %
2008	31200	8.33 %	2013	34390	1.69 %
2009	31000	-0.64 %	2014	34500	0.32 %
2010	31700	2.26 %	2015	34600	0.29 %











2.5 World rice cultivation and production

Rice has been cultivated for more than 7,000 years (YunFei et al. 2007, Zong et al. 2007). It is grown in more than a hundred countries, with a total harvested area of approximately 158 million hectares in 2009, producing above 700 million tons annually. In February-2016, United States Department of Agriculture (USDA) has estimated that the World Rice Production 2015/2016 will be 471.09 million metric tons, around 1.59 million tons more than the previous month's projection

(<https://www.worldriceproduction.com/default.asp>). The world's leading rice producing countries are China, India, Indonesia, Bangladesh, Vietnam and Thailand. The largest three exporting countries are Thailand (26% of world exports), Vietnam (15%), and the United States (11%), while the largest three importers are Indonesia (14%), Bangladesh (4%), and Brazil (3%). China and India are the top two largest producers of rice in the world (**Table 2**).

More than 90% of the world's rice is grown and consumed in Asia, where 60% of global population lives. Rice accounts for 35-60% of the calories consumed by 3 billion people in Asia alone. Rapid growth in population throughout the world is boosting demand for a corresponding increase in grain yield (Liang et al. 2010) and there is need to increase production 50% more by 2025 (Khush 2001). There is need to produce 40% more rice for rice consuming countries by the year 2030 (Khush 2005, Zhu et al. 2010).

Table 2: Top 10 largest rice producers in the world

Rank	Country	Production (1000 MT)	
1	China	145,770	
2	India	100,000	
3	Indonesia	36,300	
4	Bangladesh	34,600	
5	Vietnam	28,200	
6	Thailand	16,400	
7	Myanmar	12,200	
8	Philippines	11,500	
9	Brazil	8,000	
10	Japan	7,900	

MT = Metric ton, Source: United States Department of Agriculture, 2015.

<http://www.indexmundi.com/agriculture/?commodity=milled-rice&>

2.6 Morphology of rice

The following description on morphology of rice plants is based on McDonald (1979): Rice is a typical grass, forming a fibrous root system bearing erect culms and developing long flat leaves. It forms multiple tillers, consisting of a culm and leaves, with or without a panicle. The panicle emerges on the uppermost node of a culm, from within a flag-leaf sheath and bears the flowers in spikelets (**Fig. 1**). The culm consists of a number of nodes and hollow internodes that increase in length and decrease in diameter up the length of the culm. Primary tillers emerge from nodes near the base of the main culm and secondary and tertiary tillers emerge sequentially from these. Single leaves develop alternately on the culm, consisting of a sheath which encloses the culm and a flat leaf blade. The leaf forms a collar or junctura between the sheath and blade and a ligule and two auricles develop on the inside of the junctura and base of the leaf blade respectively. Cultivars can vary widely in the length, width, colour and pubescence of the leaves.

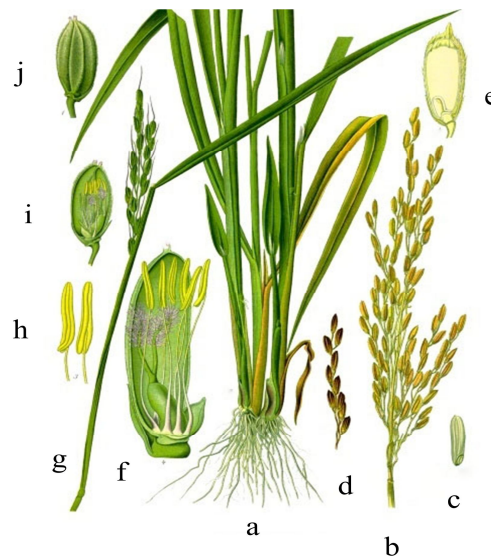


Fig. 1 (a-j): Dissected parts of rice plant showing the morphology. a) rice plant, b) panicle with mature seeds, c) rice grain, d) primary branch of panicle, e) longitudinal section (LS) of mature seed, f) LS of rice flower, g) panicle with hair bearing seed, h) anther, i-j) immature seeds.

Source: <https://en.wikipedia.org/wiki/Rice>

The panicle emerges from the flag-leaf sheath and consists of a central rachis with up to four primary branches at each node. Primary and secondary branches bear the flower spikelets. Each spikelet has a single floret and two glumes. It is enclosed by a rigid, keeled lemma, which is sometimes extended to form an awn and partially envelops the smaller palea. The floret contains six stamens and a single plumose ovary with two branches. At anthesis, two lodules at the base of the floret swell and force the lemma and palea apart as the stamens elongate and emerge. The stigma is sometimes exposed as well. The fertilized ovary is a caryopsis, meaning a small, single-seeded dry fruit with the pericarp and seed coat fused. It is commonly called a grain. The grain consists of an embryo, endosperm, pericarp and testa, surrounded by the husk or hull (the lemma and palea). Grain length varies with cultivar between 5 and 7 mm, and grains can be round, bold or slender.

2.7 Genomes of rice plant

Rice is now becoming a model cereal plant for molecular genetic research, primarily because of its small genome size (4×10^8 bp) (Arumuganathan and Earle 1991), well developed linkage maps (Causse et al. 1994), and high level of synteny and colinearity of genes with other cereal genomes (Ahn et al. 1993, Moore et al. 1995). A diploid cell of rice carried 24 chromosomes (12 pairs). The genomes size of rice (*Oryza sativa* L. japonica subspecies) is 420 Mb; and suggested the genome contain 32000 to 50000 genes (Goff et al. 2002). The size of rice genome is one sixth of the maize genome and 40 times smaller than the wheat genome (IRGSP 2005). The species of the genus *Oryza* was grouped according to the compatibility of their genomes (**Table 3**). *O. sativa* has an AA-type genome, which means that its chromosomes can pair correctly at meiosis with other AA-type species (Vaughan and Morishima 2003). By definition, gene flow through conventional sexual hybridization is limited to *O. sativa* varieties and to the AA-type genome species within this genus. Gene flow between more distantly related species, particularly those outside of the *Oryza* genus, is restricted to artificial breeding methods such as embryo rescue and somatic hybridization, the regeneration of plants following the fusion of two protoplasts (Liu et al. 1999, Multani et al. 2003).

Table 3: Genome types of *Oryza* species

Oryza species	Genome	Oryza species	Genome	Oryza species	Genome
<i>O. sativa</i>	AA	<i>O. punctata</i>	BB, BBCC	<i>O. latifolia</i>	CCDD
<i>O. glaberrima</i>	AA	<i>O. malampuzhaensis</i>	BBCC	<i>O. australiensis</i>	EE
<i>O. barthii</i>	AA	<i>O. minuta</i>	BBCC	<i>O. brachyantha</i>	FF
<i>O. glumaepatula</i>	AA	<i>O. eichingeri</i>	CC	<i>O. granulata</i>	GG
<i>O. longistaminata</i>	AA	<i>O. officinalis</i>	CC	<i>O. meyeriana</i>	GG
<i>O. meridionalis</i>	AA	<i>O. rhizomatis</i>	CC	<i>O. longiglumis</i>	HHJJ
<i>O. nivara</i>	AA	<i>O. alta</i>	CCDD	<i>O. ridleyi</i>	HHJJ
<i>O. rufipogon</i>	AA	<i>O. grandiglumis</i>	CCDD		

2.8 Growth stages of rice

The growth of the rice plant is divided into three phases. Rice is generally a short living plant, with an average life span of 3-7 months; its life span depends on the climate and the variety. The life span of rice comprises three basically distinct sequential growth stages that are vegetative growth stage, reproductive growth stage, and ripening growth stage (Yoshida and Hasegawa 1982).

2.8.1 Germination and vegetative growth

After imbibition of the seed, germination begins with the emergence of the coleorhiza and coleoptile from the pericarp. The radicle gives rise to the seminal root system, which has limited branching. Germination can occur under aerobic or anaerobic conditions. Under anaerobic conditions, the coleoptile emerges first, as it is the only part of the embryo that can grow under energy derived solely from fermentation (Moldenhauer and Gibbons 2003).

2.8.2 Reproductive development

The reproductive stage begins with panicle initiation. The timing of this may be linked to specific photoperiods and is highly cultivar-dependent (McDonald 1979). Panicle initiation occurs at the growing tip of the tiller. As the panicle grows inside the flag-leaf sheath, senescence of the lower leaves begins (Moldenhauer and Gibbons 2003). In this stage when rice is most sensitive to cold temperatures (McDonald 1979).

2.8.3 Ripening development

The ripening period is characterized by grain growth-increase in size and weight, changes in grain color, and senescence of leaves. At the early stages of ripening, the grains are green; they turn yellow as they mature. The texture of the grains changes from a milky, semi-fluid stage to a hard solid. On the basis of such changes the ripening period is subdivided into milky, dough, yellow ripe, and maturity stages.

2.9 Vegetative regeneration

Rice plants have the ability to propagate vegetatively, through the production of new growth at internodes after grain maturation. These secondary growths are called ratoons and under favorable conditions, can provide a second harvest. *O. sativa* cultivars range in growth habit from perennial to strictly annual, with the majority of crops grown as annuals. Some of the South American *Oryza* species form ratoons that can break off the parent plants and disperse by floating down rivers, forming mats of regenerative material that can take root on reaching land (Vaughan et al. 2003). In areas with a long growing season, ratoons can produce a second harvest in a single season (Street and Bollich 2003).

2.10 Nutrient values and importance of rice as a food

Rice is the major cereal crop that is primarily consumed by humans directly as harvested, and only wheat and corn are produced in comparable quantity. Rice provides more than 20% of the calories consumed by humans. It is a very nutritious grain, especially brown rice. It has high fiber, vitamin B, carbohydrate, protein etc. Rice is a great source of complex carbohydrates, which is an important source of the energy of our bodies. It has no fat, no cholesterol and is sodium free. It is an excellent food to include in a balanced diet and gluten free, so it is non-allergic, making rice the essential choice for people with gluten free dietary requirements. It is also a good source of vitamins and minerals such as thiamine, niacin, iron, riboflavin, vitamin D, calcium, and fiber. It is also a source of protein containing eight amino acids. It is the most important food grain in the diets of hundreds of millions of Asians, Africans, and Latin Americans living in the tropics and subtropics. Here is presented the nutrient composition of white and brown rice (**Table 4**). Rice is the predominant dietary energy source for 17 countries in Asia and the Pacific, 9 countries in North and South America and 8 countries in Africa. Rice provides 20% of the world's dietary energy supply, while wheat supplies 19% and maize (corn) 5% (FAO 2004).

Table 4: Nutrient content of white and brown rice

Element	White	Brown	Element	White	Brown
Water (g)	12.0	10.00	Selenium (µg)	15.1	-
Energy (kj)	1528	1549.0	Vitamin C (mg)	0.00	0.00
Protein (g)	7.10	7.90	Thiamin (B1) (mg)	0.07	0.40
Fat (g)	0.66	2.92	Riboflavin (B2) (mg)	0.05	0.09
Carbohydrates (g)	80.0	77.0	Niacin (B3) (mg)	1.60	5.09
Fiber (g)	1.30	3.50	Pantothenic acid (B5) (mg)	1.01	1.49
Sugar (g)	0.12	0.85	Vitamin B6 (mg)	0.16	0.51
Calcium (mg)	28.0	23.0	Folate Total (B9) (µg)	8.00	20.0
Iron (mg)	0.80	1.47	Vitamin A (IU)	0.00	0.00
Magnesium (mg)	25.0	143.0	Vit-E, alpha-tocopherol (mg)	0.11	0.59
Phosphorus (mg)	115.0	333.0	Vitamin K1 (µg)	0.10	1.90
Potassium (mg)	115.0	223.0	Beta-carotene (µg)	0.00	-
Sodium (mg)	5.00	7.00	Saturated fatty acids (g)	0.18	0.58
Zinc (mg)	1.09	2.02	Monounsaturated fatty acids (g)	0.21	1.05
Copper (mg)	0.22	-	Polyunsaturated fatty acids (g)	0.18	1.04
Manganese (mg)	1.09	3.74			

Source: <https://en.wikipedia.org/wiki/Rice>

2.11 Golden rice

The Golden rice is a genetically modified rice which able to produce Vitamin A in the grain. It is produced through genetic engineering to biosynthesize beta-carotene, a precursor of vitamin A, in the edible parts of rice (Ye et al. 2000). In the early 1990s Ingo Potrykus and Peter Beyer started the research to develop Golden rice and by the year 1999 they were been succeed. The grain of Golden rice carries beautiful yellow color having usual taste while some genetically modified rice have various plant pigments (**Fig. 2**). This type of rice differs from its parental strain by the addition of three beta-carotene biosynthesis genes. The rice plant can naturally produce beta-carotene in its leaves, where it is involved in photosynthesis. However, the plant does not normally produce the pigment in the endosperm, where photosynthesis does not occur. In 2005, Golden Rice-2 was announced, which produces up to 23 times more beta-carotene than the original golden rice (Paine et al. 2005). Studies have found that

golden rice poses no risk to human health, and multiple field tests have taken place with no adverse side-effects to participants (https://en.wikipedia.org/wiki/Golden_rice). In 2009, results of a clinical trial of golden rice with adult volunteers from the US were published in the American Journal of Clinical Nutrition. The trial concluded that beta carotene derived from golden rice is effectively converted to vitamin A in humans (Tang et al. 2009). Clinical and subclinical vitamin A deficiency is still a problem, affecting 250 million school children worldwide (WHO 1995, Underwood and Arthur 1996). To prevent clinical vitamin A deficiency in developing countries, chemically synthesized vitamin A supplements have been distributed periodically to deficient populations (West et al. 1991, Ribaya-Mercado et al. 2004). In 2005, 190 million children and 19 million pregnant women, in 122 countries, were estimated to be affected by vitamin A deficiency (VAD) (Staff 2009). VAD is responsible for 1-2 million deaths, 500,000 cases of irreversible blindness and millions of cases of xerophthalmia annually (Humphrey et al. 1992).



Fig. 2 (A-D): Rice of genetically modified genotypes carrying various colors. A) white, B) brown (white arrow indicated); C) black (blue arrow indicated) brown, silver, red indica rice and D) golden rice.

Source: <http://www.goldenrice.org/> and <https://en.wikipedia.org/wiki/Rice>

2.12 Green revolution improving of rice

The story of the 'Green Revolution' in rice actually began in India, moved to the Philippines and then throughout Southeast Asia. As early as 1949, the new United Nations Food and Agriculture Organization (FAO) set up a rice breeding program in Cuttack, India. Rice has traditionally been the most important food source across Asia. In India, local scientists crossed japonica rice with taller indica and produce two good strains known as ADT-27 and Mahsuri that yielded well and were adapted to the Indian environment. ADT-27, in particular, created the first phase of the Green Revolution in rice.

In 1949, Dee-geo-woo-gen, a semi-dwarf indica, was crossed with Tsai-yuanchung, a tall, disease-resistant variety. Taichung Native I (TN1) was selected from this cross and released in 1956, the first high yielding indica variety. In 1960, IRRI (International Rice Research Institute) was established in the Philippines. In 1962, IRRI began research and breeder crossed Dee-geo-woo-gen and peta and from the cross high yielding indica rice variety IR8 was selected and released in 1966. It was 10 times the traditional rice yield. IR8 was hailed as "Miracle Rice" and helped to spark what is now known as the Green Revolution.

In 1990, IRRI produced and sent modern varieties around the world. India, Pakistan, Bangladesh, China, Brazil, Argentina, Sri Lanka, Taiwan, Malaysia, Korea, Burma, and even the U.S. use IRRI varieties and agricultural techniques. IRRI is currently working on Super Rice plants that produce 60% grain and 40% straw. The Super Rice also has a vigorous root system, and IRRI scientists are working on improving its resistance to disease and insects. Current high-yielding rice varieties produce around 100 grains per panicle. The prototype Super Rice, on the other hand, produced 250 to 300 grains per panicle. Scientists are also trying to improve the Super Rice grain quality by incorporating genes for disease and insect resistance.

2.13 Tissue culture of rice

In vitro tissue culture is an experimental approach for basic and applied research. It is the most important step of plant regeneration and gene transfer among modern methods. Embryogenic calli, rather than direct tissues such as shoot spices, immature inflorescences, roots and leaves are used for genetic transformation and regeneration

of rice plants because the callus culture, compared with organogenesis, is much more suitable for the gene delivery and regeneration of transgenic rice plants (Aananthi et al. 2010). *In vitro* culture of plant cells and tissue has attracted considerable interest over recent years because it provides the means to study plant physiological and genetic processes in addition to offering the potential to assist in the breeding to improve cultivars by increasing genetic variability. Regenerated plants are expected to have the same genotype as the donor plant; however, in some cases somaclonal variants have been found among regenerated plants (Karp et al. 1987).

2.13.1 Totipotency of cells

The character of totipotency of plant cells allow that any differentiated cells that retains its nucleus has the ability to regenerate an entire new plant by somatic embryogenesis (SE) or organogenesis (Fortes and Pais 2000). Verdeil et al. (2007) mentioned that little is known of the mechanisms that induce the dedifferentiation of a somatic cell into a totipotent embryogenic cell that can either be regenerated or develop into an embryo and subsequently an entire plant. Moreover, the molecular mechanisms underlying plant cell totipotency are largely unknown (Chupeau 2013). Guzzo et al. (1994) identified the origin of cells capable to generate somatic embryos using hypocotyl explants of carrot seedlings; and reported that various tissues responded in different ways. The totipotency of different plant parts or organs is not identical (similar) in which regeneration capability is differed in various explants.

2.13.2 Somatic embryogenesis

The first documentation of somatic embryogenesis was with carrot cell suspension cultures (Steward et al. 1958, Reinert et al. 1959). Generally plant embryogenesis is the process that produces a plant embryo from a fertilized ovule by asymmetric cell division and the differentiation of undifferentiated cells into tissues and organs. Somatic embryogenesis (SE) means a process where a plant or embryo is derived from a single somatic cell or group of somatic cells. Somatic embryos are formed from plant cells that are not normally involved in the development of embryos, i.e. ordinary plant tissue. No endosperm or seed coat is formed around a somatic embryo. Two types of somatic embryogenesis are recognized: direct somatic embryogenesis (DSE) and indirect somatic embryogenesis (ISE). In case of DSE the induction of

somatic embryos are produced directly from pro-embryogenic cells from leaves, stem, microspores or protoplasts without the proliferation of calli. On the other hand, in ISE, somatic embryos are developed from friable embryogenic calli (Jiménez et al. 2001, Molina et al. 2002, Quiroz-Figueroa et al. 2006). It is a unique process in plants and it is of remarkable interest for biotechnological applications such as clonal propagation, artificial seeds and genetic engineering (Namasivayam 2007).

SE is the developmental process by which bipolar structures that resemble zygotic embryos are developed from haploid or diploid somatic cell through an orderly embryological stage without gametes fusion (Jiménez et al. 2001, Quiroz-Figueroa et al. 2006, Namasivayam 2007). Plant regeneration via SE occurs in five steps: initiation of embryogenic cultures, proliferation of embryogenic cultures, pre-maturation of somatic embryos, maturation of somatic embryos and plant development on nonspecific media. Initiation and proliferation occur on a medium rich in auxin, which induces differentiation of localized meristematic cells (George et al. 2007). In rice, SE is the most common regeneration pathway and has been obtained from roots, leaf bases of young seedlings, mature embryos, immature embryos, caryopses, microscopes, cell suspension, protoplast and young inflorescences (Chen et al. 1985, Abe and Futsuhara 1986, Raina et al. 1987, Hartke and Lörz 1989, Hoque and Mansfield 2004, Ge et al. 2006). Ghobeishavi et al. (2014) studied on the effect of various factors including sucrose, agar, PEG, AgNO₃, activated charcoal on somatic embryos induction and its subsequent regeneration was investigated using varieties Dom siah and Nemat. Somatic embryogenesis has been done in various species viz. *Chamaecyparis pisifera* Sieb. et Zucc. (Hosoi and Maruyama 2016) and Strawberry (Martins et al. 2016).

2.13.3 Effect of genotypes on somatic embryogenesis

Potentiality of somatic embryogenesis as well as callus induction and subsequent plant regeneration are affected by the genotypes, carbohydrate metabolism-source, plant growth regulators, culture medium and conditions etc. In particular genotype and explants are important factors for a successful embryogenic callus induction and regeneration of the rice plants (Rueb et al. 1994). The effect of rice genotypes on somatic embryogenesis has been investigated by several authors (Hoque and Mansfield 2004, Ramesh et al. 2009, Fook et al. 2015, Hossain et al. 2015, Din et al.

2016, Sankepally and Singh 2016). Somatic embryogenesis and plant regeneration were influenced by the genotype as well as composition of the medium used. The callus induction rate and callus morphology were different depend on genotypes (Ghobeishavi et al. 2014).

2.13.4 Effect of media and plant growth regulators (PGRs) on SE

Somatic embryos are mainly produced *in vitro* and for laboratory purposes, using either solid or liquid nutrient media which contain plant growth regulators (PGRs). The main PGRs used are auxins but can contain cytokinin in a smaller amount (George et al. 2007). Media composition mainly the hormonal balance is an important factor influencing *in vitro* culture initiation and plant regeneration from embryos (Jiang et al. 1998). The auxin typically used is 2, 4-dichlorophenoxy acetic acid (2,4-D). Once transferred to a medium with low or no auxin, these cells can then develop into mature embryos. Germination of the somatic embryo can only occur when it is mature enough to have functional root and shoot (George et al. 2007). The auxin 2,4-D alone or in combination with cytokinins, is widely used to enhance callus induction and maintenance (Šerhantová et al. 2004, Ge et al. 2006, Zhao et al. 2011, Din et al. 2016, Sankepally and Singh 2016). Genetic factors are considered to be a major contributor to the *in vitro* response of cultured tissues. Differences in the production of embryogenic calli and the regenerated plantlets have been observed, depending on the genotype and source of the explants (Ganeshan et al. 2003). Mannan et al. (2013) investigated the effect of various growth regulators on callus induction and plant regeneration efficiency in two Bangladeshi traditional aromatic rice var. Kalijira and Chinigura. They also investigated that MS medium supplemented with 2 mg/l of 2,4-D was the most effective in callus induction for both cultivars.

2.13.5 Organogenesis

The development of adventitious organs or primordia from undifferentiated cell mass in tissue culture by the process of differentiation is called organogenesis (<http://agriinfo.in/?page=topic&superid=3&topicid=1885>). The main objective in plant cultures is to regenerate a plant or plant organ from the callus culture. The regeneration of plant or plant organs only taken place by the expression of cellular totipotancy of the callus tissues. Scattered areas of actively dividing cells, known as

meristematic centres, develop as a result of differentiation and their further activity results in the production of root and shoot primordia. These processes can be controlled by adjusting the cytokinins: auxin ratio in culture medium. The production of adventitious roots and shoots from cells of tissue is called organogenesis. Several tissues are organized together to form an organ, such as leaves, roots, flowers and the vascular system. Some author present organogenesis and embryogenesis, occurring simultaneously, as the regeneration pathway (Boissot et al. 1990, Gairi and Rashid 2004).

In plant tissue culture, organogenesis is an important way to regenerate plants from the culture. *In vitro* plant regeneration in rice via organogenesis has been reported from different explants, such as root (Hoque and Mansfield 2004), leaf (Ramesh et al. 2009), coleoptile (Ghobeishavi et al. 2014), mature embryo (Pons et al. 2000) and protoplast (Jelodar et al. 2002). Several factors including plant growth regulators, explants, culture conditions and plant genotypes affect rice somatic embryogenesis and subsequent plant regeneration (Deo et al. 2010).

Some authors used various explants for organogenesis such as epicotyl in sweet orange (Filho 2001), pummelo cv. Cikoneng (Dewi et al. 2013) and hypocotyl in blackgram (Saini and Jaiwal 2002). In rice several authors also have been used the organs singly, and regenerate plant i.e. coleoptile (Sahrawat and Chand 2001) and leaf (Afrasiab and Jafar 2011). Using various explants i.e. inflorescence of rice (Chen et al. 1985) and root (Abe and Futsuhara 1985) reports on *in vitro* plant regeneration have been described.

2.13.6 Relative water content

Leaf water potential is closely related to leaf relative water content (RWC), but it is confounded by osmotic adjustment (Lafitte 2002). Drought resistant varieties showed consistently higher leaf water potential in their tissues than susceptible types under soil moisture deficit. Relatively higher RWC has been reported in drought tolerant cultivars of wheat (Martin et al. 1997). Drought is reported to cause decrease in leaf water potential and relative water content (Flores-Nimedeiz et al. 1990, Munns and Cramer 1996).

2.14 Major abiotic stresses

Abiotic stress is defined as the negative impact of non-living factors on the living organisms in a specific environment. Basic abiotic stresses are heat, cold, nutrient, wind, water, drought, salt etc. Out of them drought and high salinity show adverse effects on plants growth and the productivity of crops, resulting significant economic losses worldwide. Minimizing these losses is a major concern for all countries in the world including in Bangladesh.

2.14.1 Drought stress

Drought can be simply defined as reduction in yield due to shortage of water (Bernier et al. 2008). It is an abiotic stress that limits productivity in both rainfed and upland ecosystems (Bimpong et al. 2011). It's the major constraint to rice production in rainfed areas across Asia. It occurs when the available water in the soil is reduced and atmospheric conditions cause continuous loss of water by transpiration or evaporation. Drought stress tolerance is seen in almost all plants but its extent varies from species to species and even within species. Jeong et al. (2010) showed that rice plants significantly enhanced drought tolerance at the reproductive stage, with a grain yield increase of 25-42% over the controls under field drought conditions. Of all the cereals, rice (*Oryza sativa*) is most susceptible to damage from water deficit. Drought is a world-wide problem that seriously influences grain production. Increasing human population and global climate change make the situation more serious (Hongbo et al. 2005). It is estimated that 50% of world rice production is affected by drought (Bouman et al. 2007).

Drought resistance is a complex trait whose expression depends on action and interaction of different morphological, physiological and biochemical characteristics (Mitra 2001). A plant's first line of defense against abiotic stress is in its roots (Brussaard et al. 2007). It has been hypothesized that coarse roots have a direct role in drought resistance because larger diameter roots are related to penetration ability (Clark et al. 2008). Rice plant has several mechanisms to adapt under drought condition. Responses of plants to cope up drought situation are drought escape, drought avoidance, drought tolerance and drought recovery (Singh et al. 2012).

2.14.2 Salt stress

A saline soil is defined as having a high concentration of soluble salts, high enough to affect plant growth. According to USDA salinity laboratory, saline soil is defined as the soil having an electrical conductivity (EC_e) of solution extracted from the water-saturated soil is 4 dS/m (~ 40 mM) NaCl or more. Plant salt stress is a condition where excessive salts in soil solution cause inhibition of plant growth or plant death.

2.14.2.1 Salt affected area

The term ‘salt-affected’ refers to soils that are saline or sodic. Much of the world’s land is not cultivated, but a significant proportion of cultivated land is salt-affected. According to FAO Land and Plant Nutrition Management Service, over 6% of the world’s land is affected by either salinity or sodicity. Salinity covers over 800 million hectares, which are over 3.1% saline and 3.4% sodic soil of the world land area (**Table 5**). Worldwide, more than 45 million hectares of irrigated land have been damaged by salt, and 1.5 million hectares are taken out of production each year as a result of high salinity levels in the soil (Munns and Tester 2008).

Table 5: Regional distribution of salt-affected soils, in million hectares (Mha)

Regions	Total area	Saline soils		Sodic soils	
	Mha	Mha	%	Mha	%
Africa	1,899	39	2.0	34	1.8
Asia, the Pacific and Australia	3,107	195	6.3	249	8.0
Europe	2,011	7	0.3	73	3.6
Latin America	2,039	61	3.0	51	2.5
Near East	1,802	92	5.1	14	0.8
North America	1,924	5	0.2	15	0.8
Total	12,781	397	3.1%	434	3.4%

Source: FAO Land and Plant Nutrition Management Service.

http://www.plantstress.com/articles/salinity_i/salinity_i.htm

2.14.2.2 Physiology of plants to salinity

High salinity affects plants in two main ways, a) salts in the soil disturb the capacity of roots to extract water and b) salts within the plant itself can be toxic, resulting in an inhibition of many physiological and biochemical processes such as nutrient uptake and assimilation (Munns et al. 1995). Together, these effects reduce plant growth, development and survival ability. Another report expressed that during the onset and development of salt stress within a plant, all the major processes such as photosynthesis, protein synthesis and energy and lipid metabolism are affected (Parida and Das 2005). However, Munns et al. (1995) proposed a two-phase model describing the osmotic and ionic effects of salt stress to plants growth (**Fig. 3**).

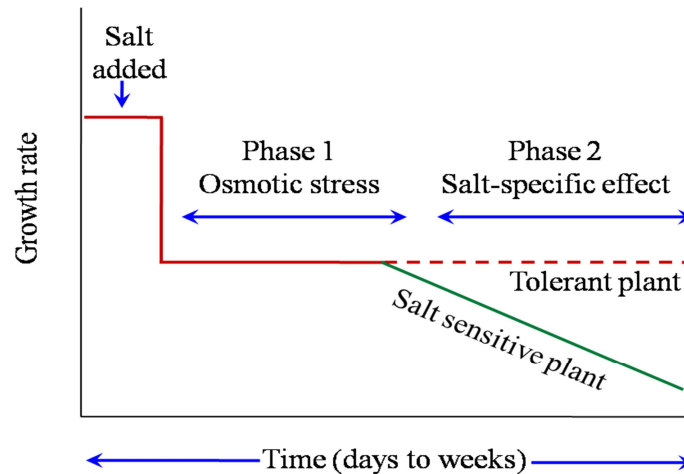


Fig. 3: Diagrammatic representation of consequence to salt stress of plants proposed by Munns et al. (1995).

Time scale is days, weeks or months, depending on the species and the salinity level. Shoot growth is more sensitive than root to salt-induced osmotic stress (Munns and Tester 2008). In photosynthetic tissues, in fact, Na^+ accumulation affects photosynthetic components such as enzymes, chlorophylls, and carotenoids (Davenport et al. 2005). High concentrations of Na^+ in the soil solution may depress nutrient-ion activities and produce extreme ratios of $\text{Na}^+/\text{Ca}^{2+}$ or Na^+/K^+ (Grattana and Grieveb 1999).

2.14.2.3 Complexity of tolerance to salt stress

The mechanisms of genetic control to salt tolerance in plants have not yet fully understood because of its complexity. The tolerance or susceptibility to salinity in plants is a coordinated action of multiple stress responsive genes (Silva and Gerós 2009). Overexpression of regulatory genes in signaling pathways also increases plant salt tolerance (Chen et al. 2010). In fact several genes are involved to control salinity tolerance in different species. Thus, genetic variation can only be demonstrated indirectly, by measuring the responses of different genotypes (Allen et al. 1994). The overexpression of the vacuolar Na^+/H^+ antiport has shown to improve salinity tolerance in several plants (Silva and Gerós 2009). Salinity tolerances may increase or decrease depending on the plant species and/or environmental factors. For some species, salt sensitivity may be greatest at germination, whereas for other species, sensitivity may increase during reproduction (Howat 2000). Plants have evolved several mechanisms to acclimatize in salinity. It is possible to distinguish three types of plant response or tolerance: i) tolerance to osmotic stress, ii) exclusion of Na^+ from leaf blades and iii) tolerance of tissues (Munns and Tester 2008). James et al. (2011) reported that exclusion of Na^+ from leaves was associated with salt tolerance in cereal crops including rice, durum wheat, bread wheat and barley. It has been demonstrated that the plant's response to the osmotic stress is independent of nutrient levels in the growth medium.

2.15 Improvement of plants by genetic transformation

A commonly accepted definition of plant transformation is, "the introduction of exogenous genes into plant cells, tissues or organs employing direct or indirect means developed by molecular and cellular biology" (Jenes et al. 1993). Integrative events are confirmed by molecular and genetical analysis, are correctly designated as genetic transformation (Potrykus 1990). However, the capacity to introduce and express diverse foreign genes in plants, first described for tobacco in 1984.

Now a day, many countries have field tests of transgenic plants. As of December-1995, genetically engineered plants as numbers of 3647 were field tested worldwide: United States (1952), Canada (486), France (253), United Kingdom (133), Holland (113), Argentina (78), Italy (69), Germany (49), Australia (46), Chile (39), Mexico

(38), Spain (30), Japan (25), South Africa (22), Sweden (18), Cuba (18) and Russia (11) among many others. The main transgenic tested field crops are maize, oilseed rape, potato, tomato, soybean, cotton and tobacco, and the most frequent genetically introduced traits are herbicide tolerance (35%), product quality (20%), insect resistance (18%), virus resistance (11%), fungi resistance (3%) and others (13%). The last corresponds to nematode and bacteria resistance and marker or reporter gene transference (Fontes and Carvalho 1997). Thereafter, we have many examples of salts and/or drought tolerance transgenics which developed using biotechnological techniques. Transgenic rice exhibited higher salt tolerance overexpressing the Na^+/H^+ antiporter gene clone OsNHX1 (Fukuda et al. 2004, Wu et al. 2004). Transgenic tomato plants were able to grow, flower and produce fruit in presence of 200 mM NaCl by overexpressing AtNHX1 gene (Zhang et al. 2001). In *Petunia hybrida* overexpression of AtNHX1 enhanced salt and drought tolerance which accumulated more Na^+ , K^+ and proline in their leaf tissue, maintaining high water contents and high ratio of K^+/Na^+ (Xu et al. 2009). By introgressing Nax genes from *Triticum monococcum* into hexaploid bread wheat (*Triticum aestivum*), the leaf blade Na^+ concentration was reduced by 60% and the proportion of Na^+ stored in leaf sheaths was increased. It has been proved that Nax genes have the potential to improve the salt tolerance to bread wheat (James et al. 2011).

To produce the transgenic plants, numerous methods have been described for introduction of exogenous gene into plant genomes (Potrykus 1990). The methods are classified into two groups, such as a) indirect gene transfer- where exogenous DNA is introduced using biological vector and b) direct gene transfer- where DNA introduction is based on physical and chemical processes. Some of the proposed methods are not more efficient to be used. However, the following methods are commonly used for genetic transformation.

2.15.1. Methods of genetic transformation

2.15.1.1 PEG-mediated

In 1986, Uchimiya et al. first obtained transgenic calli by polyethylene glycol (PEG) and induced DNA uptake of nptII gene into root-derived protoplast, followed by selection on kanamycin. The first transgenic rice plants were recovered by and Datta et al. (1990) published the first report on recovery of transgenic indica rice of cultivar Chinsurah BoroII using PEG method.

2.15.1.2 Electroporation-mediated

This method is widely used and Toriyama et al. (1988) produced first transgenic in rice cultivar Yamahoushi via anther culture-derived protoplast with *aph(3')II* (aminoglycoside phosphotransferase) gene confirming resistance to antibiotics kanamycin. Moreover, Gad et al. (1990) and Negrutiu et al. (1990) are concern with electroporation procedures.

2.15.1.3 Microprojectile bombardment

It is also known as biolistic or particle method and this method has been described in detail by Sanford (1988). Christou et al. (1992) bombarded the scutellar region of embryo and bombarded tissues were planted on regeneration method. Several reports has been published for different plants related to microparticle bombardment system viz. Becker et al. (1994), Vasil et al. (1992), Sanford et al. (1993) and Christou (1995).

2.15.1.4 *Agrobacterium*-mediated genetic transformation

2.15.1.4.1 *Agrobacterium* species and host range

The genus *Agrobacterium* has a number of species. Among them *A. radiobacter* is an “virulent” species, *A. tumefaciens* causes crown gall disease, *A. rhizogenes* causes hairy root disease, and *A. rubi* causes cane gall disease. More recently, a new species has been proposed, *A. vitis*, which causes galls on grape and a few other plant species (Otten et al. 1984). However, Two species of *Agrobacterium* viz. *A. tumefaciens* and *A. rhizogenes* are be used as transformation delivery system. Both species had the unique ability to transfer DNA into plants genome (Zambryski et al. 1989). The mechanism by which they transfer DNA is same, while the resulting phenotypes are attributed to the presence of a Ti plasmid in *A. tumefaciens* and Ri plasmid in *A. rhizogenes*. The species *A. tumefaciens* is capable to infect almost any plant tissue, and therefore the bacterium has become the primary choice for genetic transformation in many plants. Many plant species are routinely transformed using this bacterium, and the list of species that is susceptible to *Agrobacterium*-mediated transformation seems to grow daily while twenty-five years ago, the concept of using *Agrobacterium tumefaciens* as a vector to create transgenic plants was viewed as a prospect.

2.15.1.4.2 Molecular basis of *Agrobacterium*-mediated transformation

The molecular basis of genetic transformation of plant cells by *Agrobacterium* is transfer from the bacterium and integration into the plant nuclear genome of a region of a large tumor-inducing (Ti) or rhizogenic (Ri) plasmid resident in *Agrobacterium*. Ti plasmids are on the order of 200 to 800 kbp in size (Fortin et al. 1993). The transferred DNA (T-DNA) is referred to as the T-region when located on the Ti or Ri plasmid. The processing of the T-DNA from the Ti plasmid and its subsequent export from the bacterium to the plant cell result in large part from the activity of virulence (*vir*) genes carried by the Ti plasmid (Stachel and Zambryski 1986).

2.15.1.4.3 T-DNA transfer to plant cells from *Agrobacterium*

Many proteins encoded by Vir genes play essential roles in the *Agrobacterium*-mediated transformation process. Some of these roles have been discussed in several excellent review articles (Gelvin 2000, Tzfira et al. 2000) and the roles of Vir proteins that may serve as points of manipulation for the improvement of the transformation process. VirA and VirG proteins function as members of a two component sensory-signal transduction genetic regulatory system.

The *Agrobacterium*-method is well established for plant transformation, especially dicotyledonous plants. It is still preferred carrying some advantages, such as i) easy to handle, ii) higher efficiency, iii) more predictable pattern of foreign DNA integration and iv) low copy number of integration. Raineri et al. (1990) described first producing transformed japonica cultivar by *Agrobacterium*. Using *Agrobacterium*-mediated transformation system several reports have been published in rice (Hiei et al. 1994, Sahoo et al. 2011, Tuteja et al. 2013, Banu et al. 2014, Sahoo and Tuteja 2014, Amin et al. 2016). This method also has been used for the genetic transformation of various plant species viz. *Gossypium hirsutum* L. (Bajwa et al. 2014, Awan et al. 2015, Chen et al. 2016), wheat (Risacher et al. 2009, Sparks et al. 2014), Barley (Tingay et al. 1997, Holme et al. 2008), maize (Ishida et al. 1996, Miller et al. 2002). However, Den Nijs et al. (2004) reported that gene transfer can occur within a species or between different species or other genera. Some selected reports on genetic transformation using the specific genes are given in tabular form (**Table 6**).

Table 6: Selective reports of studies on genetic transformation in plants

Variety	Explants	Resistance to stresses/Targets	References
Using electroporation-method			
Tainung67 (japonica rice)	Protoplasts	Plant virus	Ou-Lee et al. (1986)
Radon (japonica), IR54 (indica rice)	Immature embryos, Suspension cells	Herbicide	Rao (1995)
PEG-mediated DNA uptake method			
IR72 (indica rice)	Protoplast	Herbicide	Datta et al. (1992)
Radon, Nortai (japonica rice)	Protoplast	Herbicide	Rathore et al. (1993)
IR 72 (indica rice)	Immature embryos	Bacterial blight	Tu et al. (1998)
Using particle gun-mediated transformation method			
Taipei 309 (japonica rice)	Suspensions cells	Dehydrogenase protein producer	Wang et al. (1988)
Taipei 309 (japonica rice)	Suspensions cells	Herbicide	Cao et al. (1992)
Agrobacterium-mediated transformation method			
Rice (<i>Oryza sativa</i> L.)			
Nipponbare (japonica)	Scutellar callus	Salt and cold	Sakamoto et al. (1998)
PB-1 (indica)	Embryo derived calli	salt, drought, cold	Garg et al. (2002)
Kasalath (indica)	Mature embryo	Agronomically useful traits	Saika and Toki (2010)
Nipponbare (japonica)	Derived primary calli	Salinity	Sahoo et al. (2011)
IR64, CSR10, PB1 and Swarna (indica)	Mature embryo derived calli		
IR64, Jaya, Basmati370 (indica) and AC41039 (japonica)	Mature seeds	Efficient protocol	Puhan et al. (2012)
IR64 (indica)	Mature embryo derived calli	Salinity	Tuteja et al. (2013)
Binnatoa (indica)	Embryo derived calli	Salt tolerance	Amin et al. (2016)

Contd. (Table 6)

Wheat

<i>Triticum aestivum</i> L cv. Veery5	Immature embryo-derived calli	Efficient protocol	Khanna and Daggard (2003)
<i>T. aestivum</i> L cv. Shi4185	-	Heat and drought	Wang GP et al. (2010)

Tobacco

<i>N. tabacum</i> cv. NC89	-	Heat	Yan et al. (2008)
<i>N. tabacum</i> L.	-	Heat	Xu et al. (2014)

Cotton

<i>Gossypium hirsutum</i> L.	Shoot tips	Fiber expansion	Bajwa et al. (2014)
<i>G. hirsutum</i> L. var. MNH-786	Mature embryos	Insect and Herbicide	Awan et al. (2015)
<i>G. hirsutum</i> L.	Hypocotyl segment	Salinity	Chen et al. (2016)

Tomato

<i>Solanum lycopersicum</i> cv. Moneymaker	-	Heat	Li et al. (2011)
<i>S. lycopersicum</i> cv. Ailsa Craig	-	Heat and <i>Botrytis cinerea</i> infection	Chen et al. (2013)

Others

<i>Brassica chinensis</i>	-	Temperature, salinity	Wang QB et al. (2010)
<i>Arabidopsis thaliana</i> L. ecotype Columbia	-	Thermo tolerance	Chae et al. (2013)
<i>Arabidopsis thaliana</i>	-	Salinity	Tuteja et al. (2014)
<i>Arabidopsis thaliana</i>	-	Thermotolerant	Wang et al (2014)
<i>Rorippa indica</i>	Seeds	Salt and drought	Xu et al. (2016)

2.16 Biosafety issue for genetically modified organisms

Biosafety is defined as the procedures intended to protect humans or animals against disease or harmful biological agents. Biosafety is the prevention of large-scale loss of biological integrity, focusing both on ecology and human health (UNEP 1972). These prevention mechanisms include conduction of regular reviews of the biosafety in laboratory settings, as well as strict guidelines to follow. To keep the earth in safety and security many organizations are constituted in the world, such as American Biological Safety Association (ABSA), Asia-Pacific Biosafety Association (A-PBA), European Biological Safety Association (EBSA), International biosafety Working Group (IBWG), International Veterinary Biosafety Workgroup (IVBWG) etc. Besides, WHO, UN, World Organization for Animal Health (OIE), Food and Agriculture Organization (FAO) and Organization for Economic Cooperation and Development (OECD) work on biosafety and biosecurity.

A report on global health agenda has expressed that most of the countries around the world do not have the capability to address potential pandemics or bioterrorist events, a situation related to international health security threat (Jenkins 2015). Many countries including Bangladesh have their biosafety guidelines to save the nation from the negative impact of modern Biotechnology as well as the genetically modified organisms and foods. The regulation carries the guideline covering aspects of risk assessment and safety requirements needed for undertaking the laboratory work, field trial and commercial use, involving microorganisms, plants and animals. The guidelines for laboratory work specify the experiments to be categorized as belonging to different biosafety levels like work bearing minimal risk.

For millennia, farmers have relied on selective breeding and cross-fertilization to modify plants and animals and encourage desirable traits that improve food production and satisfy other human needs. While modern biotechnology may have great potential, it must be developed and used with adequate safety measures, particularly for the environment. The global use of biotechnology will be safe if each and every country actively promotes biosafety at the national level. National policymakers and legislators have a vital role to play in establishing and strengthening laws and standards for reducing the potential risks of GMOs (www.biodiv.org/biosafety).

Chapter III

Materials and Methods (General)



3. Materials and Methods (General)

The experiments for the present studies were conducted during 2011-2015 in Plant Genetic Engineering Lab., Institute of Biological Sciences (IBSc), University of Rajshahi (RU), Rajshahi, Bangladesh. Some partial works have been conducted in 2013 for Bioinformatics, cloning of targeted gene and its confirmation by sequencing, restriction enzyme (RE) analysis with Dr. Narendra Tuteja in the Laboratory of PMB, ICGEB, New Delhi, India.






3.1 Plant Materials

In this study a total number of seventeen Bangladeshi indica rice (*Oryza sativa* L.) varieties were used as the plant materials (**Fig. 4**). The mature seeds of all the varieties were collected from Bangladesh Rice Research Institute (BRRI), Regional Station, Shyampur, Rajshahi, Bangladesh.

3.1.1 Salient features of studied rice genotypes

The studied varieties were selected considering their popularity in agricultural sector in Bangladesh and/ or some features on abiotic stresses specially related to drought and salt. The important features of seventeen studied rice varieties described by BRRI (2011) are mentioned in **Fig. 4**.

Fig. 4: Important features of selected varieties

Sl	Variety (popular/local name)	Features	Morphological picture
01.	BR3 (Biplob)	<ul style="list-style-type: none"> Life length 170d. Yield 6.5 ton/hector. Placed 1st international yield competition, 1974. 	
02.	BR4 (Brrishail)	<ul style="list-style-type: none"> Light sensitive. Lift length 145 d. Yield 5.0 ton/hector. Placed 1st international yield competition, 1976. 	
03.	BR8 (Asha)	<ul style="list-style-type: none"> Lift length 145 d. Yield 5.0 ton/hector. Stem hardy. Popular to cultivation. 	
04.	BR10 (Progoti)	<ul style="list-style-type: none"> Lift length 150 d. Yield 5.5 ton/hector. Popular south west region of Bangladesh. Internationally to listed as high yielding variety. 	
05.	BR11 (Mukta)	<ul style="list-style-type: none"> Lift length 145 d. Yield 5.5 ton/hector. Popular around whole Bangladesh. 	

Contd. (Fig. 4)

06. BR23 (Dishari)
- Lift length 150 d.
 - Stem hardy.
 - Slight salt resistant.
 - Yield 5.5 ton/hector.



07. BR24 (Rahamot)
- Lift length 105 d.
 - Stem very hardy.
 - Yield 3.5 ton/hector.



08. BRRI dhan27
- Lift length 115 d.
 - Stem high and hardy.
 - Yield 4.0 ton/hector.



09. BRRI dhan28
- Lift length 140 d.
 - Yield 6.0 ton/hector.
 - Very popular as eating rice.




10. BRRI dhan29
- Lift length 160 d.
 - Stem hardy.
 - Yield 7.5 ton/hector (Highest among BRRI dhan).
 - Qualitatively best among modern BRRI dhan.



11. BRRI dhan32
- Lift length 130 d.
 - Yield 5.0 ton/hector.
 - No light sensitivity.



Contd. (Fig. 4)

12. BRRI dhan34	<ul style="list-style-type: none"> • Lift length 135 d. • Light sensitive. • Yield 3.5 ton/hector. • Aromatic. 	
13. BRRI dhan37	<ul style="list-style-type: none"> • Lift length 140 d. • Yield 3.5 ton/hector. • Aromatic • The end of seed (=keel) is little curve with awn. 	
14. BRRI dhan42	<ul style="list-style-type: none"> • Lift length 100 d. • Moderately drought resistant. • Yield 3.5 ton/hector. 	
15. BRRI dhan43	<ul style="list-style-type: none"> • Lift length 100 d. • Stem hardy. • Moderately drought resistant. • Yield 3.5 ton/hector. 	
16. BRRI dhan47	<ul style="list-style-type: none"> • Lift length 156 d. • Yield 6.0 ton/hector. • 12-14 DS/m salt resistant at plantlet, 6 DS/m whole life. 	
17. BRRI dhan57	<ul style="list-style-type: none"> • Lift length 103 d. • Moderately drought resistant • Yield 4.0-4.5 ton/hector. 	

Source: BRRI (www. brri.gor.bd)

3.2 Methods

3.2.1 Sterilization of seeds

Mature seeds were dehusked and surface sterilized with 70% (v/v) ethanol for 1 minute. Then seeds were sterilized with 0.1% (v/v) mercuric chloride (HgCl_2) for 5 minutes. After that sterilized seeds were washed by autoclaved distilled water for 4-5 times and inoculated them on different culture media for germination, callus induction and other experimental works (**Table 7**).

3.2.2 Preparation and sterilization of culture media

The used different tissue culture media for growth and development of explants were consisted of various inorganic and organic nutrients. Several macro-elements such as nitrogen (N), oxygen (O), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulphur (S) and the micro-elements i.e. iron (Fe), manganese (Mn), zinc (Zn), boron (B), iodine (I) etc. consisted the inorganic nutrients of the media. The organic nutrients were sucrose (sugar) which was the source of carbon (C) and various growth substances of vitamins i.e. amino acids, thiamine, pyridoxine etc. The compositions of the used media containing inorganic and organic nutrients were followed by the basal media of MS and N6 and SK1 are shown in **Table 7**. The media were solidified with 0.8% agar (Sigma). The pH of media was adjusted to 5.8. Then the media were autoclaved at 15 pound square inch (psi) for 20 minutes at 121°C . Inoculation was carried out under a sterilized environment after applying ultra-violet rays in a laminar air flow cabinet following Haque et al. (2013).

Table 7: Composition of used media for plant tissue culture

Nutrients group/ Others	Components	Media (mg/l)			
		MS	N6	LS	SK1
Macro	KNO ₃	1900.00	2830.00	1900.00	3150.00
	NH ₄ NO ₃	1650.00	-	1650.00	-
	KH ₂ PO ₄	170.00	400.00	170.00	540.00
	CaCl ₂ .2H ₂ O	440.00	166.00	332.02	150.00
	MgSO ₄ .7H ₂ O	370.00	185.00	180.54	370.00
	(NH ₄) ₂ SO ₄	-	463.00	-	-
Micro	MnSO ₄ .4H ₂ O	22.30	4.40	16.90	16.90
	H ₃ BO ₃	6.20	1.60	6.20	6.20
	ZnSO ₄ .4H ₂ O	8.60	1.50	8.60	10.00
	KI	0.83	0.80	0.83	1.00
	Na ₂ MoO ₄	0.25	-	0.25	0.250
	CuSO ₄ .5H ₂ O	0.025	-	0.025	0.025
	CoCl ₂ .6H ₂ O	0.025	-	0.025	-
	AlCl ₃	-	0.03	-	-
	NiCl ₂	-	0.03	-	-
Iron	FeSO ₄ .7H ₂ O	27.80	27.80	-	37.25
	Na ₂ EDTA	37.30	37.30	-	27.85
Organics/ Vitamins	Glycine	2.00	2.00	-	2.00
	Nicotonic acid	0.50	1.00	-	2.00
	Pyridoxine	0.50	0.50	-	2.00
	Thiamine HCl	0.10	0.50	0.40	2.00
	Myo-inositol	100.00	100.00	100.00	100.00
Carbon	Sucrose	30000.00	30000.00	30000.00	30000.00

MS (Murashige and Skoog 1962), N6 (Chu et al. 1975), LS (Linsmaier and Skoog 1965), SK1 (Raina 1989).

3.2.3 Culture conditions for callus induction and plant regeneration

For callus induction sterilized explants were inoculated on CIM and the petri dishes were sealed with parafilm and kept them at dark condition. The temperature of the culture room or growth chamber was maintained at $25 \pm 1^\circ\text{C}$. For plant regeneration the calli with vigorous growth were placed on regeneration medium (RM) and the petri dishes were kept under white fluorescent lamp of 32 Watt with light intensity of 2000 lux (Hoque et al., 2013) at 16/8 hours light/dark cycle. The temperature of the culture room or growth chamber was maintained at $25 \pm 1^\circ\text{C}$.

3.2.4 Data recording and statistical analysis

Data were recorded regarding the specific methods of different experiments and the results were converted into percentage values. The average or mean values were computed from 5 replications with standard error (\pm SE) using Microsoft Office Excel 2007 statistical tools. The experiments were laid out as completely randomized design (CRD). Analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) (Duncan 1955) were done using the SPSS17.0 software. The software was operated at 5% significance level for post hoc analysis as well as to test the homogeneity of the means values and DMRT. To calculate the values of mean, variance, standard deviation, standard error and other parameters the following formulae were used.

$$\text{i) Mean } (\bar{X}) = \frac{\sum X_i}{n} \quad i = 1, 2, 3, 4, \dots, n$$

$$\text{ii) Variance } (S^2) = \frac{\sum X_i^2 - \frac{(\sum X_i)^2}{n}}{n-1}$$

S = Standard deviation
n = Number of observations

$$\text{iii) Standard deviation } (S) = \sqrt{S^2}$$

S^2 = Variance
n = Number of observations

$$\text{iv) Standard error (SE)} = \frac{S}{\sqrt{n}}$$

S = Standard deviation
n = Number of observations

$$\text{v) Student (t)} = \frac{|\bar{X}_1 - \bar{X}_2|}{S \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \quad S = \frac{S_1^2(n_1-1) + S_2^2(n_2-1)}{n_1 + n_2 - 2}$$

3.2.5 Genetic transformation mediated by *Agrobacterium*

3.2.5.1 Preparation of competent cells and transformation

DH5 α competent cells were made by the protocol of Hanahan (1985) [In: DNA cloning, Volume 1, Glover, D. ed. IRL Press Ltd., London, U.K.]. A single colony of DH5 α was picked up and inoculated into 5 ml LB medium (Luria-Broth) and grown overnight at 37°C and next day 1 ml of this was inoculated freshly into 100 ml of LB and grown at 37°C for 3 hours, till the OD (optical density) of 0.5-0.6 was obtained. The cells were harvested by centrifugation at 3000 \times g for 10 min. The pellet was re-suspended in 10 ml of ice-cold 0.1M CaCl₂ and stored on ice. Then swirl the tube on ice for at least 1-2 hrs and pellet down the centrifuge at 2500 rpm for 10 minutes. Remove the CaCl₂ and re-suspended in 2 ml of 0.1M CaCl₂, swirl properly if needed. Add 2 ml of 50% glycerol and mix well. Then the cell suspension (0.1 ml) was aliquoted into Eppendorf tubes, frozen immediately in liquid nitrogen and stored at -80°C.

3.2.5.2 Ligation of targeted gene

DNA fragments were ligated overnight to the appropriate vectors by using T₄ DNA ligase at 4°C or 16°C (sub-cloning). The ligation mix was added to the competent cells and mixed by tapping and then incubated for 30 minutes at 4°C. All steps were carried in a laminar hood under sterile conditions. This was subjected to heat shock by incubating at 42°C for 90 sec and then immediately transferred to 4°C for 10-20 min followed by addition of 900 μ l of LB and then grown at 37°C with slow shaking. Different aliquots of these transformed competent cells were plated on to LB plate containing 100 μ g/ml of Ampicillin or 100 μ g/ml Kanamycin. For Blue and White selection, 0.1 M IPTG (5 μ l) and 20 % X-gal (5-bromo-4-chloro-3-Indolyl-4-isopropyl- β D-galactoside, 5 μ g) per plate was spread prior to plating the cells. The plates were dried and incubated overnight at 37°C.

3.2.5.3 Purification of DNA fragment from agarose gel

After restriction enzyme digestion of plasmid, the digested product was electrophoresed on an agarose gel. By using standard molecular wt. marker (1 Kb ladder) the desired fragment was identified, cut and purified by one of the following methods:

3.2.5.3.1 By phenol-freeze-fracture method

The cut pieces of agarose gel containing the band were macerated by passing through a needle attached to a syringe and to this was added an equal volume of Tris-HCl, pH 8.0 saturated phenol, vortexed and frozen at -80°C for 1 hour. After thawing, the samples were centrifuged at $10,000 \times g$ for 5 min at room temperature. The upper aqueous phase was collected and precipitated by adding 0.1 volume of 3 M Na-Acetate (pH 4.8) and 2.5 volume of ethanol and kept for 1 hour at -80°C . The DNA was collected by centrifugation at $14,000 \times g$ for 15 min. at 4°C washed with 70 % alcohol, air dried and dissolved in TE or doubled distilled water.

3.2.5.3.2 By QIAquick gel extraction protocol (by Qiagen kit)

To the cut pieces of agarose gel containing DNA fragment were added 3 volumes of buffer as supplied with QIAquick gel extraction kit and dissolved by heating to 50°C for 10 min. The mixture was loaded on to QIAquick spin column and spun briefly. The flow through was discarded and the column was washed twice with buffer PE. The purified DNA fragment was eluted with (50 μl) 10 mM Tris-HCl, pH 8.0.

3.2.5.4 Targeted fragment/gene clone to pGEM-T vector

Targeted gene was inserted into plasmids, using PCR. Then ligate the PCR product from the gene with the pGEM-T vector, which was 3'T overhangs that bond with the 5'A overhangs that get added to the ends by thermo stable polymerases and transform this ligation product into bacteria, which plated on selective media.

3.2.5.5 Isolation of plasmid DNA

This method was adopted from Sambrook et al. (1989). The overnight grown culture of the desired bacterial cells was harvested by centrifugation at $5000 \times g$ for 10 min. at 4°C. The pellet was then suspended in Solution I (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose) and then 2 ml of freshly prepared Solution II (0.2 N NaOH, 1% SDS) was added to this and mixed by inverting. This was incubated at room temperature for 5 minutes and then 1.5 ml of Solution III (3 M Potassium acetate, pH 4.8) was added to it, mixed by inversion and incubated on ice for 20 min. This mixture was centrifuged at $12,000 \times g$ for 3 min at 4°C. The supernatant was collected and treated with RNase A (10 mg/ ml) and incubated at 37°C for 3 min. After this the supernatant containing plasmid was extracted twice with phenol: chloroform: isoamyl alcohol (24:24:1). The aqueous phase was taken and used for further purification by PEG precipitation. The plasmid DNA isolated by kit also.

3.2.5.6 Standardization of PCR protocol

The PCR protocol for genotyping was standardized as follows:

1. The reagents and sterile water were divided into aliquots to minimize the number of sampling errors.
2. To avoid cross contamination, via the electrophoresis equipment, the gel combs and casting trays were washed properly.
3. If there was any doubt about a critical result, the experiment was repeated until the reach of unambiguous results.
4. Various control strategies have been followed to carry out PCR reaction successfully. Important controls used in the standardization of the PCR reactions (Newton, 1995).
 - a. PCR in the absence of exogenously added DNA was used as negative control to check the DNA-free status of reagents and solutions.

- b. PCR with positive control DNA was used to check the completeness of PCR mixture (to check the quantity of essential components including MgCl_2 in the cocktail).
- c. PCR with sufficient quantity of positive control DNA was used to amplify weak but consistent amplicons and to identify sensitivity and efficiency of PCR.
- d. Negative and positive controls were used to check for spurious background bands and reaction specificity and to identify PCR parameters (includes annealing temperature and number of cycles) that were suitable for amplifying expected products.

PCR amplification

The cocktail for PCR amplification was prepared as follows:

Reaction mixture (15 μl)

Stock	Aliquot	Final concentration
DNA 25 ng/ μl	2.00 μl	50 ng
dNTPs (2.5 mM)	0.60 μl	100.0 μM
Forward primer (10 μM)	1.50 μl	1 μM
Reverse primer (10 μM)	1.50 μl	1 μM
Assay buffer (10 X)	1.50 μl	1 X
<i>Taq</i> DNA polymerase (3 units/ μl)	0.10 μl	0.02 units
Sterile distilled H_2O	12.80 μl	-
Total	20.00 μl	-

dNTPs, assay buffer and *Taq* DNA polymerase used were obtained from Bangalore Genei Ltd., India and Promega, USA.

The reaction mixture was given a momentary spin for thorough mixing of the cocktail components. Then 200 µl PCR tubes were loaded in a thermal cycler.

- The reaction in thermal cycler (Eppendorf Master Cycler Gradient) was programmed as follows:

Profile 1:	94°C for 4 minutes	Initial denaturation
Profile 2:	94°C for 1 minute	Denaturation
Profile 3:	55°C for 1 minute	Annealing
Profile 4:	72°C for 1 minute	Extension
Profile 5:	72°C for 5 minutes	Final extension
Profile 6:	4°C for 5 minutes	Hold the samples

Profiles 2, 3 and 4 were programmed to run for 30 cycles. Annealing temperature differs for various primers.

3.2.5.7 Separation of PCR amplified product by agarose gel electrophoresis

Agarose gel electrophoresis was performed to separate PCR amplified products.

Materials

- Loading dye

Glycerol 50% (v/v)

Bromophenol blue 0.5% (w/v)

- 5X TBE (Tris Borate EDTA buffer)

Tris base	107.75 g
Boric acid	55.03 g
EDTA (Na ₂ .2H ₂ O)	8.19 g

Dissolved in 800 ml of sterile water and made up to 2000 ml.

Steps

- a. Open ends of the gel casting plate were sealed with cello tape and placed on a horizontal perfectly leveled platform.
- b. 0.8% agarose was added to 0.5X TBE buffer and boiled till the agarose dissolved completely and then cooled to 50-60°C. Ethidium bromide was used as a staining agent at the final concentration of 1 µg/ml.
- c. Agarose gel was poured in to the gel casting tray. The comb was placed properly and allowed to solidify.
- d. After solidification of the agarose, the comb and cello tape were removed.
- e. DNA samples were mixed well with 3-4 µl of loading dye and were loaded into the gel wells.
- f. The gel was run at 100 volts for 30-45 minutes and bands were visualized and documented in gel documentation system.

3.2.5.8 Isolation of plants genomic DNA

Genomic DNA was isolated from the rice varieties are as following Gawel and Jarret, (1991).

Requirements

- Leaf samples (leaf samples were collected at vegetative stage and were kept immediately at -70°C till DNA was isolated).
- Cetyl Trimethyl Ammonium Bromide (CTAB) Extraction buffer

CTAB	2 % (w/v)
Tris HCl (pH 8.0)	1M
Sodium chloride	4M
EDTA (pH 8.0)	0.2M

Tris, Sodium chloride and EDTA were autoclaved and 2% CTAB was added after autoclaving and preheated before using the buffer.

- Tris EDTA (TE) Buffer

Tris HCl (pH 8.0)	1M 1.0 ml
EDTA (pH 8.0)	0.2M 0.5 ml

(Volume was made up to 100 ml, autoclaved and stored at 4°C).

- Ice cold 2-propanol
- Chloroform: Isoamyl alcohol - 24:1 (v/v)
- Sodium acetate (3.0M, pH 5.2)
- Ethanol (70 and 100%)
- RNase A (10 mg/ml)

RNase A was dissolved in TE buffer and boiled for 15 minutes at 100°C to destroy DNase and stored at -20 °C.

Procedure

1. Two gram of leaf samples were cut into small pieces with the help of sterile scissors and transferred to pre-chilled mortar.
2. The leaf tissues were frozen using liquid nitrogen and grinded to fine powder.
3. The fine powder was allowed to thaw in 10 ml of pre-heated extraction buffer in polypropylene centrifuge tubes and incubated for 30 minutes at 65°C in water bath with occasional mixing.
4. The tubes were removed from the water bath and equal volume of chloroform: Isoamyl alcohol mixture (24:1) was added and mixed by inversion for 15 minutes.
5. It was centrifuged at 10,000 rpm for 10 minutes at room temperature.
6. The clear aqueous phase was transferred to a new sterile tube.
7. Equal volume of ice cold propanol was added and mixed gently by inversion and then kept in the freezer until DNA was precipitated out.

8. Using blunt end tips, the precipitated DNA was spooled out in to an eppendorf tube.
9. The spooled DNA was centrifuged at 10,000 rpm for 10 minutes and air dried after removing the supernatant.
10. 200 µl of TE buffer was added to dissolve the DNA and kept at room temperature for sometimes or overnight.
11. 10 µl of RNase was added and incubated at 37°C for 1 hour and 500 µl of Chloroform: Isoamyl alcohol mixture was added and centrifuged at 10,000 rpm for 15 minutes.
12. Aqueous phase was transferred to another eppendorf without disturbing the inner phase.
13. 2.5 volume of absolute alcohol (~1 ml) and 1/10 volume (~50 µl) of sodium acetate were added and kept for overnight incubation.
14. Then it was centrifuged at 10,000 rpm for 10 minutes and the supernatant was discarded. To this 500 µl of 100 per cent ethanol was added and centrifuged at 10,000 rpm for 1 minute.
15. The alcohol was discarded and DNA was completely air-dried.
16. Then the DNA pellet was dissolved in 50 µl TE buffer/sterile distilled water and stored at 4°C.

3.2.5.9 DNA quality check by agarose gel electrophoresis

The quality of DNA was verified using 0.8% agarose gel (Sambrook et al. 1989). The protocol briefly mentioned previously (Section 3.2.5.3).

3.2.5.10 Quantification of DNA

Materials

- NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA.)
- DNA sample
- Sterile water
- Tris EDTA (TE) buffer - blank sample (carrier liquid used with samples)

Tris HCl (pH 8.0) 1M

EDTA (pH 8.0) 0.2 M

This was dissolved and made up to 100 ml, autoclaved and stored at 4°C.

Protocol

1. Sampling arm's upper and lower pedestals were wiped using a sterile tissue paper.
2. Water sample (2 µl) was loaded onto the sampling arm to clean the sample column and to initialize spectrometer ready for use.
3. Sterile distilled water or 2 µl TE buffer (blank sample) was loaded for initial blank measurement and both the pedestals were wiped after the blank value was obtained.
4. 2 µl of DNA samples were loaded sequentially and the readings on concentration of DNA in ng/µl were stored as image file with sample details and spectral measurements.

3.2.5.11 Polymerase chain reaction (PCR)

Taq-polymerase, dNTPs (deoxynucleotide triphosphate) and convergent primers achieved amplification of the DNA fragment. After 30 cycle of amplification an aliquot of this reaction mixture was loaded onto a 0.8 % agarose gel and checked.

3.2.5.12 Cloning and selection of PCR products

10 µl of the PCR products were examined on a 1.2% EtBr agarose gel and visualized under UV illumination. Where PCR products more than 300 bp were visualized as generated from an adaptor library (even if multiple products were seen to have been amplified), an aliquot of the PCR product (1-5 µl, depending on the quantity of the PCR product in the reaction) was sub-cloned into pGEM-T vector using a TA cloning kit for cloning (Promega) in accordance with the manufacturer's instructions. Following transformation, colonies were picked into 1 ml of liquid LB-medium and incubated at 37°C for overnight. Screening by blue and white selection was used to transfer a white colony to 0.5 ml PCR tube containing in each 10 µl of PCR mixture as follows: 1 µl of 10xTaq DNA polymerase buffer and 0.1 µl of *Taq* DNA polymerase, 1 µl of dNTPs (2 mM each), 1 µl of T7 primer (10 pM/µl), and gene specific primer (10 pM/µl) in a final volume of 20 µl. The total PCR reaction was examined on a 1% EtBr agarose gel. Those colonies that produced a PCR product of unique size were selected for sequencing.

**Chapter
IV**

**Somatic
Embryogenesis**



4. Somatic Embryogenesis

4.1 Effect of PGRs on callus induction and regeneration

4.1.1 Introduction

The first documentation of somatic embryogenesis was reported with carrot cell suspension cultures by Steward et al. (1958). For quick micropropagation and *in vitro* screening, an efficient standard protocol is essential, particularly for callus induction, callus multiplication and plantlet regeneration. Wang et al. (1987) studied regeneration in rice through somatic embryogenesis (SE). Potentiality for callus induction and plantlets regeneration in culture condition depends on a number of factors, like genotype of the donor plants, physiological and biochemical status of the explants, concentration and composition of different ingredients of culture medium etc. Among these factors, genotypic difference is the most important one (Abe and Futsuhara 1986, Rueb et al. 1994, Ge et al. 2006). The effect of rice genotypes on SE has been investigated by several authors (Hoque and Mansfield 2004, Vega et al. 2009, Ramesh et al. 2009, Ghobeishavi et al. 2014). Genotype and nutrient composition affect the variability in tissue culture (Kunnuvatchaidach et al. 1995, Khanna and Raina 1998). Induction of callus and subsequent plant regeneration are affected by the genotypes, carbohydrate metabolism-source, plant growth regulators, culture medium and conditions etc. In particular genotype and explants are important factors for a successful embryogenic callus induction and regeneration of the rice plants (Rueb et al. 1994). Puan et al. (2012) studied on different methods to regenerate plant using a set of 12 rice accessions representing indica, japonica, aromatic and wild groups; and reported that the key factors for enhancing successful regeneration are genotypes, tissue source of explants, combination concentration of growth regulators and culture conditions. Genetic factors are considered to be a major contributor to the *in vitro* response of cultured tissues. Differences in the production of embryogenic calli and the regenerated plantlets have been observed, depending on the genotype and source of the explants (O'Toole 1982, Rashid et al. 1996, Seraj et al. 1997, Wang et al. 2003, Lutts et al. 2004, Mahajan and Tuteja 2005, Khalequzzaman et al. 2005, Islam et al. 2013). However, *in vitro* plants were regenerated through androgenesis in wheat (Kunz et al. 2000a, Kunz et al. 2000b, Redha et al. 2000, Islam et al. 2001, Islam 2010a, Islam 2010b, Redha and Islam 2010).

It has been observed that rice produces two types of calli viz. embryogenic and non-embryogenic. Non-embryogenic callus would not be able to regenerate plant; whereas, embryogenic callus develop granular form and produce plants *in vitro* after developmental stages. The yield of callus and somatic embryos depends on the composition of the medium (Fennel et al. 1996) and explants sources (Ozias-Akins and Vasil 1982). The yield also depends on genotype and age of embryos (He et al. 1988). It was reported that induction and proliferation of callus varies with genotype, auxin source and concentration in rice (Aananthi et al. 2010, Khatun et al. 2012). Zafar et al. (1992) obtained highest callus formation in basmati rice cv.370 on MS with 2 mg/l of 2,4-D. Somatic embryogenesis was achieved on either N6 or MS medium with 2.0 mg/l each of 2,4-D and Kinetin. However, this callus was non-embryogenic and addition of 50 mg/l Tryptophan induced embryogenic callus (Bano et al. 2005). Sah et al. (2014) reported a protocol to produce embryogenic callus and plant regeneration rapidly in *Oryza sativa* cv. Kitaake a japonica rice cultivar. They used MS medium supplemented with 2, 4-D (3.0 mg/l), BAP (0.25 mg/l) for callus induction and regeneration (82.66%) occurred in 18 days on MS supplemented with BAP (3.0 mg/l) and NAA (0.2 mg/l). Moreover, effect of carbon sources in barley (Haque et al. 2015, Haque and Islam 2015), in banana (Hossain et al. 2009); light in tobacco (Yanjie 2004, Siddique and Islam 2015); silver nitrate in barley (Haque et al. 2015); callus age and size in barley (Haque and Islam 2015), in wheat (Islam 2010a) were investigated in some somatic embryogenic studies.

For somatic embryogenesis MS (Murashige and Skoog 1962) is most widely used as basal medium both for indica and japonica varieties. Besides, N6 (Chu et al. 1975), LS (Linsmaier and Skoog 1965) and SK1 (Raina 1989) media gave an additional response for rice genotypes. In addition to the composition of culture media, the concentrations of plant growth regulators also influence the process of callus induction, callus multiplication and somaclonal variation. A number of reports have shown that the N6 and LS media gave an additional response for some japonica rice lines. Aananthi et al. (2010) tested two basal media MS and N6 for induction in five indica rice (*Oryza sativa* L.) cultivars viz. ASD 16, White Ponni, Pusa Basmati 1, Pusa Sugandh 4 and Pusa Sugandh 5. They found highest regeneration (56.03%) potential for Pusa Basmati 1 and the lowest for White Ponni (30.37%). It has been reported that indica subspecies are more specific than japonica to tissue culture conditions (Ge et al. 2006). To enhance anther culture frequency SK1 (Raina 1989) basal medium was used in rice (Raina and Zapapa 1997, Khanna and Raina 1998).

Tissue culture of monocots is difficult compared to dicots (Morel and Wetmore, 1951). It is often difficult to establish embryogenic cell cultures and to regenerate plants from the cultured cells specially those belonging to indica subspecies (Jain 1997). A number of reports have shown that most of the indica lines are less responsive to callus induction and regeneration as compared to japonica lines (Abe and Fursuhara 1984, Reddy et al. 1994, Mikami and Kinoshita 1988). Even not all the indica subspecies have the equal potentiality for *in vitro* responses (Seraj et al. 1997, Khanna and Raina 1998). While efficient plant regeneration is essential for the successful utilization of biotechnology in rice crop improvement (Hoque 2002). Mannan et al. (2013) investigated that the effect of various growth regulators on callus induction and plant regeneration efficiency in two Bangladeshi traditional aromatic rice var. Kalijira and Chinigura; and showed that MS medium supplemented with 2 mg/l of 2,4-D was the most effective in callus induction for both cultivars. Somatic embryogenesis has been reported for six elite Bangladeshi indica rice cultivars such as BR14, BRRI dhan28, BRRI dhan29, BRRI dhan38, BRRI dhan39 and BRRI dhan40 (Hoque et al. 2007). So far as it is known, till now efficient regeneration for Bangladeshi indica rice is not reported enough. Hence, present work has been carried out on following objectives.

- Screening of suitable rice cultivars for *in vitro* tissue culture as well as to evaluate the potentiality of high yielding Bangladeshi indica rice varieties for callus induction and plant regeneration.
- To find out the effect of media and plant growth regulators to the rice genotypes for efficient callus induction.
- Standardization of media and growth regulators for efficient plant regeneration.

4.1.2 Materials and Methods

4.1.2.1 Plant materials

As described previous Chapter III, page 36.

4.1.2.2 Methods

4.1.2.2.1 Sterilization of seeds

As described previous Chapter III, page 40.

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

Callus induction medium (CIM) were prepared by MS basal supplemented with fifteen different concentration and combinations of PGRs were used either singly or in combinations (T₁ - T₁₅) as shown in **Table 8**.

Table 8: Concentration and combinations of PGRs for callus induction

Treatment	Concentrations and combinations of PGRs	
	Auxin	Cytokinin
	2,4-D	Kinetin
T ₁	0.5	-
T ₂	1.0	-
T ₃	1.5	-
T ₄	2.0	-
T ₅	2.5	-
T ₆	3.0	-
T ₇	3.5	-
-	-	-
Treatment	Concentrations and combinations of PGRs	
	Auxin	Cytokinin
	2,4-D	Kinetin
T ₈	0.5	0.5
T ₉	1.0	0.5
T ₁₀	1.5	0.5
T ₁₁	2.0	0.5
T ₁₂	2.5	0.5
T ₁₃	3.0	0.5
T ₁₄	3.5	0.5
T ₁₅	4.0	0.5

300 mg/l casein hydrolysate + 400 mg/l L-proline + 30000 mg/l sucrose was added with all hormonal combinations as constant.

4.1.2.2.3 Inoculation of sterilized seeds

The sterilized seeds were inoculated on CIMs and then the petri dishes were incubated at $25 \pm 1^\circ\text{C}$ in dark condition to induce callus. The age of callus was counted from inoculation day and 3 w old calli were considered to determine the frequency of callus induction (CI). Ten (10) days old calli induced in different media for different varieties were transferred onto the same fresh medium for sub-culture as well as better proliferation at 1 w interval. The pH of all media adjusted at 5.8.

4.1.2.2.4 Test of basal media for callus induction

To observe the effect of media on callusing, the suitable hormonal concentration and combination ($T_5 = 2.5 \text{ mg/l } 2, 4\text{-D}$) was considered as constant growth regulator, and added separately with four basal media, such as MS, N6, LS and SK1 to prepare the CIMs. Sterilized mature seeds of all the studied varieties were inoculated on the CIMs and cultured for callus induction.

4.1.2.2.5 Culture media and plant growth regulators (PGRs) for regeneration

For regeneration, media were prepared by MS basal supplemented with fifteen different concentration and combinations of PGRs singly and/ or combined ($H_1 - H_{15}$) as shown in **Table 9**. The calli age of 4 w with vigorous growth, induced in $2.5 \text{ mg/l } 2, 4\text{-D}$ (T_5) of all the studied varieties were placed onto the regeneration media in the petri dishes or culture vessels and cultured for plant regeneration (PR). The vessels were kept under white fluorescent lamp of 36 Watt with light intensity of 5000 lux at 16/8 hours light/dark cycle. The temperature of the culture room or growth chamber was maintained at $25 \pm 1^\circ\text{C}$. The calli which produced the shoots were counted and considered to determine the frequency of plant regeneration.

Table 9: Concentration and combinations of PGRs for plant regeneration

Treatment	Concentrations and combinations of PGRs		
	Cytokinin		Auxins
	BAP	Kin	NAA
H ₁	0.5	-	-
H ₂	1.0	-	-
H ₃	1.5	-	-
H ₄	2.0	-	-
H ₅	2.5	-	-
H ₆	3.0	-	-
H ₇	3.5	-	-
-	-	-	-

Treatment	Concentrations and combinations of PGRs		
	Cytokinin		Auxins
	BAP	Kin	NAA
H ₈	0.5	0.1	0.5
H ₉	1.0	0.1	0.5
H ₁₀	1.5	0.1	0.5
H ₁₁	2.0	0.1	0.5
H ₁₂	2.5	0.1	0.5
H ₁₃	3.0	0.1	0.5
H ₁₄	3.5	0.1	0.5
H ₁₅	4.0	0.5	0.5

300 mg/l Casein hydrolysate + 400 mg/l L-proline + 30000 mg/l sucrose was added with all hormonal combinations as constant.

4.1.2.2.6 Test of basal media for plant regeneration

To observe the effect of media on plant regeneration, the suitable hormonal concentration and combination H₁₁ (2.0 mg/l BAP + 1.0 mg/l Kin + 1.0 mg/l NAA) was considered as constant growth regulator, and added separately with four basal media, such as MS, N6, LS and SK1 to prepare the regeneration media and cultured the calli for plant regeneration.

4.1.2.2.7 Root induction

To induce sufficient roots half strength MS basal medium supplemented with 0.5 mg/l IBA was used as root induction media (RIM). The induced shoots, length of 3 - 5 cm placed into culture vessels or test tubes containing RIM. The culture vessels were kept under white fluorescent lamp of 36 Watt with light intensity of 5000 lux at 16/8 hours light/dark cycle. The temperature of the culture room or growth chamber was maintained at $25 \pm 1^{\circ}\text{C}$. The shoots which produced roots considered for determining the frequency of root induction (RI).

4.1.2.2.8 Acclimatization, hardening and field culture

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

4.1.2.2.9 Data recording and statistical analysis

Data were recorded as number of calli to determine the frequencies of callus induction and plant regeneration (%) and calculate using the formulae followed by Zaidi et al. (2006). Mean as well as the average values were determined from 5 (five) replicates. Analysis of variance (ANOVA), DMRT and test of homogeneity were done using SPSS 17.0 software.

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

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

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

4.1.3 Results

4.1.3.1 Effect of PGRs on callus induction

Fifteen types of hormonal combinations ($T_1 - T_{15}$) were added to MS medium and tested their effectiveness on callus induction for the studied seventeen rice varieties.. The results indicated that all the responding genotypes showed well performance to callus induction (CI). Out of seventeen genotypes, BRRI dhan29 performed the highest number of CI (78.67%) in T_5 (2.5 mg/l 2,4-D) followed by BRRI dhan32 (77.33%) in T_5 , BRRI dhan29 (74.67%) in T_6 (3.0 mg/l 2,4-D), BRRI dhan29 (73.33%) in T_4 (2.0 mg/l 2,4-D), BRRI dhan32 (72.00%) in T_6 , BRRI dhan32 72.00% in T_4 , BRRI dhan29 (70.67%) in T_{12} (2.5 mg/l 2,4-D + 0.5 mg/l Kin.), BR10 (69.33%) in T_6 and BRRI dhan28 (69.33%) in T_5 (**Table 10**). On the other hand BR11 and BRRI dhan27 both varieties gave the lowest number of CI 33.33% in T_1 (0.05 mg/l 2, 4-D). On an average of the growth regulators (treatments) BRRI dhan29 showed the highest callusing performance (64.01%); followed by 62.49, 60.98, 58.40 and 57.69% for BRRI dhan32, BR10, BRRI dhan28 and BRRI dhan34 respectively (**Fig. 5**). Beside, average of seventeen rice varieties showed that the hormonal concentration T_5 influenced the varieties to produce callus at the highest rate (59.76%) and the lowest (39.45%) in T_1 (**Table 10**). Analysis of variance showed the significant differences within the studied genotypes and the media examined at $p \leq 0.001$ (**Table 11**).

4.1.3.2 Test of basal media effect on callus induction

To test the effect of media on callus induction, a suitable hormonal concentration T_5 (2.5 mg/l 2, 4-D) was considered as constant and added separately with four basal media namely MS, N6, LS and SK1; and all the studied varieties were cultured to induce calli. The results showed that all the varieties performed the maximum callusing in MS medium. BRRI dhan29 induced calli at the highest rate (77.33%); and followed by 74.67% 72.00% and 68.00% for BRRI dhan32, BR10 and BRRI dhan28 in MS respectively (**Table 12**). Average 59.76% callusing was recorded from MS the highest value out of four media; and the lowest was in SK1 (46.98%). Within the medium the genotypes showed significant difference at $p \leq 0.001$ (**Table 13**).

Table 10: Effect of PGRs on callus induction for the seventeen rice varieties

Variety	Callus induction (% \pm SE)			
	T ₁	T ₂	T ₃	T ₄
BR3	34.67 \pm 1.33	41.33 \pm 1.33	49.33 \pm 2.67	49.33 \pm 2.67
BR4	38.67 \pm 1.33	40.00 \pm 2.11	41.33 \pm 1.33	44.00 \pm 2.67
BR8	36.00 \pm 2.67	44.00 \pm 1.63	52.00 \pm 2.49	53.33 \pm 2.98
BR10	46.67 \pm 2.11	56.00 \pm 2.67	61.33 \pm 2.49	68.00 \pm 2.49
BR11	33.33 \pm 2.11	38.67 \pm 2.49	46.67 \pm 2.11	48.00 \pm 3.27
BR23	36.00 \pm 2.67	41.33 \pm 3.27	44.00 \pm 2.67	45.33 \pm 3.89
BR24	34.67 \pm 2.49	40.00 \pm 2.98	45.33 \pm 2.49	46.67 \pm 3.65
BRRi dhan27	33.33 \pm 2.98	38.67 \pm 2.49	41.33 \pm 1.33	44.00 \pm 2.67
BRRi dhan28	48.00 \pm 2.49	57.33 \pm 2.67	58.67 \pm 3.27	66.67 \pm 2.98
BRRi dhan29	48.00 \pm 2.49	56.00 \pm 2.67	65.33 \pm 2.49	73.33 \pm 3.65
BRRi dhan32	49.33 \pm 1.63	54.67 \pm 2.49	64.00 \pm 2.67	72.00 \pm 3.27
BRRi dhan34	42.67 \pm 2.67	56.00 \pm 2.67	60.00 \pm 2.98	65.33 \pm 3.27
BRRi dhan37	40.00 \pm 2.11	53.33 \pm 2.98	56.00 \pm 2.67	62.67 \pm 2.67
BRRi dhan42	34.67 \pm 1.33	36.00 \pm 2.67	37.33 \pm 2.67	41.33 \pm 2.49
BRRi dhan43	38.67 \pm 2.49	52.00 \pm 3.27	53.33 \pm 3.65	60.00 \pm 3.65
BRRi dhan47	38.67 \pm 2.49	45.33 \pm 1.33	52.00 \pm 2.49	58.67 \pm 2.49
BRRi dhan57	37.33 \pm 1.63	46.67 \pm 2.11	54.67 \pm 1.33	56.00 \pm 1.63
Mean \pm SE	39.45 \pm 1.33	46.90 \pm 1.83	51.92 \pm 2.04	56.16 \pm 2.56
F-value	5.98	8.89	10.97	12.28
Significance	0.000	0.000	0.000	0.000
LSD _{0.05}	3.429	3.866	3.882	4.616

Contd. (Table 10)

Variety	Callus induction (% \pm SE)			
	T ₅	T ₆	T ₇	T ₈
BR3	52.00 \pm 1.33	49.33 \pm 1.63	45.33 \pm 3.27	36.00 \pm 2.67
BR4	46.67 \pm 2.11	42.67 \pm 2.67	41.33 \pm 1.33	34.67 \pm 3.27
BR8	60.00 \pm 2.98	52.00 \pm 3.27	48.00 \pm 2.49	40.00 \pm 2.11
BR10	73.33 \pm 2.11	69.33 \pm 2.67	61.33 \pm 3.27	50.67 \pm 2.67
BR11	50.67 \pm 1.63	46.67 \pm 2.98	42.67 \pm 1.63	33.33 \pm 2.11
BR23	50.67 \pm 1.63	48.00 \pm 2.49	45.33 \pm 1.33	37.33 \pm 2.67
BR24	52.00 \pm 1.33	48.00 \pm 2.49	44.00 \pm 1.63	34.67 \pm 2.49
BRR I dhan27	49.33 \pm 1.63	46.67 \pm 2.11	45.33 \pm 1.33	33.33 \pm 2.11
BRR I dhan28	69.33 \pm 2.67	66.67 \pm 3.65	57.33 \pm 4.00	42.67 \pm 2.67
BRR I dhan29	78.67 \pm 3.89	74.67 \pm 2.49	66.67 \pm 3.65	49.33 \pm 1.63
BRR I dhan32	77.33 \pm 2.67	72.00 \pm 2.49	64.00 \pm 3.40	53.33 \pm 2.11
BRR I dhan34	64.00 \pm 2.67	62.67 \pm 2.67	60.00 \pm 3.65	48.00 \pm 3.27
BRR I dhan37	64.00 \pm 1.63	60.00 \pm 2.11	57.33 \pm 2.67	42.67 \pm 1.63
BRR I dhan42	45.33 \pm 2.49	41.33 \pm 1.33	38.67 \pm 2.49	34.67 \pm 2.49
BRR I dhan43	58.67 \pm 2.49	57.33 \pm 2.67	54.67 \pm 1.33	41.33 \pm 1.33
BRR I dhan47	62.67 \pm 3.40	57.33 \pm 2.67	54.67 \pm 2.49	42.67 \pm 2.67
BRR I dhan57	61.33 \pm 2.49	54.67 \pm 2.49	52.00 \pm 3.27	42.67 \pm 2.67
Mean \pm SE	59.76 \pm 2.55	55.84 \pm 2.51	51.69 \pm 2.08	41.02 \pm 1.55
F-value	19.00	16.16	10.00	6.84
Significance	0.000	0.000	0.000	0.000
LSD _{0.05}	3.687	3.943	4.141	3.736

Contd. (Table 10)

Variety	Callus induction (% \pm SE)			
	T ₉	T ₁₀	T ₁₁	T ₁₂
BR3	42.67 \pm 1.63	48.00 \pm 1.33	49.33 \pm 1.63	54.67 \pm 1.33
BR4	37.33 \pm 1.63	38.67 \pm 2.49	42.67 \pm 1.63	44.00 \pm 1.63
BR8	44.00 \pm 2.67	49.33 \pm 1.63	50.67 \pm 1.63	54.67 \pm 1.33
BR10	56.00 \pm 2.67	60.00 \pm 2.98	64.00 \pm 2.67	66.67 \pm 3.65
BR11	38.67 \pm 2.49	45.33 \pm 2.49	48.00 \pm 2.49	53.33 \pm 2.11
BR23	40.00 \pm 2.98	42.67 \pm 2.67	45.33 \pm 2.49	49.33 \pm 1.63
BR24	38.67 \pm 2.49	44.00 \pm 2.67	48.00 \pm 2.49	50.67 \pm 2.67
BRRi dhan27	34.67 \pm 1.33	38.67 \pm 2.49	41.33 \pm 1.33	48.00 \pm 1.33
BRRi dhan28	54.67 \pm 3.27	61.33 \pm 2.49	62.67 \pm 2.67	65.33 \pm 3.89
BRRi dhan29	56.00 \pm 2.67	60.00 \pm 2.98	65.33 \pm 3.27	70.67 \pm 3.40
BRRi dhan32	57.33 \pm 2.67	58.67 \pm 2.49	62.67 \pm 2.67	68.00 \pm 3.27
BRRi dhan34	57.33 \pm 3.40	62.67 \pm 2.67	64.00 \pm 2.67	66.67 \pm 3.65
BRRi dhan37	50.67 \pm 2.67	57.33 \pm 2.67	58.67 \pm 2.49	62.67 \pm 2.67
BRRi dhan42	36.00 \pm 1.63	38.67 \pm 2.49	41.33 \pm 1.33	42.67 \pm 1.63
BRRi dhan43	49.33 \pm 2.67	54.67 \pm 1.33	56.00 \pm 1.63	61.33 \pm 1.33
BRRi dhan47	53.33 \pm 2.11	57.33 \pm 1.63	60.00 \pm 2.11	61.33 \pm 2.49
BRRi dhan57	46.67 \pm 3.65	52.00 \pm 3.27	54.67 \pm 1.33	58.67 \pm 2.49
Mean \pm SE	46.67 \pm 1.99	51.14 \pm 2.08	53.80 \pm 2.07	57.57 \pm 2.11
F-value	10.02	12.12	14.72	11.66
Significance	0.000	0.000	0.000	0.000
LSD _{0.05}	3.960	3.770	3.411	3.897

Contd. (Table 10)

Variety	Callus induction (% \pm SE)		
	T ₁₃	T ₁₄	T ₁₅
BR3	48.00 \pm 1.33	42.67 \pm 1.63	37.33 \pm 1.63
BR4	42.67 \pm 1.63	41.33 \pm 1.33	32.00 \pm 2.49
BR8	48.00 \pm 1.33	44.00 \pm 2.67	37.33 \pm 1.63
BR10	65.33 \pm 1.33	61.33 \pm 2.49	54.67 \pm 2.49
BR11	46.67 \pm 2.11	41.33 \pm 1.33	34.67 \pm 1.33
BR23	46.67 \pm 2.11	42.67 \pm 1.63	38.67 \pm 2.49
BR24	48.00 \pm 2.49	44.00 \pm 1.63	40.00 \pm 2.98
BRRi dhan27	45.33 \pm 2.49	41.33 \pm 1.33	37.33 \pm 2.67
BRRi dhan28	62.67 \pm 1.63	56.00 \pm 2.67	46.67 \pm 2.98
BRRi dhan29	68.00 \pm 3.27	65.33 \pm 1.33	61.33 \pm 2.49
BRRi dhan32	64.00 \pm 1.63	62.67 \pm 2.67	57.33 \pm 2.67
BRRi dhan34	58.67 \pm 2.49	52.00 \pm 3.89	45.33 \pm 2.49
BRRi dhan37	56.00 \pm 1.63	50.67 \pm 2.67	44.00 \pm 2.67
BRRi dhan42	38.67 \pm 1.33	37.33 \pm 2.67	34.67 \pm 1.33
BRRi dhan43	53.33 \pm 2.98	49.33 \pm 2.67	40.00 \pm 2.11
BRRi dhan47	54.67 \pm 2.49	52.00 \pm 3.89	41.33 \pm 1.33
BRRi dhan57	50.67 \pm 2.67	46.67 \pm 3.65	38.67 \pm 2.49
Mean \pm SE	52.79 \pm 2.07	48.86 \pm 2.03	42.43 \pm 2.02
F-value	15.85	11.10	12.84
Significance	0.000	0.000	0.000
LSD _{0.05}	3.283	3.850	3.553

Table 11: Analysis of variances (ANOVAs) of fifteen different treatments for callus induction

ANOVA of treatment	Source of Variation	Sum of Squares	df	Mean Square	F-value	Sig.
T ₁	Variety	2401.627	16	150.102	5.981	0.000
	Error	1706.694	68	25.098	-	-
	Total	4108.321	84	-	-	-
T ₂	Variety	4537.830	16	283.614	8.890	0.000
	Error	2169.405	68	31.903	-	-
	Total	6707.235	84	-	-	-
T ₃	Variety	5643.754	16	352.735	10.967	0.000
	Error	2187.121	68	32.164	-	-
	Total	7830.874	84	-	-	-
T ₄	Variety	8938.597	16	558.662	12.282	0.000
	Error	3092.987	68	45.485	-	-
	Total	12031.584	84	-	-	-
T ₅	Variety	8822.579	16	551.411	19.003	0.000
	Error	1973.121	68	29.016	-	-
	Total	10795.700	84	-	-	-
T ₆	Variety	8584.686	16	536.543	16.163	0.000
	Error	2257.289	68	33.195	-	-
	Total	10841.975	84	-	-	-
T ₇	Variety	5857.039	16	366.065	10.001	0.000
	Error	2489.085	68	36.604	-	-
	Total	8346.124	84	-	-	-

df = degrees of freedom, Sig. = Significance

Contd. (Table 11)

ANOVA of treatment	Source of Variation	Sum of Squares	df	Mean Square	F-value	Sig.
T ₈	Variety	3262.897	16	203.931	6.844	0.000
	Error	2026.080	68	29.795	-	-
	Total	5288.978	84	-	-	-
T ₉	Variety	5369.138	16	335.571	10.024	0.000
	Error	2276.418	68	33.477	-	-
	Total	7645.556	84	-	-	-
T ₁₀	Variety	5883.521	16	367.720	12.120	0.000
	Error	2063.138	68	30.340	-	-
	Total	7946.659	84	-	-	-
T ₁₁	Variety	5847.092	16	365.443	14.717	0.000
	Error	1688.552	68	24.832	-	-
	Total	7535.643	84	-	-	-
T ₁₂	Variety	6048.455	16	378.028	11.661	0.000
	Error	2204.436	68	32.418	-	-
	Total	8252.891	84	-	-	-
T ₁₃	Variety	5831.958	16	364.497	15.846	0.000
	Error	1564.169	68	23.002	-	-
	Total	7396.127	84	-	-	-
T ₁₄	Variety	5617.169	16	351.073	11.095	0.000
	Error	2151.716	68	31.643	-	-
	Total	7768.885	84	-	-	-
T ₁₅	Variety	5533.570	16	345.848	12.837	0.000
	Error	1831.956	68	26.941	-	-
	Total	7365.526	84	-	-	-

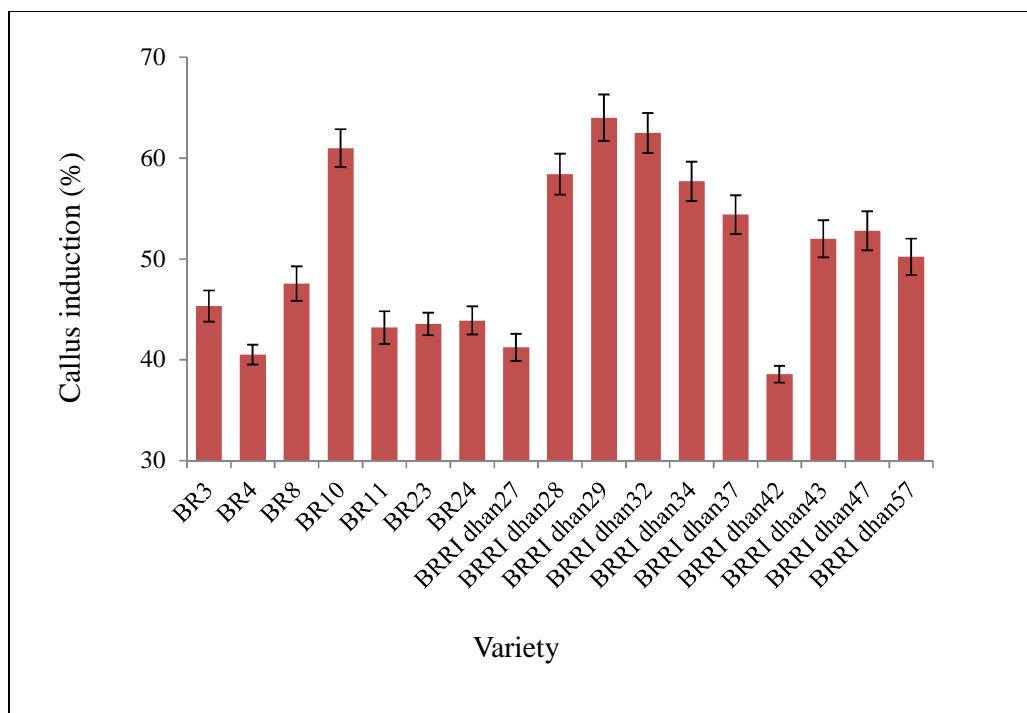


Fig. 5: Callus induction efficiency of studied rice varieties on an average of tested hormonal concentration and combinations.

Table 12: Effect of four basal media to callus induction

Variety	Callus induction (% \pm SE)			
	MS	N6	LS	SK1
BR3	50.67 \pm 1.63	45.33 \pm 2.49	46.67 \pm 2.98	38.67 \pm 2.49
BR4	45.33 \pm 2.49	42.67 \pm 1.63	40.00 \pm 2.11	37.33 \pm 2.67
BR8	58.67 \pm 3.27	52.00 \pm 2.49	50.67 \pm 2.67	45.33 \pm 1.33
BR10	72.00 \pm 2.49	66.67 \pm 2.98	61.33 \pm 2.49	57.33 \pm 1.63
BR11	52.00 \pm 1.33	49.33 \pm 2.67	45.33 \pm 2.49	40.00 \pm 2.11
BR23	53.33 \pm 2.11	46.67 \pm 3.65	42.67 \pm 1.63	36.00 \pm 2.67
BR24	53.33 \pm 2.11	50.67 \pm 2.67	44.00 \pm 2.67	40.00 \pm 2.98
BRRi dhan27	50.67 \pm 1.63	45.33 \pm 2.49	40.00 \pm 2.11	37.33 \pm 2.67
BRRi dhan28	68.00 \pm 1.33	65.33 \pm 3.27	60.00 \pm 2.98	54.67 \pm 2.49
BRRi dhan29	77.33 \pm 3.40	72.00 \pm 3.89	66.67 \pm 2.11	61.33 \pm 2.49
BRRi dhan32	74.67 \pm 2.49	69.33 \pm 3.40	65.33 \pm 2.49	61.33 \pm 1.33
BRRi dhan34	64.00 \pm 1.63	62.67 \pm 1.63	58.67 \pm 2.49	60.00 \pm 2.11
BRRi dhan37	64.00 \pm 1.63	61.33 \pm 1.33	60.00 \pm 2.11	49.33 \pm 2.67
BRRi dhan42	48.00 \pm 3.27	42.67 \pm 1.63	45.33 \pm 1.33	38.67 \pm 2.49
BRRi dhan43	60.00 \pm 2.98	57.33 \pm 2.67	54.67 \pm 3.27	46.67 \pm 2.11
BRRi dhan47	64.00 \pm 2.67	61.33 \pm 2.49	56.00 \pm 2.67	52.00 \pm 2.49
BRRi dhan57	60.00 \pm 2.11	56.00 \pm 1.63	48.00 \pm 1.33	42.67 \pm 2.67
Mean \pm SE	59.76 \pm 2.32	55.69 \pm 2.34	52.08 \pm 2.14	46.98 \pm 2.22
F-value	16.30	13.44	13.45	14.99
Significance	0.000	0.000	0.000	0.000
LSD _{0.05}	3.620	4.036	3.687	3.621

Table 13: Analysis of variances of four basal media for callus induction

ANOVA of Media	Source of Variation	Sum of Squares	df	Mean Square	F-value	Sig.
MS	Variety	7294.018	16	455.876	16.298	0.000
	Error	1902.072	68	27.972	-	-
	Total	9196.090	84	-	-	-
N6	Variety	7475.266	16	467.204	13.437	0.000
	Error	2364.329	68	34.770	-	-
	Total	9839.595	84	-	-	-
LS	Variety	6248.235	16	390.515	13.454	0.000
	Error	1973.707	68	29.025	-	-
	Total	8221.942	84	-	-	-
SK1	Variety	6712.267	16	419.517	14.989	0.000
	Error	1903.192	68	27.988	-	-
	Total	8615.459	84	-	-	-

4.1.3.3 Effect of PGRs on regeneration

Fifteen types of hormonal combinations (H_1 - H_{15}) were added to basal medium MS which known as regeneration medium (RMs) and the calli of studied varieties were transferred on RMs to test their effectiveness on plant regeneration (**Table 14**). By the results it was observed that the tested varieties showed significant difference at $p \leq 0.001$, considering their efficiency to regenerate plants (**Table 15**). Among the genotypes, BRRI dhan29 performed highest number of plant regeneration (70.67%) in H_{11} (2.0 mg/l BAP + 1.0 mg/l Kin + 1.0 NAA) and the lowest value was found for BRRI dhan32 (22.67%) in H_{15} (4.0 mg/l BAP + 0.5 mg/l Kin + 0.5 mg/l NAA).

Average of fifteen tested hormonal concentration and combinations for BRRI dhan29 (59.38%) expressed that the variety was the most efficient genotype for plant regeneration (**Fig. 6**). In case of other varieties the values were recorded as 53.42, 52.71, 51.73, 51.73, 48.27, 47.11, 45.78, 45.69, 44.18, 41.69, 39.02, 38.58, 37.95, 35.47 and 33.33% for BRRI dhan28, BR11, BR3, BR8, BRRI dhan57, BRRI dhan27, BRRI dhan43, BR24, BRRI dhan37, BRRI dhan34, BR23, BRRI dhan42, BR4, BRRI dhan47 and BR10 respectively. The variety BRRI dhan32 showed the lowest frequency to regenerate plant (26.93%) when the calli were transferred to regeneration medium.

On an average, the treatment H_{11} (2.0 mg/l BAP + 1.0 mg/l Kin + 1.0 NAA) carried the highest value (51.53%) that expressed its effectiveness to influence the varieties for producing plants (**Table 14**). The lowest value of plant regeneration (35.92%) was recorded from H_{15} (4.0 mg/l BAP + 0.5 mg/l Kin + 0.5 mg/l NAA). However, the comparison of the genotypes on the efficiency of callus induction and regeneration, regarding the average values of PGRs tested, the frequency curve of the results showed that BRRI dhan32 was more efficient to induce callus having the frequency of 62.49%; whereas, it performed with lowest regeneration (26.93%) efficiency (**Fig.7**).

Table 14: Effect of PGRs on plant regeneration for the seventeen rice varieties

Variety	Plant regeneration (% \pm SE)			
	H ₁	H ₂	H ₃	H ₄
BR3	46.67 \pm 2.98	49.33 \pm 2.67	53.33 \pm 2.98	58.67 \pm 1.33
BR4	28.00 \pm 1.33	33.33 \pm 2.11	38.67 \pm 2.49	45.33 \pm 2.49
BR8	46.67 \pm 2.98	49.33 \pm 2.67	53.33 \pm 2.98	58.67 \pm 3.27
BR10	28.00 \pm 1.33	34.67 \pm 1.33	38.67 \pm 2.49	45.33 \pm 2.49
BR11	45.33 \pm 3.27	50.67 \pm 2.67	54.67 \pm 2.49	62.67 \pm 1.63
BR23	32.00 \pm 2.49	36.00 \pm 2.67	38.67 \pm 2.49	44.00 \pm 2.67
BR24	45.33 \pm 2.49	48.00 \pm 2.49	50.67 \pm 1.63	52.00 \pm 2.49
BRRI dhan27	45.33 \pm 1.33	46.67 \pm 2.98	49.33 \pm 1.63	56.00 \pm 1.63
BRRI dhan28	46.67 \pm 2.11	49.33 \pm 2.67	53.33 \pm 2.11	61.33 \pm 3.27
BRRI dhan29	49.33 \pm 1.63	56.00 \pm 2.67	60.00 \pm 2.98	65.33 \pm 3.27
BRRI dhan32	21.33 \pm 1.33	24.00 \pm 2.67	28.00 \pm 2.49	33.33 \pm 0.00
BRRI dhan34	37.33 \pm 1.63	41.33 \pm 1.33	45.33 \pm 2.49	48.00 \pm 1.33
BRRI dhan37	41.33 \pm 1.33	45.33 \pm 2.49	49.33 \pm 1.63	52.00 \pm 2.49
BRRI dhan42	33.33 \pm 2.11	37.33 \pm 2.67	42.67 \pm 2.67	45.33 \pm 2.49
BRRI dhan43	46.67 \pm 2.11	48.00 \pm 2.49	50.67 \pm 1.63	53.33 \pm 2.11
BRRI dhan47	30.67 \pm 1.63	34.67 \pm 1.33	36.00 \pm 1.63	38.67 \pm 1.33
BRRI dhan57	42.67 \pm 1.63	45.33 \pm 2.49	49.33 \pm 2.67	54.67 \pm 2.49
Mean \pm SE	39.22 \pm 2.09	42.9 \pm 2.01	46.59 \pm 2.01	51.45 \pm 2.12
F-value	17.11	11.63	12.18	14.24
Significance	0.000	0.000	0.000	0.000
LSD _{0.05}	3.188	3.720	3.637	3.552

Contd. (Table 14)

Variety	Plant regeneration (% \pm SE)			
	H ₅	H ₆	H ₇	H ₈
BR3	54.67 \pm 1.33	52.00 \pm 2.49	45.33 \pm 1.33	42.67 \pm 1.63
BR4	41.33 \pm 1.33	37.33 \pm 2.67	32.00 \pm 1.33	29.33 \pm 1.63
BR8	54.67 \pm 3.27	52.00 \pm 2.49	45.33 \pm 2.49	42.67 \pm 1.63
BR10	41.33 \pm 1.33	37.33 \pm 2.67	32.00 \pm 1.33	29.33 \pm 1.63
BR11	57.33 \pm 3.40	57.33 \pm 2.67	52.00 \pm 1.33	46.67 \pm 2.11
BR23	42.67 \pm 2.67	40.00 \pm 2.11	37.33 \pm 2.67	33.33 \pm 2.98
BR24	49.33 \pm 1.63	46.67 \pm 2.11	44.00 \pm 2.67	41.33 \pm 2.49
BRR1 dhan27	50.67 \pm 1.63	49.33 \pm 1.63	44.00 \pm 2.67	41.33 \pm 1.33
BRR1 dhan28	56.00 \pm 1.63	54.67 \pm 1.33	50.67 \pm 1.63	48.00 \pm 1.33
BRR1 dhan29	61.33 \pm 2.49	58.67 \pm 2.49	53.33 \pm 2.98	52.00 \pm 2.49
BRR1 dhan32	29.33 \pm 1.63	26.67 \pm 2.98	25.33 \pm 2.49	22.67 \pm 1.63
BRR1 dhan34	45.33 \pm 2.49	42.67 \pm 2.67	38.67 \pm 2.49	34.67 \pm 1.33
BRR1 dhan37	46.67 \pm 2.98	45.33 \pm 2.49	41.33 \pm 2.49	38.67 \pm 2.49
BRR1 dhan42	42.67 \pm 2.67	38.67 \pm 2.49	36.00 \pm 2.67	29.33 \pm 2.67
BRR1 dhan43	49.33 \pm 1.63	45.33 \pm 2.49	42.67 \pm 2.67	40.00 \pm 2.11
BRR1 dhan47	37.33 \pm 1.63	36.00 \pm 1.63	32.00 \pm 1.33	29.33 \pm 1.63
BRR1 dhan57	50.67 \pm 1.63	48.00 \pm 1.33	42.67 \pm 1.63	42.67 \pm 1.63
Mean \pm SE	47.69 \pm 1.97	45.18 \pm 2.07	40.8624 \pm 1.88	37.88 \pm 1.95
F-value	13.66	13.40	12.21	16.22
Significance	0.000	0.000	0.000	0.000
LSD _{0.05}	3.356	3.569	3.393	3.051

Contd. (Table 14)

Variety	Plant regeneration (% \pm SE)			
	H ₉	H ₁₀	H ₁₁	H ₁₂
BR3	52.00 \pm 2.49	57.33 \pm 2.67	60.00 \pm 2.11	56.00 \pm 1.63
BR4	37.33 \pm 2.67	41.33 \pm 1.33	46.67 \pm 2.11	48.00 \pm 1.33
BR8	52.00 \pm 2.49	57.33 \pm 2.67	61.33 \pm 3.27	56.00 \pm 1.63
BR10	29.33 \pm 1.63	32.00 \pm 1.33	36.00 \pm 1.63	34.67 \pm 1.33
BR11	53.33 \pm 2.98	56.00 \pm 2.67	58.67 \pm 2.49	56.00 \pm 1.63
BR23	41.33 \pm 1.33	42.67 \pm 1.63	45.33 \pm 2.49	46.67 \pm 2.11
BR24	42.67 \pm 2.67	46.67 \pm 2.11	52.00 \pm 2.49	48.00 \pm 1.33
BRRi dhan27	44.00 \pm 1.63	50.67 \pm 2.67	54.67 \pm 2.49	50.67 \pm 2.67
BRRi dhan28	54.67 \pm 3.89	57.33 \pm 2.67	62.67 \pm 2.67	58.67 \pm 2.49
BRRi dhan29	57.33 \pm 2.67	65.33 \pm 3.27	70.67 \pm 3.40	66.67 \pm 2.98
BRRi dhan32	25.33 \pm 2.49	30.67 \pm 1.63	34.67 \pm 1.33	29.33 \pm 1.63
BRRi dhan34	41.33 \pm 1.33	45.33 \pm 2.49	49.33 \pm 1.63	46.67 \pm 2.11
BRRi dhan37	42.67 \pm 2.67	45.33 \pm 2.49	50.67 \pm 1.63	46.67 \pm 2.11
BRRi dhan42	41.33 \pm 1.33	44.00 \pm 1.63	46.67 \pm 2.11	41.33 \pm 1.33
BRRi dhan43	46.67 \pm 2.11	49.33 \pm 1.63	50.67 \pm 2.67	46.67 \pm 2.11
BRRi dhan47	34.67 \pm 1.33	36.00 \pm 1.63	38.67 \pm 1.33	42.67 \pm 1.63
BRRi dhan57	46.67 \pm 2.11	53.33 \pm 2.11	57.33 \pm 1.63	54.67 \pm 1.33
Mean \pm SE	43.69 \pm 2.14	47.69 \pm 2.32	51.53 \pm 2.376	48.79 \pm 2.188
F-value	14.24	18.46	18.36	22.23
Significance	0.000	0.000	0.000	0.000
LSD _{0.05}	3.569	3.411	3.500	2.928

Contd. (Table 14)

Variety	Plant regeneration (% \pm SE)		
	H ₁₃	H ₁₄	H ₁₅
BR3	53.33 \pm 2.11	49.33 \pm 1.63	45.33 \pm 3.27
BR4	41.33 \pm 1.33	36.00 \pm 2.67	33.33 \pm 2.98
BR8	53.33 \pm 2.11	49.33 \pm 1.63	44.00 \pm 1.63
BR10	30.67 \pm 2.67	26.67 \pm 2.11	24.00 \pm 2.67
BR11	52.00 \pm 2.49	46.67 \pm 2.11	41.33 \pm 3.89
BR23	40.00 \pm 2.11	34.67 \pm 2.49	30.67 \pm 2.67
BR24	46.67 \pm 2.11	37.33 \pm 2.67	34.67 \pm 1.33
BRRi dhan27	45.33 \pm 2.49	41.33 \pm 1.33	37.33 \pm 2.67
BRRi dhan28	54.67 \pm 1.33	49.33 \pm 1.63	44.00 \pm 1.63
BRRi dhan29	62.67 \pm 2.67	58.67 \pm 2.49	53.33 \pm 2.11
BRRi dhan32	26.67 \pm 2.11	24.00 \pm 2.67	22.67 \pm 1.63
BRRi dhan34	41.33 \pm 1.33	36.00 \pm 2.67	32.00 \pm 3.89
BRRi dhan37	44.00 \pm 2.67	38.67 \pm 2.49	34.67 \pm 1.33
BRRi dhan42	38.67 \pm 2.49	32.00 \pm 2.49	29.33 \pm 2.67
BRRi dhan43	45.33 \pm 2.49	38.67 \pm 2.49	33.33 \pm 3.65
BRRi dhan47	41.33 \pm 1.33	34.67 \pm 2.49	29.33 \pm 1.63
BRRi dhan57	50.67 \pm 1.63	44.00 \pm 1.63	41.33 \pm 1.33
Mean \pm SE	45.18 \pm 2.17	39.84 \pm 2.16	35.92 \pm 1.96
F-value	17.33	15.48	9.93
Significance	0.000	0.000	0.000
LSD _{0.05}	3.283	3.465	3.928

Table 15: Analysis of variances (ANOVAs) of fifteen different treatments for regeneration

ANOVA of PGRs	Source of Variation	Sum of Squares	df	Mean Square	F-value	Sig.
H ₁	Variety	5938.766	16	371.173	17.113	0.000
	Error	1474.872	68	21.689	-	-
	Total	7413.637	84	-	-	-
H ₂	Variety	5498.649	16	343.666	11.634	0.000
	Error	2008.685	68	29.539	-	-
	Total	7507.333	84	-	-	-
H ₃	Variety	5502.089	16	343.881	12.181	0.000
	Error	1919.654	68	28.230	-	-
	Total	7421.743	84	-	-	-
H ₄	Variety	6134.674	16	383.417	14.238	0.000
	Error	1831.129	68	26.928	-	-
	Total	7965.803	84	-	-	-
H ₅	Variety	5253.723	16	328.358	13.656	0.000
	Error	1635.005	68	24.044	-	-
	Total	6888.728	84	-	-	-
H ₆	Variety	5829.197	16	364.325	13.398	0.000
	Error	1849.112	68	27.193	-	-
	Total	7678.309	84	-	-	-
H ₇	Variety	4799.501	16	299.969	12.207	0.000
	Error	1671.023	68	24.574	-	-
	Total	6470.524	84	-	-	-

df = degrees of freedom, Sig. = Significance

Contd. (Table 15)

ANOVA of PGRs	Source of Variation	Sum of Squares	df	Mean Square	F-value	Sig.
H ₈	Variety	5156.607	16	322.288	16.221	0.000
	Error	1351.049	68	19.868	-	-
	Total	6507.656	84	-	-	-
H ₉	Variety	6196.267	16	387.267	14.245	0.000
	Error	1848.712	68	27.187	-	-
	Total	8044.978	84	-	-	-
H ₁₀	Variety	7333.818	16	458.364	18.455	0.000
	Error	1688.900	68	24.837	-	-
	Total	9022.719	84	-	-	-
H ₁₁	Variety	7679.327	16	479.958	18.359	0.000
	Error	1777.689	68	26.142	-	-
	Total	9457.016	84	-	-	-
H ₁₂	Variety	6506.934	16	406.683	22.225	0.000
	Error	1244.276	68	18.298	-	-
	Total	7751.210	84	-	-	-
H ₁₃	Variety	6379.055	16	398.691	17.333	0.000
	Error	1564.143	68	23.002	-	-
	Total	7943.198	84	-	-	-
H ₁₄	Variety	6344.843	16	396.553	15.477	0.000
	Error	1742.312	68	25.622	-	-
	Total	8087.155	84	-	-	-
H ₁₅	Variety	5234.431	16	327.152	9.932	0.000
	Error	2239.894	68	32.940	-	-
	Total	7474.325	84	-	-	-

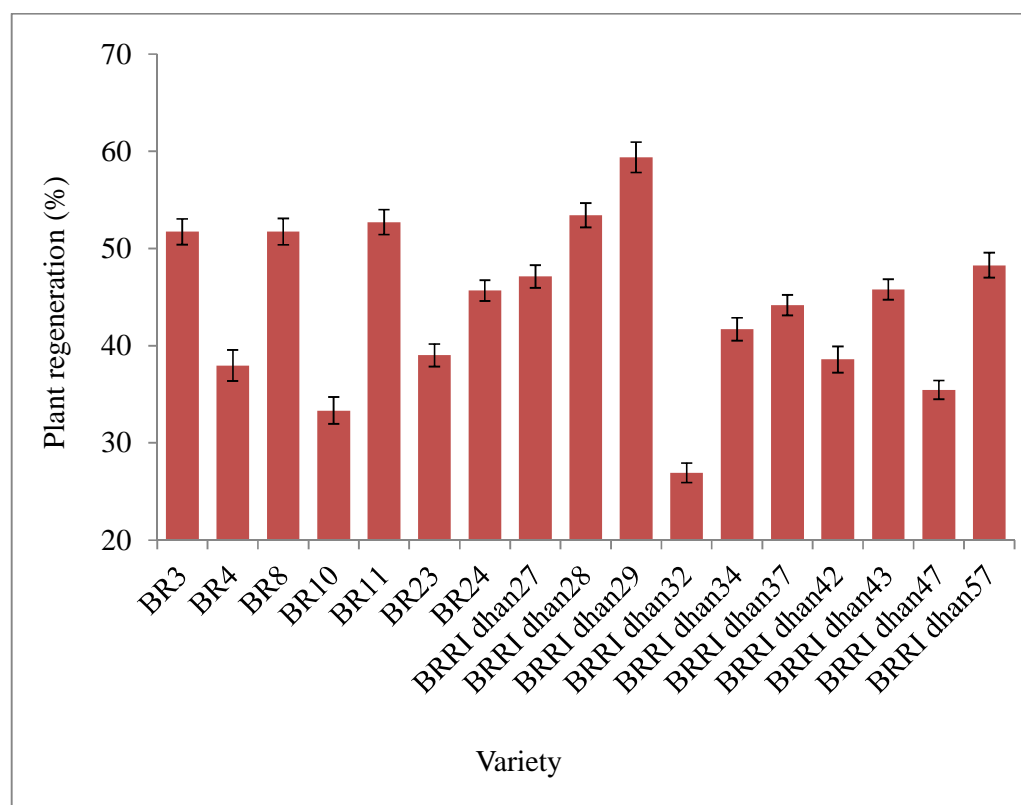


Fig. 6: Efficiency of plant regeneration of studied rice varieties on an average of tested hormonal concentration and combinations.

4.1.3.4 Effect of media on regeneration

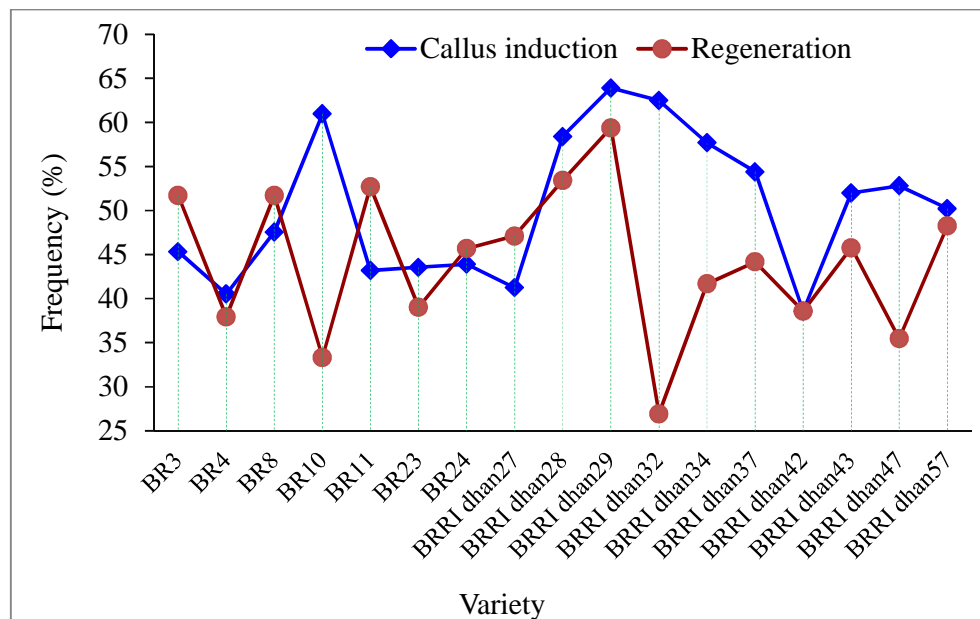
To test the effect of media on regeneration, a suitable hormonal combinations H_{11} (2.0 mg/l BAP + 1.0 mg/l Kin + 1.0 NAA) was considered as constant and added separately with four basal media MS, N6, LS and SK1; and all the studied varieties were cultured to regenerate plants. The results showed that all the varieties performed the maximum regeneration in MS medium. The calli of BRR1 dhan29 regenerate plant at the highest rate (69.33%) in MS; and followed by BRR1 dhan29 (62.67%) in N6 and BRR1 dhan28 (61.33%) in MS; and the lowest was 24.00% for BR10 in LS (**Table 16**). Within the medium the genotypes showed significant difference at $p \leq 0.001$ (**Table 17**). Average regeneration was recorded as 51.14% from MS the highest value out of four media; and the lowest was in LS (40.23%).

Table 16: Effect of four basal media to plant regeneration

Variety	Plant regeneration (% \pm SE)			
	MS	N6	LS	SK1
BR3	60.00 \pm 3.65	57.33 \pm 2.67	50.67 \pm 2.67	56.00 \pm 3.40
BR4	48.00 \pm 1.33	45.33 \pm 3.27	37.33 \pm 2.67	41.33 \pm 2.49
BR8	60.00 \pm 2.11	54.67 \pm 3.27	48.00 \pm 3.27	52.00 \pm 3.89
BR10	34.67 \pm 1.33	32.00 \pm 2.49	24.00 \pm 2.67	26.67 \pm 2.98
BR11	57.33 \pm 2.67	53.33 \pm 2.11	45.33 \pm 3.89	49.33 \pm 2.67
BR23	45.33 \pm 2.49	46.67 \pm 2.11	34.67 \pm 3.27	36.00 \pm 2.67
BR24	53.33 \pm 2.11	57.33 \pm 2.67	40.00 \pm 3.65	46.67 \pm 2.11
BRRi dhan27	53.33 \pm 2.11	46.67 \pm 2.11	40.00 \pm 3.65	42.67 \pm 2.67
BRRi dhan28	61.33 \pm 3.27	56.00 \pm 2.67	49.33 \pm 2.67	53.33 \pm 3.65
BRRi dhan29	69.33 \pm 4.52	62.67 \pm 3.40	56.00 \pm 2.67	61.33 \pm 3.89
BRRi dhan32	36.00 \pm 1.63	33.33 \pm 2.11	25.33 \pm 2.49	28.00 \pm 2.49
BRRi dhan34	50.67 \pm 2.67	46.67 \pm 3.65	37.33 \pm 4.00	44.00 \pm 2.67
BRRi dhan37	49.33 \pm 2.67	56.00 \pm 3.40	46.67 \pm 2.98	49.33 \pm 2.67
BRRi dhan42	45.33 \pm 2.49	41.33 \pm 1.33	33.33 \pm 2.11	36.00 \pm 2.67
BRRi dhan43	52.00 \pm 2.49	48.00 \pm 3.27	42.67 \pm 2.67	44.00 \pm 2.67
BRRi dhan47	38.67 \pm 2.49	37.33 \pm 2.67	29.33 \pm 2.67	30.67 \pm 2.67
BRRi dhan57	54.67 \pm 1.33	53.33 \pm 2.11	44.00 \pm 2.67	48.00 \pm 1.33
Mean \pm SE	51.14 \pm 2.26	48.83 \pm 2.21	40.23 \pm 2.18	43.84 \pm 2.38
F-value	13.19	10.46	8.80	11.77
Significance	0.000	0.000	0.000	0.000
LSD _{0.05}	3.926	4.185	4.629	4.385

Table 17: Analysis of variances (ANOVAs) of four basal media for regeneration

ANOVA of Media	Source of Variation	Sum of Squares	degrees of freedom (df)	Mean Square	F-value	Significance
MS	Variety	6950.269	16	434.392	13.187	0.000
	Error	2239.947	68	32.940	-	-
	Total	9190.216	84	-	-	-
N6	Variety	6259.667	16	391.229	10.464	0.000
	Error	2542.285	68	37.387	-	-
	Total	8801.951	84	-	-	-
LS	Variety	6439.708	16	402.482	8.798	0.000
	Error	3110.703	68	45.746	-	-
	Total	9550.411	84	-	-	-
SK1	Variety	7731.516	16	483.220	11.774	0.000
	Error	2790.703	68	41.040	-	-
	Total	10522.219	84	-	-	-

**Fig. 7:** Comparison to callus induction and plant regeneration of seventeen genotypes.

4.1.3.5 Root induction

To observe the efficiency of root induction 3-5 cm length of shoot of all the varieties were placed into the culture vessels or test tubes containing half strength MS medium supplemented with 0.5 mg/l IBA. Cent percent (100%) root induction was recorded for four varieties viz. BRRI dhan29, BRRI dhan42, BRRI dhan43 and BRRI dhan47; and the lowest value (94.67%) was found for BRRI dhan23 and BRRI dhan37 both (**Table 18**). The results showed that the varieties were not significantly differed on respect to induction rate of roots. On the other hand a significance differences were found on the feature of roots length at $p \leq 0.001$. The varieties BRRI dhan42, BRRI dhan43, BRRI dhan47 and BRRI dhan57 showed higher length of roots than the others studied ones. The highest length was measured for BRRI dhan47 as 13.52 cm; followed by BRRI dhan42 (12.79 cm), BRRI dhan43 (12.68 cm), BRRI dhan29 (12.44 cm), BR3 (12.33 cm) and BR10 (12.01 cm). Whereas, the lowest length of roots were observed for BRRI dhan37 (7.31 cm) when the plantlets were cultured for 8 weeks after placed on root induction medium. At the same significance level ($p \leq 0.001$) a wide range of variations were found in the number of roots per plant. In this case, the highest average number of roots per plant was recorded for BRRI dhan47 (6.41); followed by BRRI dhan43 (5.88), BR23 (5.65), BRRI dhan57 (5.61), BRRI dhan29 (5.57), BRRI dhan42 (4.31) and BRRI dhan27 (4.01). The lowest average value was recorded for BRRI dhan32 (2.36).

Table 18: Efficiency of seventeen genotypes to root induction, length of roots and number of roots per plant

Variety	Frequency of root induction (%)	Length of roots (cm)	No. of roots per plant
BR3	98.67 ± 1.33	12.33 ± 0.24abc	3.24 ± 0.09ef
BR4	96.00 ± 2.67	11.24 ± 0.26cd	2.97 ± 0.14efg
BR8	98.67 ± 1.33	10.45 ± 0.23de	3.35 ± 0.13ef
BR10	97.33 ± 1.63	12.01 ± 0.25bc	3.21 ± 0.10ef
BR11	97.33 ± 2.67	11.32 ± 0.21cd	3.17 ± 0.07ef
BR23	94.67 ± 3.27	10.65 ± 0.24de	5.65 ± 0.14b
BR24	96.00 ± 2.67	9.67 ± 0.11ef	2.76 ± 0.14fg
BRR1 dhan27	96.00 ± 4.00	8.87 ± 0.18fg	4.01 ± 0.10cd
BRR1 dhan28	98.67 ± 1.33	7.89 ± 0.23gh	3.24 ± 0.09ef
BRR1 dhan29	100.00 ± 0.00	12.44 ± 0.23abc	5.57 ± 0.15b
BRR1 dhan32	98.67 ± 1.33	9.56 ± 0.22ef	2.36 ± 0.08g
BRR1 dhan34	96.00 ± 2.67	10.53 ± 0.23de	2.88 ± 0.12efg
BRR1 dhan37	94.67 ± 3.27	7.31 ± 0.28h	3.44 ± 0.16de
BRR1 dhan42	100.00 ± 0.00	12.79 ± 0.26ab	4.31 ± 0.12c
BRR1 dhan43	100.00 ± 0.00	12.68 ± 0.24ab	5.88 ± 0.08ab
BRR1 dhan47	100.00 ± 0.00	13.52 ± 0.45a	6.41 ± 0.17a
BRR1 dhan57	98.67 ± 1.33	11.56 ± 0.27bcd	5.61 ± 0.14b
Mean ± SE	97.73 ± 0.45	10.87 ± 0.43	4.00 ± 0.31
F-value	0.770	50.115	113.470
Significance	0.713	0.000	0.000

4.1.4 Discussion

4.1.4.1 Effect of PGRs to CI

Upadhyaya et al. (2015) used different concentrations of 2, 4-D (1.0, 1.5, 2.0, 2.5 mg/l) for callus induction from mature embryos of three japonica rice cultivars viz. Sita, Rupali and Masuri. They recorded 63.36% to 92.23% callus induction and mentioned that all the varieties produced calli at the highest rate in 2.0 mg/l 2, 4-D concentration. Sah et al. (2014) reported a protocol to produce embryogenic callus in *Oryza sativa* var. Kitaake a japonica rice cultivar. They used MS medium supplemented with 3.0 mg/l 2, 4-D + 0.25 mg/l BAP for callus induction. Tiwari et al. (2012) reported that for maximum CI, the optimum hormonal combination was 1.5 mg/l 2, 4-D + 0.1 mg/l NAA + 0.1 mg/l BAP with MS medium. They have recorded 85% and 90% CI for indica rice varieties of Pusa Basmati1 and Kalanamak respectively. Mannan et al. (2013) induced calli from Bangladeshi aromatic rice (*Oryza sativa* L.) var. Kalijira and Chinigura using MS medium supplemented with different concentrations of 2,4-D (1.0, 1.5, 2.0, 2.5 mg/l). They recorded highest 97.22 and 94.44% callus induction in 2.0 mg/l 2, 4-D for Kalijira and Chinigura respectively.

In the present study, seven different concentrations of 2, 4-D (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mg/l) singly and/ or combined with Kin (0.5 mg/l) were added to MS and examined the effect on callus induction for studied varieties. The results showed that the frequencies of callus induction was ranged as 33.33 - 78.67% (**Table 10**); and the highest efficiency was found for BRRI dhan29 in $T_5 = 2.5$ mg/l 2, 4-D. Mannan et al. (2013) reported that on the combined effect of 2.0 mg/l 2,4-D with Kin (0.25, 0.5, 1.0, 1.5 mg/l) to induce calli; and recorded the highest values for the varieties Kalijira (97.22%) and Chinigura (94.44%) in 2.0 mg/l 2,4-D singly; while they reported that callus initiation days were decreased at combined condition of 2.0 mg/l 2,4-D + 0.5 mg/l Kin. However, in this study, it was examined the combined effect of 2, 4-D and Kin; where 0.5 mg/l Kin was added to 2,4-D concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mg/l) and recorded highest 70.67% callus induction for BRRI dhan29 in $T_{12} = 2.5$ mg/l 2,4-D + 0.5 mg/l Kin; while the same variety produced 78.67% callus induction in 2.5 mg/l 2,4-D singly. Hence, the present investigation claimed that 2.5 mg/l 2, 4-D individually influenced better to induce efficient calli than combined with

Kin for BRRI dhan29. Present reports argue with the previous findings and mentioned that due to different genotype, growth regulators and culture conditions, the dissimilarities might be occurred. Abe and Futsuhara (1986) mentioned that potentiality of callus induction depends on a number of factors, like genotype, physiological and biochemical status of the explants, composition and concentration of different ingredients of culture medium etc. while among these factors, genotypic difference is the most important one. Zuraida et al. (2010) reported that CI frequency depends on genotype and most indica rice cultivars had poor callusing potentiality. It was reported that all the indica sub-species have no equal potentiality for *in vitro* responses (Seraj et al. 1997, Khanna and Raina 1998). Genotypic effect also reported by several authors in indica rice (Khatun et al. 2010, Islam et al. 2013), maize (Morshed et al. 2014), wheat (Islam 2000, Islam et al. 2001, Islam 2010c, Saha et al. 2015), sugarcane (Roy et al. 2011) and *Rorippa indica* (Xu 2016). Makerly et al. (2012) recorded highest percentage of CI in MS supplemented with NAA for Malaysian indica rice cultivars MR232 (41%) and MR220 (37%); and also reported that the varieties responded lower in 2,4-D. Upadhyaya et al. (2015) used 2.0 mg/l 2,4-D with MS and induced calli in 10 hrs light and 14 hrs of dark cycle. Three varieties of indica rice, Sita, Rupali and Masuri resulted optimum frequency of callus initiation at 2.0 mg/l 2, 4-D concentration whereas at a high or low concentration the cultivars showed similar tendency of decrease in callus initiation. Therefore, they stated that the use of 2, 4-D with 2 mg/l was adequate for production of high amount of callus in rice. Present investigation differs with their reports, and mentioned that studied varieties responded with high efficiency of callus induction in 2, 4-D. However, a wide range of capability for inducing callus was observed in this study where the genotypes showed significant difference at $p \leq 0.001$.

4.1.4.2 Effect of media to CI

It was observed that the basal medium MS performed the best callus induction than N6, LS and SK1 when a suitable hormonal concentration (2.5 mg/l 2, 4-D) was added to CIM. The variety BRRI dhan29 gave the maximum CI (77.33 %) in MS; while the highest values were 72.00, 66.67 and 61.33% in N6, LS and SK1 respectively (**Table 12**). Aananthi et al. (2010) tested two basal media MS and N6 for induction of callus in five indica rice (*Oryza sativa* L.) cultivars viz. ASD 16, White Ponni, Pusa Basmati 1, Pusa Sugandh 4 and Pusa Sugandh 5. They found the highest callus induction for

White Ponni (88.75%) in MS + 2.0 mg/l 2, 4-D and Pusa Basmati 1 (84.97%) in MS + 2.5 mg/l 2,4-D. They described that MS was better than N6 basal medium for callus induction in rice. The present study investigated the similar findings and noticed that all the tested varieties produced better callusing in MS medium than N6 with the supplement of 2.5 mg/l 2,4-D. Moreover, MS was not only better than N6 but also the best among the four media tested.

4.1.4.3 Effect of PGRs to regeneration

The range of plant regeneration efficiency was 22.67 - 70.67% for the seventeen tested varieties in fifteen types of hormonal combinations H₁ - H₁₅ examined (**Table 14**). The highest value was found for BRRI dhan29 in H₁₁ (2.0 mg/l BAP + 0.1 mg/l Kin + 0.5 mg/l NAA) and the lowest for BRRI dhan32 in H₁₅ (4.0 mg/l BAP + 0.1 mg/l Kin + 0.5 mg/l NAA). Therefore, the analysis of variance indicated that the studied varieties were significantly differed at $p \leq 0.001$ on respect of variability to regenerate plant *in vitro*. On the other hand, the concentration and combinations of plant growth regulators which tested to investigate the effect on plant regeneration also showed significant difference influencing the varieties to regenerate plant.

However, the variety BR24 (52.00%) and BRRI dhan47 (38.67%) gave their highest plant regeneration in H₄ (2.0 mg/l BAP) and H₁₁ (2.0 mg/l BAP + 0.1 mg/l Kin + 0.5 mg/l NAA). Out of the seventeen varieties, five gave their highest regeneration in H₄ and other ten showed highest efficiency in H₁₁. Hence, it could be suggested that the treatment H₄ and H₁₁ were the most effective combination of plant growth regulators to produce rice plants *in vitro* through somatic embryogenesis for studied genotypes. It was observed that BAP (2.0 mg/l) singly played a vital role to regenerate plant without combination of Kin.

Zinnah et al. (2013) measured the highest frequency of regeneration 80% for BRRI dhan38 and 60% for Chini Kanai in MS medium supplemented with 2.0 mg/l Kin + 1.0 mg/l NAA + 2 mg/l BA. Reddy et al. (2013) used the combination of growth regulators as 0.5 mg/l NAA + 2 mg/l Kin + 0.50 mg/l BA with MS medium for plant regeneration from 'Gorah' an indigenous rice line in South Bengal, India. Haque et al. (2013) added 1.0 mg/l Kin and 1.0 mg/l NAA along with four concentrations of BA (1.0, 2.0, 3.0 and 4.0 mg/l) that supplemented with MS medium for plant regeneration

from BRRI dhan53; and recorded highest (75%) regeneration with 3.0 mg/l BA. Mannan et al. (2013) measured 83.33 and 91.67% regeneration in 0.5 mg/l BAP + 0.1 mg/l IBA for the rice varieties Kalijira and Chinigura respectively. By using the hormonal concentration and combination of MS + 2.0 mg/l BAP + 0.5 mg/l NAA + 1.0 mg/l Kin maximum 31.75, 38.10 and 30.16% regeneration was recorded for BR10, BRRI dhan32 and BRRI dhan47 respectively (Siddique et al. 2014). Alam et al. (2012) examined four rice varieties and mentioned that among four varieties the highest regeneration was found for BRRI dhan29 (84.33%) which required only 14.80 days for green bud formation and the lowest in BINAdhan-7 (39.67%) required maximum 15.47 days for green bud formation. BRRI dhan29 produced highest number of shoots (4.67) per callus while BINAdhan-7 showed minimum number of shoots (2.87) per callus. They noticed that BRRI dhan47 was the best performer considering efficiency to induce calli in MS supplemented with 2 mg/l 2 4-D + 0.8 mg/l BAP. Moreover, BRRI dhan29 performed best to regenerate plants in MS with supplements of 6.0 mg/l Kin + 0.5 mg/l NAA. This variety also showed the best performance in producing maximum roots per shoot as well as plant establishment in MS supplemented with 0.5 mg/l IBA. The present findings couldn't be agreed with the previous reports and claimed that such dissimilarities might be happened due to different genotypes, hormonal concentration and combinations and/or other unknown factors.

Hoque et al. (2007) reported that on plant regeneration for Bangladeshi indica rice varieties viz. BR14, BRRI dhan28, BRRI dhan38, BRRI dhan39 and BRRI dhan40 and mentioned highest 44.30% regeneration for BRRI dhan38 by using MS basal medium supplemented with 1.0 mg/l Kin + 1.0 NAA mg/l. However, they expressed that embryogenic and non-embryogenic callus formation as well as plant regeneration is reported to be genetically determined as described for different cereals including rice (Khanna and Raina 1998, Hoque and Mansfield 2004, Khalequzzaman et al. 2005, Khatun et al. 2010) and in wheat (Özgen et al. 1996) and barley (Powell and Dunwell 1987, Haque and Islam 2014). Hoque et al. (2007) further stated that Bangladeshi indica rice cultivar BRRI dhan38 and BRRI dhan39 performed with lower efficiency than the Japonica rice cultivar Taipei-309. Abe and Futsuhara (1986) reported that many Japonica rice cultivars respond better than the indica varieties. It is therefore, recommended that suitable genotypes along with suitable growth regulators

should be used to increase the plant regeneration frequency in rice tissue culture. Upadhyaya et al. (2015) used 2, 4-D (2.0 mg/l) with MS to induce calli in 10 hrs light and 14 hrs of dark cycle. After proliferation of calli with somatic embryogenic features were identified and sub-cultured in the same media and concentration of growth regulator for regeneration i.e. shoot, root or plantlet formation. In addition 2.0 mg/l concentration was also found to be more suitable for plant regeneration i.e. shoot initiation or plantlet formation. This may happen due to different genotypic efficiency of cultivars for callus induction and reports showed dissimilarities with the present findings.

4.1.4.4 Root induction

After regeneration of shoots, root induction was needed to produce plantlets as well as entire plants *in vitro*. For this purpose half strength MS + 0.1 mg/l IBA was used to observe the efficiency of root induction for all of the studied varieties. Among the genotypes the range of root induction rates was 94.67-100.00%. Considering F-value (0.770) as well as analysis of variance indicated that the varieties were not significantly varied considering the capability of root induction (**Table 18**). Hence, it could be claimed that the studied genotypes were genetically able to produce sufficient root for taking nutrient supplements in their body, resulting be adapted in adverse environmental conditions. However, the varieties showed significant difference regarding the length of roots. The range of root length was recorded as 7.31 - 13.52 cm when the plantlets were 8 weeks old after placing the shoots on root induction medium. The highest length of roots was found for BRRI dhan47 and the variety BRRI dhan32 carried the lowest. Furthermore significance differences were found among the varieties considering the feature of the number of roots per plant. In this case, the values of average root number per plant were ranged as 2.36 - 6.41 where BRRI dhan37 carried the minimum and the maximum was for BRRI dhan47.

Evangelista (2009) reported 26.10% well developed root in half strength MS medium from LX278 an elite line of japonica \times indica cross. Alam et al. (2012) reported the highest number of roots for BRRI dhan29 (3.66) and lowest for BINAdhan7 (3.11). By using half strength of MS medium three weeks old *in vitro* regenerated plants produced vigorous roots for Bangladeshi indica rice BRRI dhan14, BRRI dhan28, BRRI dhan29, BRRI dhan38 BRRI dhan39 BRRI dhan40 and Japonica rice Taipei-309 (Hoque et al. 2007).

4.1.5 Conclusion

Our findings provide a simple protocol on *in vitro* production of high frequency callus formation and its subsequent regeneration of Bangladeshi indica rice. For callus induction fifteen types of concentration and combinations of growth regulators which influenced the explants to produce callus were tested for all the seventeen rice varieties. Among them T₅ (2.5 mg/l 2,4-D) and T₁₁ (2.0 gm/l 2,4-D + 0.5 mg/l Kin.) were remarkably effective for most of the cases. Considering the capability of callusing the variety BRRI dhan29 (78.67%) showed supremacy to others in T₅. Furthermore, for plant regeneration fifteen different hormonal combinations were tested and the same variety BRRI dhan29 (70.67%) considered as the supreme member regarding its remarkable ability to regenerate plant *in vitro*. On the other hand, the variety BRRI dhan32 ranked as 2nd position by callusing, while its poor ability to plant regeneration considered as less important. Therefore, further experiments could be conducted to increase the regeneration ability of BRRI dhan32 resulting the new protocol would be helpful to improve varietal level through advance Biotechnological research in Bangladesh.

4.2 Enhancement of plant regeneration efficiency by various abiotic stress pre-treatments factors

4.2.1 Introduction

Improvement of rice is needed to respect on productivity, adaptive capability in adverse habitat related to abiotic stress, especially in drought and saline condition. Abiotic stress is defined as the negative impact of non-living factors on the living organisms in a specific environment (Brussaard et al. 2007). *In vitro* selection for abiotic stress tolerant rice is one of the most common and reliable way for improvement, but this requires standardized protocols (Reddy et al. 2013). Tolerant lines in salt could be screened via *in vitro* culture and such screening has been conducted in wheat a cereal crop (Karadimova and Djambova 1993).

The screening and identification of useful cultivars for callusing and its subsequent regeneration of rice is *in vitro* key steps for genetic improvement (Hoque and Mansfield 2004, Islam et al. 2005). Joyia and Khan (2013) optimized the responsive age of the cells to regeneration which was a prime character for efficient plant regeneration. Verma et al. (2011) developed highly reproducible reproduction system in rice. Many factors e.g. genotype, developmental stage and hormonal composition affect *in vitro* regeneration (Kyozyuka et al. 1988, Jain 1997, Islam and Tuteja 2012a). Successful plant regeneration has been reported by several species rice (Vasil 1983, Croughan and Chu 1991, Khatun et al. 2010), maize (Morshed et al. 2014), wheat (Islam 2010a).

Many abiotic stresses are played negative role in rice and other crop production (Krasensky and Jonak 2012, Trivedi et al. 2013). Salt stress negatively affects germination, root development and biomass production, resulting in the significant yield loss (Kawasaki et al. 2001) and may induce alterations in biochemical pathways and physiological responses (Walia et al. 2005). It can lead to changes in development, growth, and productivity. Certain crops reduced their production capabilities in high saline conditions (Rani et al. 2012). Rice is a salt sensitive monocot (Singh and Sengar 2014), and reduced 50% yield as a result of reduction in growth and development (Nozulaidi et al. 2015). More than one million hectares coastal areas are affected in Bangladesh. The conductivities of soil vary from 4-20

dS/m and rice is sensitive above a soil conductivity of 3 dS/m. Brussaard (2007) reported that plant's first line of defense against abiotic stress is in its roots. In tolerant plants, there are many defense mechanisms such as osmoregulation, ion homeostasis, antioxidant and hormonal systems, helping plants to stay alive and development prior to their reproductive stages (Hasegawa et al. 2000). Drought and salinity adversely affect the overall metabolic activities and cause plant demise (Roychoudury et al. 2008, Yadav et al. 2012). Biochemical and physiological changes have been broadly investigated in rice, growing under salt or water-deficit conditions (Ashraf and Harris 2004, Castillo et al. 2007, Ashraf 2010).

Salt tolerance is a complex trait controlled by many quantitative trait loci. It is very difficult to define the genes whose products comprise a salinity-resistance signaling pathway (Kawasaki et al. 2001). To minimize the deleterious effects of salt stress, plants need to coordinate the activation and effects of several molecular mechanisms (Zhang et al. 2010). Haque et al. (2013) reported on reduction of callus induction and regeneration ability increasing mannitol stress levels. They also stated that BRRI dhan29 was relatively tolerant to drought stress as compared to BRRI dhan28. Their study serves as a base line for *in vitro* screening of drought tolerant transgenic rice.

Partial desiccation, an abiotic stress factor has been used to enhance regeneration efficiency in rice (Rance et al. 1994, Saharan et al. 2004, Haq et al. 2009). They reported that desiccation factor is beneficial for regeneration of indica rice varieties. In Bangladeshi indica rice varieties viz. BR10, BRRI dhan32 and BRRI dhan47, beneficial effect of partial air desiccation has been reported by Siddique et al. (2014). They also investigated that partially desiccated calli were gained ability to regenerate plants in salt induced stress condition than undesiccated calli. Desiccation treatment reduced hyperhydricity of the calli (Ibrahim et al. 2012). Several authors suggested that partial desiccation can be useful in stimulating regeneration response. Tsukahara and Hirosawa (1992) reported that dehydration for 24 hrs of cell suspension derived calli of japonica rice increased regeneration up to 47%. Three folds higher regeneration was reported by 24 hrs partial desiccation in indica rice (Jain et al. 1996). Chand and Sahrawat (2001) found increased regeneration of rice cv. Safari17 and Kasturi applying desiccation pretreatment.

The optimal desiccation periods were 72 and 48 hrs for Malaysian rice cultivars of MR232 and MR220 respectively, where plant regeneration enhanced up to 2-5 folds (Makerly et al. 2012). Desiccation also has been reported to promote somatic embryos differentiation and development to other crops like as soybean (Hammatt and Davey 1987, Buchheim et al. 1989), wheat (Stipešević and Kladićko 2005, Fazeli-Nasab et al. 2012, Scotti-Campos et al. 2014), spruce (Bomal and Tremblay 1999), date palm (Ibrahim et al. 2012), cassava (Mathews et al. 1993) sugarcane (Kaur and Gosal 2009), *Citrus* (Singh 2014) and maize (Deng et al. 2009).

Three and four hours desiccated calli reduced fresh weight due to reduction of water content and stimulated callus growth, globularization and embryo formation in two date palm cultivars (Ibrahim et al. 2012). Siddique et al. (2014) has reported on relation to desiccation and regeneration in NaCl stress. They also investigated the relative growth rate (RGR) and relative water content (RWC) of calli, describing cell capability to survive in stress condition. Using Bangladeshi indica rice cultivars till there is not enough reports on successful regeneration in abiotic stress *in vitro*. Therefore, the present study has been undertaken on following objectives using three Bangladeshi indica varieties.

- ⊙ Investigation of the effect of calli age on plant regeneration.
- ⊙ Effect of salt stress to cell viability, cell growth and development, and proliferation of callus.
- ⊙ Determination of relative growth rate, relative water content in abiotic stress condition.
- ⊙ To find out the relation between callus age and partial air desiccation.
- ⊙ Evaluation of the effect of partial air desiccation for enhancing *in vitro* plant regeneration.
- ⊙ To enhance the ability of callus to regenerate plant in salt stress by desiccation pretreatment.

4.2.2 Materials and Methods

4.2.2.1 Plant Materials

The mature seeds of three Bangladeshi indica rice varieties *viz.* BR10 (Progati), BRRI dhan32 and BRRI dhan47 were collected from Bangladesh Rice Research Institute (BRRI), Regional Station, Shyampur, Rajshahi, Bangladesh; and used as plant material for the experiments.

4.2.2.2 Methods

4.2.2.2.1 Sterilization of seeds and inoculation

Mature seeds of selected rice varieties were sterilized as describe in Chapter III (Page 36). Then the sterilized seeds were inoculated on callus induction medium (CIM) in petri dishes. Two basal media, MS and N6, and four types of hormonal combinations (H_1 , H_2 , H_3 and H_4) were used for experimental purpose as shown in **Fig. 9**. Petri dishes were sealed with parafilm and incubated at $25 \pm 1^\circ\text{C}$ in dark for callus induction. Ten (10) days old calli were sub-cultured using the same medium and after three weeks callus induction (CI) frequencies were recorded. The pH of all media adjusted 5.8.

4.2.2.2.2 Application of NaCl in addition to medium

Four to five weeks old embryogenic calli were pretreated and subjected to abiotic stress of NaCl. Four different concentrations of NaCl (50, 100, 150, 200 mM) were added to medium (MS + H_2). Data were recorded by one week interval and up to four weeks of culture initiation in salt stress conditions. Through visual observation, the viable calli were counted and percentage value of viability was determined as $(VC_n / IC_n) \times 100$. Calli ages of four weeks and approximately uniform size (100 mg) were weighed individually and placed to MS medium that supplemented with different hormones and NaCl. For each treatment calli were weighed individually which was known as initial fresh weight (FWi) and cultured into vessel singly. After four weeks, calli were rinsed with sterile distilled water 4-5 times. Excess water of calli was

sacked by blotting paper and fresh weight (FWf) was recorded. Relative growth rate (RGR) and tolerance index (TI) of callus was determined on fresh weight (FW) using the standard formula $(FWf - FWi) / FWi$ followed by Smith and McComb (1981). To compare variety-related responses to stress conditions, tolerance index (TI), based on RGR was computed according to formula $TI = RGR_{\text{treatment}} / RGR_{\text{control}}$ as follows by Soheilikhah et al. (2013).

4.2.2.2.3 Application of partial air desiccation to calli

To observe the effect of partial desiccation on plant regeneration, four (04) age groups of calli derived from mature seeds (3, 4, 5 and 6 w) and five (05) desiccation periods (15, 30, 45, 60 and 75 hrs) were applied to the suitable calli. For air desiccation, calli were transferred to empty petri dishes containing sterile whatman-1 filter papers followed by the standard protocol of Saharan et al. (2004). The petri dishes were sealed with parafilm and kept at $27 \pm 1^\circ\text{C}$ in dark for different desiccation period. After the pretreatment duration the calli were transferred to regeneration medium MS + 2.0 mg/l BAP + 0.5 mg/l NAA + 1.0 mg/l Kin.

4.2.2.2.4 Determination of relative water content in calli

Targeted calli were incubated at 60°C for 48hrs and after drying, dry mass of calli was weighted. The relative water content (RWC) of callus was calculated using the formula $(FW - DW) / DW$; where FW = fresh weight, DW = dry weight and percentage value was determined following the method of Al-Khayri and Al-Bahrany (2004).

4.2.2.2.5 Data recording and statistical analysis

The average or mean values were computed from three replications with standard error (SE). The experiments were laid out as completely randomized design (CRD). Analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were done by software SPSS17.0 version. To test the homogeneity of means accordance on DMRT, percentage values of replications were used. The seed derived calli without any pretreatment were considered as control.

4.2.3 Results

4.2.3.1 Effect of media on callus induction (CI)

Two basal media (MS and N6) consisting of four hormonal combinations (H_1 - H_4) were used for their effectiveness on callus induction. The results indicated that all of the responding genotypes showed well embryogenic response to induce callus. Out of three genotypes, BRRI dhan32 (64.44%) and BRRI dhan47 (52.78%) performed highest number of CI in MS + H_2 . The maximum callus induction (53.26%) was recorded for BR10 in MS + H_1 (Fig. 8). On the other hand, BR10, BRRI dhan47 and BRRI dhan32 gave minimum callusing 47.13, 47.71 and 55.34% in N6+ H_4 , respectively. It was observed that all the responding genotypes showed better performance on callusing in MS medium than N6. Analysis of variance showed the significant differences within the studied genotypes and the media examined at the significance level of $p \leq 0.01$ (Table 22).

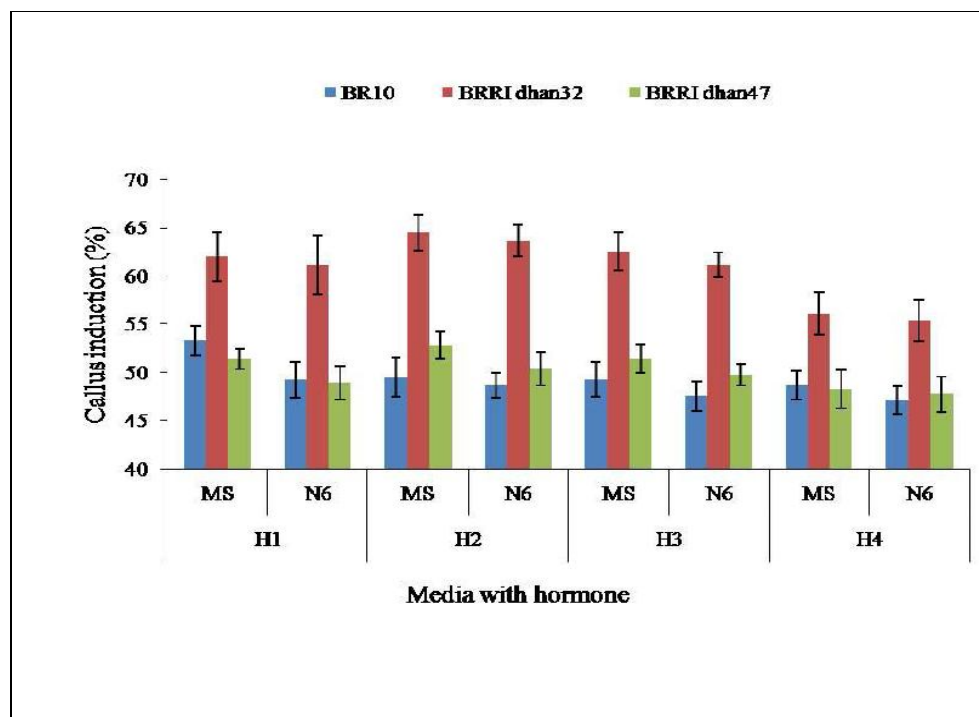


Fig. 8: Effect of media and hormonal combinations on callus induction in three rice genotypes. H_1 = 0.5 mg/l 2, 4-D + 1.0 mg/l kin, H_2 = 2.5 mg/l 2, 4-D + 1.0 mg/l kin + 300 mg/l L-proline + 400 mg/l CH, H_3 = 0.5 mg/l NAA + 2.0 mg/l kin + 400 mg/l L-proline + mg/l CH, H_4 = 2.5 mg/l IAA + 0.5 mg/l kin + 300 mg/l L-proline + 400 mg/l CH.

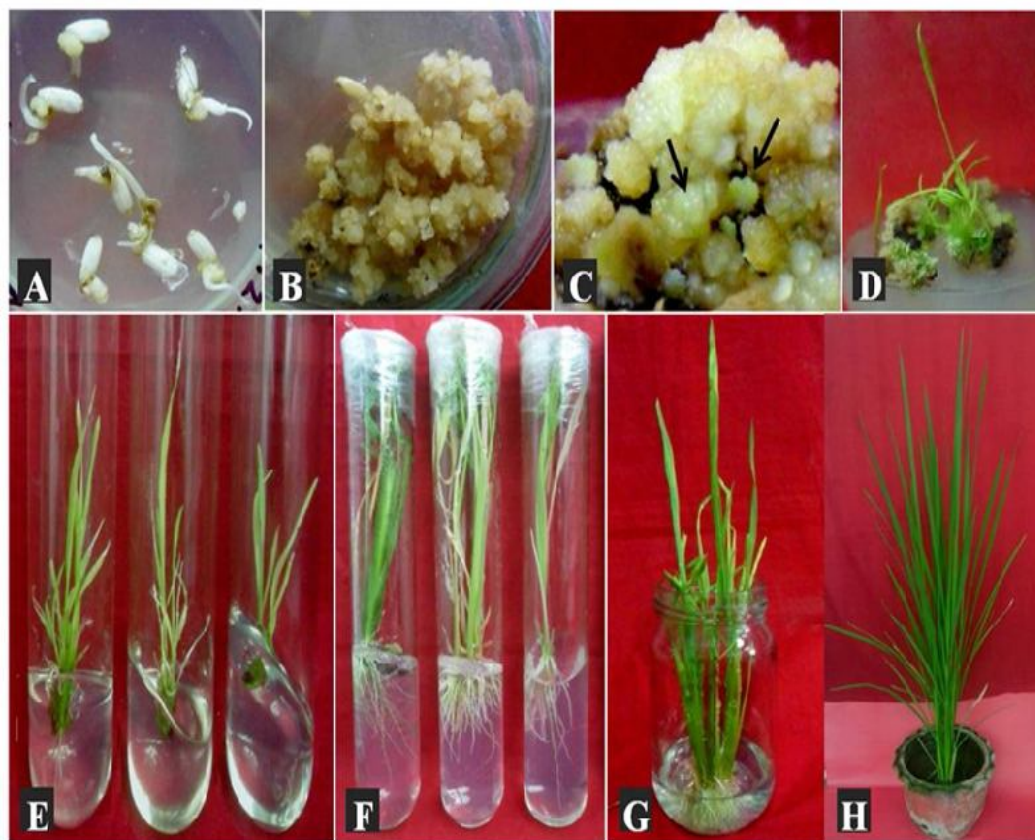


Fig. 9 (A-H): Callus induction, somatic embryogenesis (direct) and plant regeneration in rice. A) Calli derived from mature seeds after one week of culture, B) Proliferation of embryogenic calli (4-5 weeks old), C) Development of somatic embryos on mature embryo induced calli (indicated by arrows), D) Formation of shoots from germinated somatic embryos, E) Elongated shoots, F: Rooted shoots (regenerated plantlets), G) Acclimatized plantlets, H) Hardening of regenerated plantlets in pot culture.

Table 19: Effect of salt on viability of seed derived callus exposed in different concentrations of NaCl and grown one to four weeks for three genotypes

Variety	NaCl (mM)	Viable calli (% \pm SE)			
		1 w	2 w	3 w	4 w
BR10	Cont.	93.33 \pm 1.33ab	88.00 \pm 2.31a	86.67 \pm 3.53a	85.33 \pm 2.67a
	50	78.67 \pm 3.53d	77.33 \pm 2.67cd	74.67 \pm 1.33bc	73.33 \pm 1.33b
	100	41.33 \pm 1.33h	36.00 \pm 2.31g	33.33 \pm 2.67f	33.33 \pm 2.67e
	150	32.00 \pm 2.31i	32.00 \pm 1.33g	26.67 \pm 1.33f	24.00 \pm 2.31f
	200	14.67 \pm 2.67j	12.00 \pm 2.31i	12.00 \pm 2.31gh	10.67 \pm 2.67gh
BRRI dhan32	Cont.	86.67 \pm 2.67bc	84.00 \pm 2.31ab	81.33 \pm 2.67ab	81.33 \pm 2.67a
	50	62.67 \pm 3.53f	52.00 \pm 2.31f	45.33 \pm 2.67e	33.33 \pm 1.33e
	100	25.33 \pm 1.33i	22.67 \pm 1.33h	18.67 \pm 1.33g	16.00 \pm 2.31g
	150	13.33 \pm 1.33j	8.00 \pm 2.31i	8.00 \pm 2.31h	5.33 \pm 1.33hi
	200	2.67 \pm 1.33k	0.00 \pm 0.00j	0.00 \pm 0.00i	0.00 \pm 0.00i
BRRI dhan47	Cont.	94.67 \pm 1.33a	89.33 \pm 1.33a	86.67 \pm 3.53a	85.33 \pm 1.33a
	50	81.33 \pm 3.53cd	80.00 \pm 2.31bc	77.33 \pm 1.33bc	74.67 \pm 1.33b
	100	74.67 \pm 1.33de	73.33 \pm 1.33d	72.00 \pm 2.31c	72.00 \pm 2.31b
	150	70.67 \pm 3.53e	64.00 \pm 2.31e	64.00 \pm 2.31d	58.67 \pm 1.33c
	200	53.33 \pm 1.33g	49.33 \pm 1.33f	46.67 \pm 2.67e	45.33 \pm 3.53d

Culture medium was MS + 2.5 mg/l 2, 4-D + 1.0 mg/l kin + 300 mg/l L-proline + 400 mg/l CH + NaCl. For each NaCl concentration, number of used callus was 75 in 3 replications and in a column the mean values followed by same letter (s) are not significantly different at $p \leq 0.05$ according to DMRT.

4.2.3.2 Response to *in vitro* abiotic stresses

4.2.3.2.1 Viability of callus to NaCl induced stress

To observe the viability of calli four concentrations of NaCl were used and data shown in **Table 19**. The varieties BRRI dhan47, BR10 and BRRI dhan32 gave 53.33, 14.67 and 2.67% viable calli after one week cultured in the highest concentration of NaCl (200 mM) respectively. In the same salt level, the viability was decreased remarkably after four weeks of culture in BRRI dhan47 (45.33%), BR10 (10.67%) and BRRI dhan32 (0.00%). In stress condition, the survival rate of callus was significantly differed on NaCl concentrations, culture periods and rice genotypes.

4.2.3.2.2 Relative growth rate, tolerance index and relative water content in NaCl stress

Three weeks old calli were used for this experiment and grown in four concentrations of NaCl (50, 100, 150 and 200 mM) up to four weeks. Relative growth rate (RGR), tolerance index (TI) and relative water content (RWC) were determined. It was observed that in all cases, significant differences were found among the genotypes. Furthermore, significant differences were also observed in absence of NaCl in the medium (control) on RGR, TI and RWC. However, 1.03, 0.23 and 0.11 RGR values were recorded at 200 mM salt stress in BRRI dhan47, BR10 and BRRI dhan32 respectively (**Fig. 10**). On comparison to the controls, RGR values were decreased at 79.88% in BRRI dhan47, 94.26% in BR10 and 97.59% in BRRI dhan32. Since, BRRI dhan47 grew with highest capability in the top most level of NaCl stress (200 mM). The same genotype carried the highest TI (0.20) which expressed the high capability to grow in abiotic stress condition developed by NaCl (**Fig. 11**). Comparatively lower TI numbers were recorded in other two genotypes BR10 (0.02) and BRRI dhan32 (0.06). The WRC values were also lower for BR10 (7.22%) and BRRI dhan32 (7.03%) than BRRI dhan47 (10.23%) (**Fig. 12**).

On spite of showing the highest RGR and TI values, BRRI dhan47 was taken to conduct related another extended experiment. In this case, to observe the changing pattern of RGR, its values were determined in contrast of stress levels and calli exposure periods (every week up to four). The results showed that RGR was increased till two weeks of calli exposure periods, at all NaCl levels tested. After two weeks, RGR was restricted in 100, 150 and 200 mM stress levels, while in 50 mM lower rate of increment in RGR value was found (**Fig. 13**).

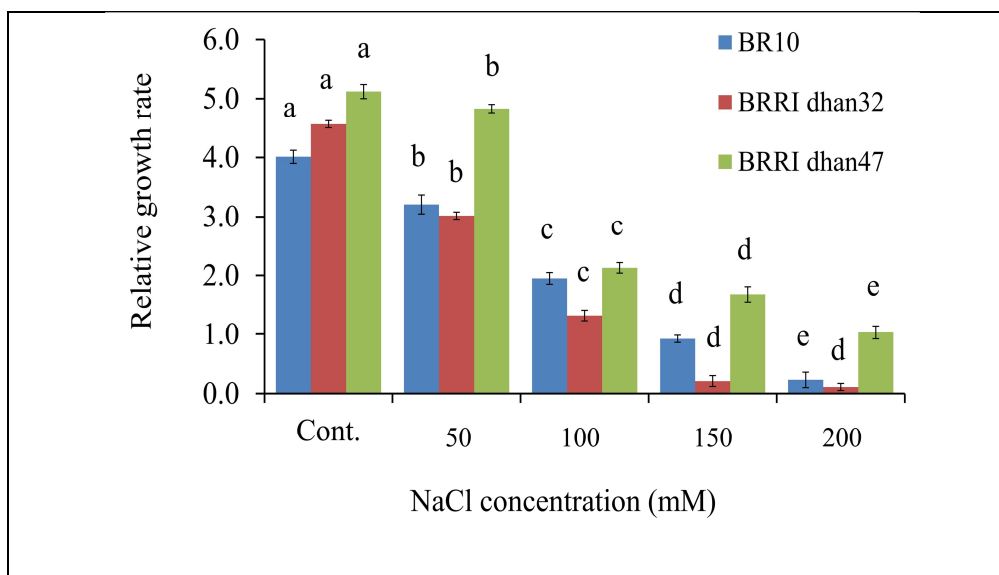


Fig. 10. Effect of different concentrations (50, 100, 150, 200 mM) of NaCl subjected to relative growth rate of three rice genotypes.

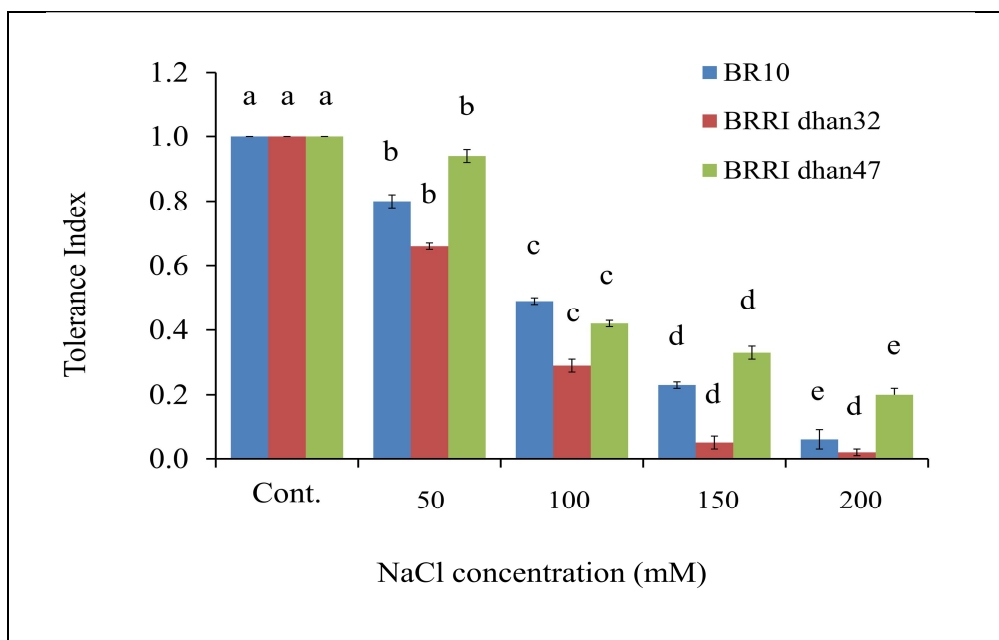


Fig. 11: Effect of different concentrations (50, 100, 150, 200 mM) of NaCl subjected to tolerance index of three rice genotypes.

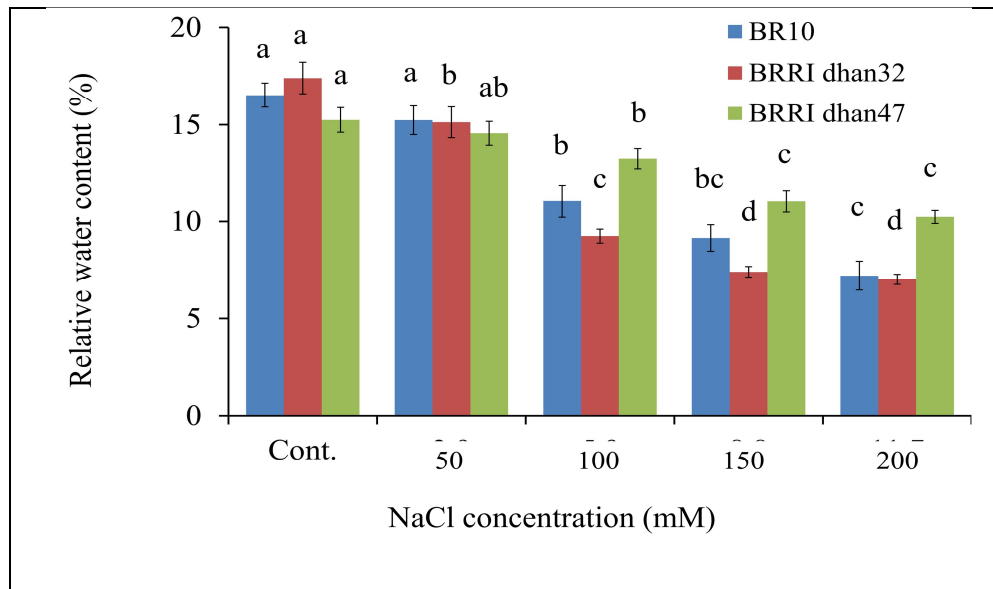


Fig. 12: Effect of different concentrations (50, 100, 150, 200 mM) of NaCl subjected to relative water content of three rice genotypes.

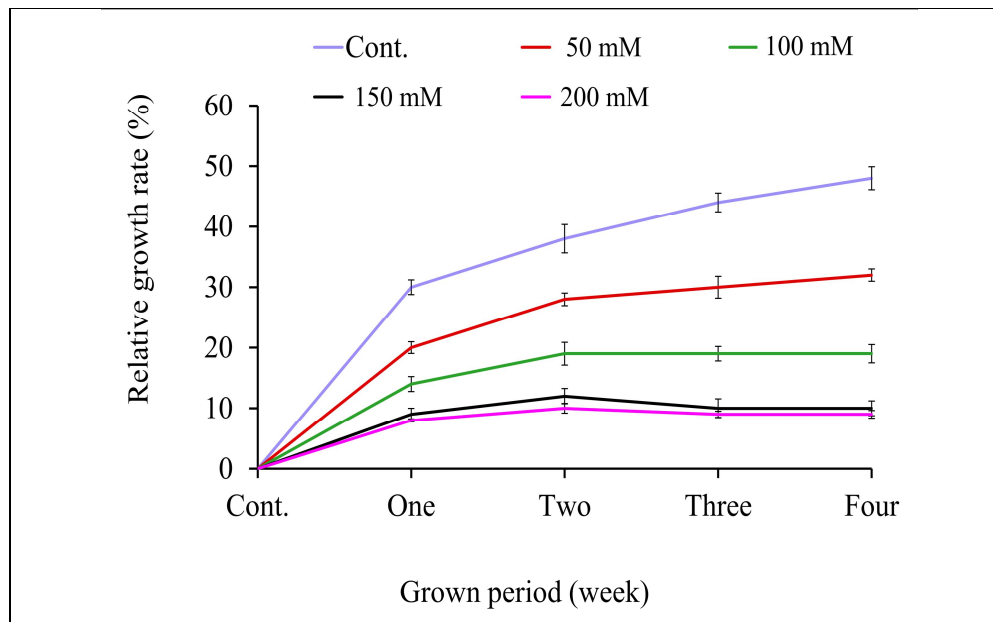


Fig. 13: Effect of different concentrations (50, 100, 150, 200 mM) of NaCl subjected to relative growth rate of BRRI dhan47 in different NaCl concentrations in contrast to calli exposure periods.

Table 20: Effect of partial air desiccation period and four age groups of calli on plant regeneration for three rice genotypes (% \pm SE)

Age of calli (weeks)	Desiccation (hrs)	Genotypes		
		BR10	BRRI dhan32	BRRI dhan47
3	0 (Cont.)	38.10 \pm 2.75de	31.75 \pm 3.17jk	33.33 \pm 2.75def
	15	36.51 \pm 1.59e	39.68 \pm 1.59ghij	36.51 \pm 3.17cde
	30	47.62 \pm 2.75c	55.56 \pm 1.59de	38.10 \pm 2.75cde
	45	50.79 \pm 3.17c	68.25 \pm 3.17b	42.86 \pm 2.75bc
	60	34.92 \pm 1.59e	41.27 \pm 1.59fghi	26.98 \pm 1.59fg
4	0 (Cont.)	31.75 \pm 1.59e	38.10 \pm 2.75hijk	30.16 \pm 1.59efg
	15	53.97 \pm 3.17c	47.62 \pm 2.75efg	34.92 \pm 3.17cdef
	30	68.25 \pm 3.17ab	65.08 \pm 4.20bc	42.86 \pm 2.75bc
	45	73.02 \pm 4.20a	76.19 \pm 2.75a	58.73 \pm 3.17a
	60	46.03 \pm 3.17cd	58.73 \pm 1.59cd	47.62 \pm 2.75bc
5	0 (Cont.)	30.16 \pm 1.59e	34.92 \pm 3.17ijk	30.16 \pm 1.59efg
	15	47.62 \pm 2.75c	47.62 \pm 2.75efg	47.62 \pm 2.75bc
	30	63.49 \pm 4.20b	55.56 \pm 1.59de	39.68 \pm 1.59bcd
	45	49.21 \pm 4.20c	49.21 \pm 3.17ef	34.92 \pm 3.17cdef
	60	34.92 \pm 1.59e	39.68 \pm 1.59ghij	31.75 \pm 1.59defg
6	0 (Cont.)	31.75 \pm 3.17e	30.16 \pm 1.59k	26.98 \pm 1.59fg
	15	38.10 \pm 2.75de	44.44 \pm 1.59fgh	34.92 \pm 3.17cdef
	30	36.51 \pm 1.59e	39.68 \pm 3.17ghij	33.33 \pm 2.75def
	45	34.92 \pm 1.59e	38.10 \pm 2.75hijk	26.98 \pm 1.59fg
	60	33.33 \pm 2.75e	31.75 \pm 3.17jk	23.81 \pm 2.75g

Used regeneration medium, MS + 2.0 mg/l BAP + 0.5 mg/l NAA + 1.0 mg/l Kin was constant for all the age of calli and partial air desiccation pretreatments. For each desiccation pretreatment number of callus was 63 in 3 replications and in a column the mean values followed by same letter (s) are not significantly different at $p \leq 0.05$ according to DMRT.

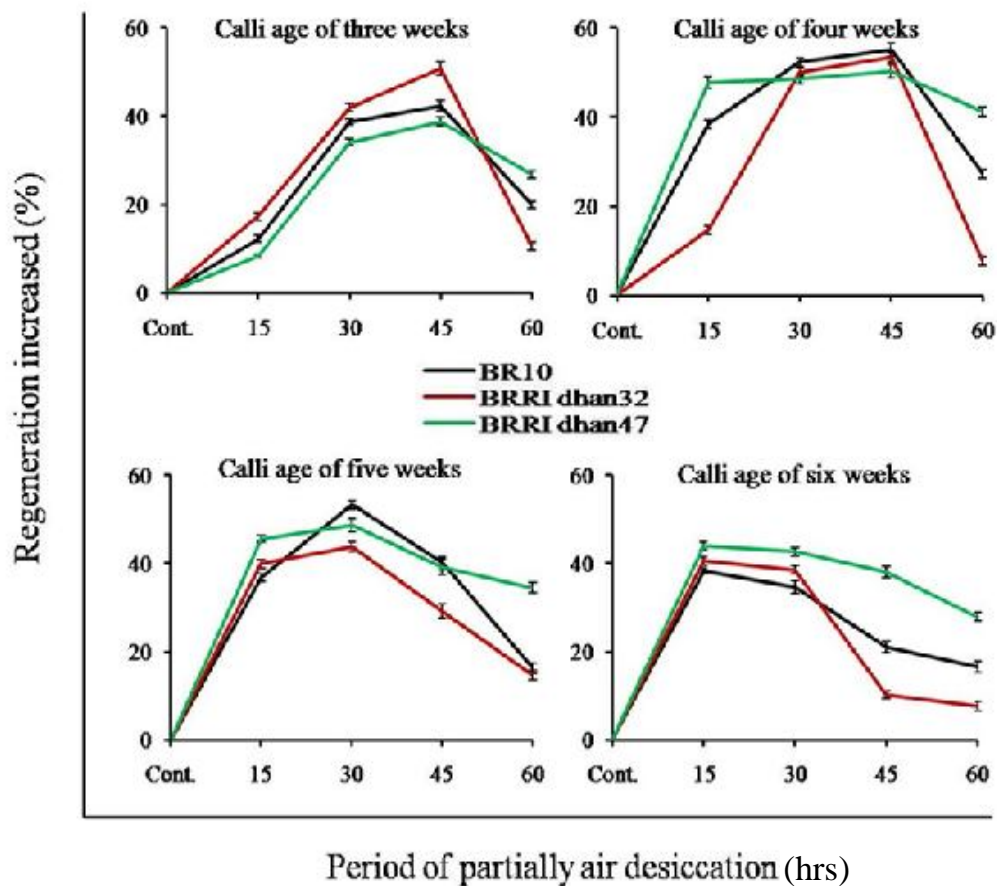


Fig. 14: Effect of different period of partial air desiccation in relation to age of calli to enhance plant regeneration. Each curve indicates the percentage value of increased regeneration compare to the control (vertical bar expressed the SE of four replicates).

4.2.3.2.3 Effect of partial air desiccation to regeneration

Calli of different age groups (3, 4, 5 and 6 w) were partially desiccated (15, 30, 45 and 60 hrs) and cultured on regeneration medium (RM). The significant differences were found within the genotypes, age groups and air desiccation period on regeneration (**Table 20, Fig. 14**). The results showed that 4 w old calli of BRRI dhan32 performed highest regeneration (76.19%) among the genotypes when it was desiccated by 45 hrs. The gained regeneration value was 2 fold higher than the control (38.10%). In the same desiccation pretreatment (45 hrs), other two genotypes BR10 and BRRI dhan47 gave 73.02% and 58.73% regeneration respectively; and the results were more than 2 fold higher than the undesiccated calli (**Fig. 14**). In contrast to the controls, desiccated calli showed around 2-3 folds higher regeneration. Considering our recorded data, the calli of lower age, needed to higher periods of desiccation pretreatment within a range than comparatively aged callus to perform their maximum regeneration. However, analysis of variance (ANOVA) showed that the effect of partial air desiccation, age of calli and rice genotype on plant regeneration differed significantly at $p \leq 0.01$ significance level (**Table 22**).

4.2.3.2.4 Regeneration response by partially desiccated calli in salt stress

Desiccated callus performed with increased regeneration in NaCl induced stress condition. The callus age of four weeks were pretreated at 45 hrs air desiccation and transferred to regeneration medium (RM) supplemented with NaCl levels (50, 100, 150, 200 mM). The variety BRRI dhan47 gave the highest regeneration (26.98%) at 200 mM after desiccation pretreatment (**Table 21**). The result was 1.89 fold higher than the control (14.29%). In the same stress level, other two varieties BR10 and BRRI dhan32 could not be regenerated plants from undedicated calli; whereas, after desiccation pretreatment they were been capable for regeneration at 11.11% and 4.76%, respectively.

Table 21: Effect of desiccation to NaCl stress on regeneration of three rice genotypes (% \pm SE)

Variety	Desiccation (hrs)	NaCl (mM)				
		0 (Cont.)	50	100	150	200
BR10	0 (Cont.)	36.51 \pm 1.59	31.75 \pm 1.59	9.52 \pm 2.75	3.17 \pm 1.59	0.00 \pm 0.00
	45	71.43 \pm 4.76	68.25 \pm 4.20	33.33 \pm 2.75	14.29 \pm 2.75	11.11 \pm 3.17
BRRI dhan32	0 (Cont.)	36.51 \pm 1.59	25.40 \pm 1.59	9.52 \pm 2.75	3.17 \pm 1.59	0.00 \pm 0.00
	45	74.60 \pm 4.20	63.49 \pm 3.17	20.63 \pm 1.59	7.94 \pm 3.17	4.76 \pm 2.75
BRRI dhan47	0 (Cont.)	30.16 \pm 1.59	28.57 \pm 2.75	25.40 \pm 3.17	23.81 \pm 2.75	14.29 \pm 2.75
	45	60.32 \pm 4.20	58.73 \pm 3.17	49.21 \pm 4.20	38.10 \pm 2.75	26.98 \pm 3.17

Regeneration medium was MS + 2.0 mg/l BAP + 0.5 mg/l NAA + 1.0 mg/l Kin + different concentration of NaCl. In each case number of used callus was 63 in 3 replications.

4.2.4 Discussion

4.2.4.1 Efficiency to callus induction

In the present study high frequency of callus induction (CI) was found in three Bangladeshi indica rice varieties viz. BR10, BRRI dhan32 and BRRI dhan47. Zuraida et al. (2010) reported that the frequency CI depends on genotype; and most *indica* rice cultivars showed poor callusing potentiality. The potentiality of callus induction of the genotypes was examined using four hormonal combinations (H₁, H₂, H₃ and H₄). The results showed that all the responding genotypes induced callus at high frequency in 2,4-D than NAA and IAA. Makerly et al. (2012) recorded the highest percentage of CI (41% and 37%) for Malaysian indica rice cultivars MR232 and MR220, respectively, in MS supplemented with NAA; and also reported that the varieties responded lower in 2,4-D. Present investigation differs with their reports, and mention that in 2, 4-D studied varieties responded high to induce calli. Tiwari et al. (2012) reported that the optimum hormonal combination as 1.5 mg/l 2, 4-D + 0.1 mg/l NAA + 0.1 mg/l BAP with MS for maximum CI. They have recorded 85% and 90% CI for indica rice varieties of Pusa Basmati1 and Kalanamak respectively. In this study, it was found that BRRI dhan32 showed the highest CI capability at 64.44% and 63.61% in MS and N6 media respectively (**Fig. 8**). Out of three responding genotypes BRRI dhan37, recorded CI was 52.78% in MS + H₂ and 50.33% in N6 + H₂. The results of present the findings differed to the previous reports with lesser frequency of CI. It might be occurred due to the effect of different media including difference in concentration and combinations of PGRs along with the genetic variability of the rice genotypes.

Table 22: Analysis of variance (ANOVA) subjected to callus induction, partial air desiccation and NaCl treatments in three rice genotypes

Stress	Subject of ANOVA (Data source)	Source of variation	DF	Mean sum of square
-	Callus Induction (CI) (Fig. 8)	Genotype (G)	2	442.476**
		Media (M)	7	2.858**
		G × M	14	-
Salt induced stress	Viability of callus (VC) (Table 19)	Genotype (G)	2	7422.85**
		Salt concentration (SC)	4	8543.69**
		Callus culture period (CCP)	3	193.27**
		G × SC	8	581.68**
		G × CCP	6	4.36 ^{NS}
		SC × CCP	12	7.27* ^{NS}
		G × SC × CCP	24	8.48
	Relative growth rate (RGR) (Fig. 10)	Genotype (G)	2	1.741**
		Salt concentration (SC)	4	9.414**
		G × S	8	0.132
	Tolerance index (TI) (Fig. 11)	Genotype (G)	2	0.039**
		Salt concentration (SC)	4	0.453**
		G × S	8	0.005
	Relative water content (RWC) (Fig. 12)	Genotype (G)	2	3.327**
		Salt concentration (SC)	4	38.644**
		G × SC	8	2.119
Partial air desiccation stress	Plant regeneration (PR) (Table 20)	Genotype (G)	2	604.34**
		Age of callus (AC)	3	803.94**
		Partial air desiccation (PAD)	4	686.17**
		G × AC	6	18.45 ^{NS}
		G × PAD	8	45.93 ^{NS}
		AC × PAD	12	124.71*
		G × AC × PAD	24	35.09

** = Significant at $p \leq 0.01$, * = Significant at $p \leq 0.05$, NS = Non-significant

4.2.4.2 Viability of callus in salt stress

Soheilikhah et al. (2013) recorded 47 - 64% decreased cell viability in NaCl induced stress for Safflower (*Carthamus tinctorius* L.) varieties, and mentioned that the accumulation of Na⁺ ions and osmolytes could play an important role in osmotic adjustment in cells under saline stress. In the present investigation significant differences were found among the rice genotypes on cell viability as well as the viability of the calli in NaCl induced stress. The calli of BRRI dhan47 exhibited with the highest viability (45.33%) after four weeks cultured in 200 mM of NaCl (**Table 19**). BR10 survived with 10.67% viable calli and BRRI dhan32 could not be exhibited viability when the calli were exposed in 200 mM up to four weeks. This study has observed that the phenomenon was happened due to presence of necrotic cells in the calli. A huge number of necrotic cells turned the calli deep brown or blackish in color, together with survival disability was appeared. It was observed that lower frequency of viable calli was appeared in higher salt concentration than the lower one. On spite of lesser osmotic potentiality and genotypic effect, the varieties might disable to adapt in salt stress condition. The calli of BR10 and BRRI dhan32 were begun to necrosis within a week, and after four weeks cultured in 200 mM of NaCl level, a few number of calli were existed for BR10; while no calli were viable for BRRI dhan32. On the other hand BRRI dhan47 could be adapted to the NaCl stress and showed the highest viability in all cases of salt stresses. Based on survival feature, BRRI dhan32 and BR10 showed sensitivity to NaCl induced *in vitro* stress; whereas, BRRI dhan47 was appeared as tolerant in nature.

4.2.4.3 Growth rate, tolerance index and relative water content

Remarkable differences were found among the genotypes examined on relative growth rate (RGR), tolerance index (TI) and relative water content (RWC). In the top level of NaCl stress (200 mM) recorded RGR values were 1.03, 0.23 and 0.11; TI were 0.20, 0.06 and 0.02; and WRC were 10.23, 7.22 and 7.03% for the genotypes BRRI dhan47, BR10 and BRRI dhan32, respectively (**Fig. 10-12**). Among three genotypes BRRI dhan47 exhibited the highest potentiality to survive in NaCl induced

abiotic stress with maximum RGR (1.03), TI (0.20), and WRC (10.23%). The recorded values of the parameters RGR, TI and WRC expressed the higher survival capability against the abiotic stress conducting the physiological activities of BRRI dhan47. On the other hand stress sensitivity was found in BRRI dhan32 and BR10 considering lower value of the parameters. The RGR, TI and RWC values were decreased in higher stress level than the lower ones. The phenomena might be occurred due to reduction of water availability and lose of turgor pressure (TP) in the cells of the calli. Such physiological causes were reported in previous investigation for *Oryza sativa* (Lutts et al. 1996), *Carthamus tinctorius* (Soheilikhah et al. 2013, Zebarjadi et al. 2010), *Saccharum sp.* (Errabii et al. 2007), *Tagetes minuta* (Mohamed et al. 2000) and *Triticum durum* (Bajji et al. 2000, Lutts et al. 2004). Errabi et al. (2007) mentioned that due to interference of Na^+ and Cl^- ions on uptake and translocation processes, nutritional imbalance might be created and the growth of callus is declined. However, NaCl treated calli of BRRI dhan47 was least affected by the highest dose of salt stress and exhibited high ability in terms of both cellular viability and growth of callus. BRRI (2011) described that BRRI dhan47 can tolerate at 12-14 dS/m (equivalent to approximately 120-140 mM) of NaCl stress and it has an ability to survive and grown in saline soil. So that it is considered as salt tolerant rice genotype. Therefore, the present investigations are in agreement with the previous reports for the genotype BRRI dhan47. Calli of B.RRI dhan47 might accumulate less Na^+ ion than salt susceptible BR10 and BRRI dhan32. In several species K^+ is a major cation and contributor to adjust osmotic potential (OP) under stress condition (Santos-Diaz and Ochoia-Alejo 1994, Bajji et al. 2000). In salt stress Na^+ concentration is increased which lead to decrease concentration of K^+ among rice genotypes. As a result an imbalance of essential ions be created and cell of salt sensitive varieties could not be survived. Such reports previously mentioned in rice (Basu et al. 2002, Lutts et al. 1996), sugarcane (Patade et al. 2008, Errabii et al. 2007), *Carthamus tinctorius* (Soheilikhah et al. 2013) and *Cynara cardunculus* (Benlloch-González 2005).

4.2.4.4 Partial air desiccation

Rance et al. (1994) reported 2 - 4 folds higher regeneration from 3hrs desiccated calli than the control in rice genotypes viz. PN1, IR72 and IR64. In another report compare to undesiccated calli, 2 and 5 folds higher regeneration was recorded from 48 and 72 hrs desiccation in MR220 and MR232 respectively (Makerly et al. 2012). Approximately similar increment of regeneration was recorded in maize (Deng et al. 2009, Stipešević and Kladić 2005), sugarcane (Kaur and Gosal 2009) and rice (Alam et al. 2012, Biswas and Mandal 2007, Saharan et al. 2004). Under this study around 2 - 3 folds higher regeneration have been recorded from desiccated calli in BR10, BRRI dhan32 and BRRI dhan47 (**Table 20, Fig. 14**). Therefore, the obtained results are in agreement with previous findings. The effect of partial air desiccation to regeneration was determined in contrast of callus age. However, an effective relationship was found, and investigated that callus of lower age (within a range) need to comparatively higher period of desiccation to perform maximum regeneration. On the other hand callus of relatively higher age gave maximum regeneration when it was pretreated at lower level of desiccation. The phenomena could be depended on water content (WC) in the cells of the calli. Callus of lower age might contain a large amount of water, while they need to higher desiccation in which the calli dehydrated at the optimum level. Makerly et al. (2012) reported that the degrees of water loss differ against same desiccation period in different rice genotype, and an optimal level of water loss (partial air desiccation) could be beneficial to plant regeneration. They also noticed that regeneration varied depending on cultivars and duration of partial desiccation. In date plum cultivar, 3 and 4 hrs partial desiccation reduced fresh weight of calli and stimulated calli growth globularization as well as embryos formation (Ibrahim et al. 2012). Under this study recorded results showed that partial air desiccation strongly influenced the regeneration and played an effective role to enhance the somatic embryogenesis. To regenerate *in vitro* plants for indica rice genotype, genetic effect along with the age of explants has been reported earlier by

Hoque and Mansfield (2004). Such effect was noticed in sugarcane (Gandonou et al. 2005), coffee (Molina et al. 2000), rice (Beena 2006, Katiyar et al. 1999) and *Primula* sp. (Schween and Schwenkel 2003). However, genetic variability, optimal air desiccation and suitable age of calli might play a vital role to enhance regeneration in rice genotypes. Although at optimum level of desiccation promote the regeneration, yet over desiccation suppressed to embryo formation as well as plant regeneration. Over desiccation created the drought abiotic stress in which regeneration frequency was decreased up to 23.81% at 60 hrs desiccation to 6 w age of calli for BRRI dhan47 (**Table 20**). Kranner et al. (2002) reported that loss of more than 20 - 50% water content of the cells is been lethal to most of the higher plants. However, the present investigation suggested that optimum period of partial air desiccation pretreatment was varied on the age of calli as well as the rice genotype significantly (**Fig. 14**).

4.2.4.5 Response of desiccated calli in NaCl stress to enhance regeneration

In case of salt stress condition, desiccated calli gave higher regeneration than the controls (**Table 21**). At the top NaCl level (200 mM), the variety BRRI dhan47 performed 88.80 per cent higher regeneration than control after pretreatment of 45 hrs desiccation. The calli of other two genotypes BR10 and BRRI dhan32 were been able to regenerate at 200 mM NaCl level; while both were not shown any regeneration without desiccation (control). Makerly et al. (2012) reported that regeneration capability was varied depending on the duration of desiccation. At optimal period of partial air desiccation characterized the cells of the callus to survive and adapt at adverse physiological stress. Because of reduction of water the cells of desiccated calli might acquired higher osmotic potential (OP). So that ability was developed to uptake water in salt stress condition and could be able to survive. However, in case of BRRI dhan47 partial air desiccation pretreatment was more effective to enhance the capability of regeneration in NaCl induced abiotic stress.

4.2.5 Conclusion

Out of three Bangladeshi indica rice genotypes, BRRI dhan32 showed better callusing in MS medium that supplanted with 2.5 mg/l 2, 4-D + 1.0 mg/l Kin + 300 mg/l L-proline + 400 mg/l CH. Present investigation also suggests that hormonal combination as 2.0 mg/l BAP + 0.5 mg/l NAA + 1.0 mg/l Kin with MS was better for obtaining maximum regeneration after partially air desiccation pretreatment for BRRI dhan32. The parameters RGR, TI, RWC and VC in NaCl induced abiotic stress, expressed the tolerance features of BRRI dhan47, while salt susceptibility was found in BR10 and BRRI dhan32. At the optimal level, partial air desiccation positively influenced the regeneration in all the genotypes, especially for BRRI dhan47. Desiccation also increased the capability of the calli to survive and adapt in NaCl stress condition.

Chapter V

Organogenesis (organ culture)



5. Organogenesis (organ culture)

5.1 Introduction

In vitro plant regeneration in rice via organogenesis or somatic embryogenesis has been reported from different explants, such as root (Hoque and Mansfield 2004), leaf (Ramesh et al. 2009), coleoptile (Ghobeishavi et al. 2014), mature embryo (Pons et al. 2000) and protoplast (Jelodar et al. 2002). Several factors including plant growth regulators, explants, culture conditions and plant genotypes affect rice somatic embryogenesis and subsequent plant regeneration (Deo et al. 2010). These factors have different interact with explants and plant genotype.

At first somatic embryo was developed successfully through callus induction in carrot (Steward et al. 1958). Verdeil et al. (2007) mentioned that little is known of the mechanisms that induce the de-differentiation of a somatic cell into a totipotent embryogenic cell that can either be regenerated or develop into an embryo and subsequently an entire plant. Moreover, the molecular mechanisms underlying plant cell totipotency are largely unknown (Chupeau et al. 2013). Guzzo et al. (1994) identified the origin of cells capable to generate somatic embryos using hypocotyl explants of carrot seedlings; and reported that various tissues responded in different ways. The totipotency of different plant parts or organs is not identical (similar) in which regeneration capability is differed in various explants.

However, for *in vitro* culture of rice mature embryos (Alam 1994, Bano et al. 2005, Islam et al. 2005, Zuraida et al. 2011) and immature embryos (Noouri-Delawar and Arzani 2001) are usually used by many scientists. Using various explants of rice i.e. inflorescence (Chen et al. 1985) and root (Abe and Futsuhara 1985) reports on *in vitro* plant regeneration have been described. Rashid et al. (2000) studied that rice seeds have more potential for callogenesis as compared to node or tip. Indirect somatic embryogenesis was done from shoot and stem in *Crassula ovata* Mill (Ahmed et al.

2014). The leaf and/or stem are also commonly used in many dicot plants such as, sour cherry (Song and Sink 2005), sweet orange (Khan et al. 2009), lime tree (Al-Bahrany 2002b), tobacco (Yanjie 2004, Ali G et al. 2007), chili (Ashrafuzzaman et al. 2009), potato (Kumlay and Ercisli 2015) etc. and various parts of pummelo cv. Cikoneng seedling were used by Rahayu et al. (2012). Deshpande et al. (2014) regenerate plants using leaf, stem and shoot apex as explants for medicinal plant *Plumbago auriculata*. In general, there is a few reports in plant regeneration through coleoptile explants in cereal, while plant regeneration from coleoptile tissue of *Poa pratensis* L. (Ke and Lee 1996), durum wheat (Benkirane et al. 2000) and rice (Akter and AL-Forkan 2010) have been reported. Besides, enough reports is not available on the totipotency of coleoptile in *indica* rice (Oinam and Kothari 1995). The coleoptile explants can be obtained easily at any time in *in vitro* culture condition through germinating the seeds.

However, the radicle, adventitious root, coleoptile, epicotyle, mesocotyl, hypocotyl, etc are not seen to be used commonly, especially for rice; while, some authors used those of the explants individually and established callus induction and its subsequent regeneration in various plants such as epicotyl in sweet orange (Filho 2001), pummelo cv. Cikoneng (Dewi et al. 2013) and hypocotyl in blackgram (Saini and Jaiwal 2002). In rice some authors also have used the organs singly, and regenerate plant e.g. coleoptile (Sahrawat and Chand 2001) and leaf (Afrasiab and Jafar 2011).

So far as it is known that no enough reports are found on organogenesis of rice using all the possible parts of seedling and the comparison of efficiency to regeneration from various explants. Hence, present study was worked out to investigate the capability of de-differentiation and re-differentiation as well as the totipotency of various explants of popular rice cultivar in Bangladesh.

5.2 Materials and Methods

5.2.1 Plant Materials

For this study mature seeds of Bangladeshi indica rice (*Oryza sativa* L.) variety BRRI dhan29 were used as plant materials which obtained from Bangladesh Rice Research Institute (BRRI), Regional Station, Rajshahi, Bangladesh.

5.2.2 Methods

5.2.2.1 Seed sterilization, germination and preparation of explants

Matured seeds of BRRI dhan29 were dehusked and sterilized surface with 70% (v/v) ethanol for 1 min and in sodium hypochlorite (NaOCl) for five minutes. Then the seeds were sterilized with 0.1% (v/v) mercuric chloride (HgCl₂) for 5 minutes. Sterilized seeds were divided into two groups. The first group was directly used as explants and the second group was inoculated on MS + 0 that produced seedlings. For organogenesis, the vegetative organs of *in vitro* grown seedlings age of 10 d were used as the source of explants i.e. radicle, adventitious root (AR), adventitious root tip (ART), coleoptile, mesocotyl nodal segment (MNOS), mesocotyl internodal segment (MINS), primary leaf (PL) and secondary leaf (SL) as shown in **Fig. 15**. In this case by using sterilized forceps and scissor, the specific organs of seedling were separately excised and cut into pieces of 1 cm in length approximately which used as explants for callus induction.

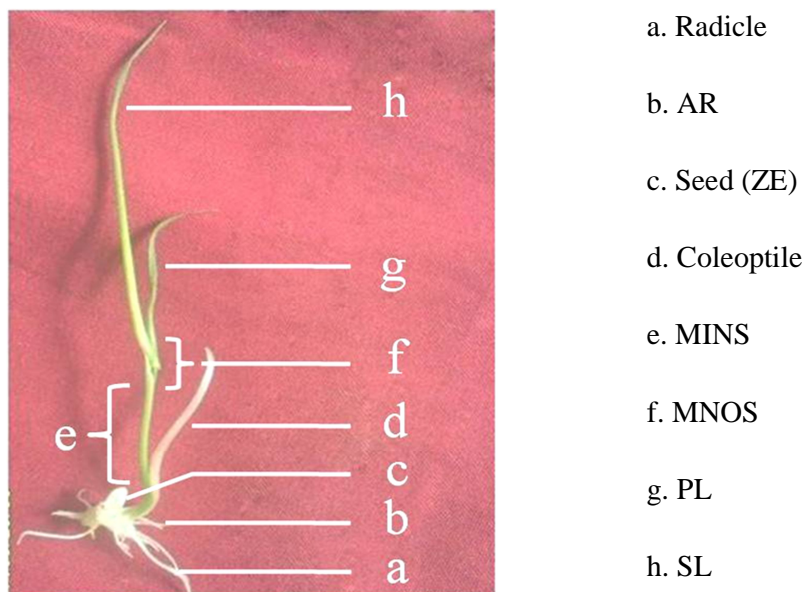


Fig. 15: *In vitro* germinated seedling age of 10 d of BRRI dhan29 in MS + 0 medium showing different explants

5.2.2.2 Callus induction from different explants

Sterilized seeds were inoculated on CIM (MS + 2.5 mg/l 2, 4-D + 1.0 mg/l kin + 300 mg/l L-proline + 400 mg/l) to induce calli derived from zygotic embryos (ZE). On to the same medium (CIM) eight types of explants which came from vegetative organs i.e. radicle, adventitious root (AR), adventitious root tip (ART), coleoptile, mesocotyl nodal segment (MNOS), mesocotyl internodal segment (MINS), primary leaf (PL) and secondary leaf (SL) were inoculated for induction of organ derive calli. Inoculated petri dishes were sealed with parafilm and incubated at $25 \pm 1^\circ\text{C}$ in dark for callus induction. The calli age of seven days were considered as primary callus. The primary calli were sub-cultured on CIM for further proliferation. The proliferated calli age of 3 weeks (w) was considered as secondary callus, and its recorded values were generally considered as the frequency of callus induction (CI).

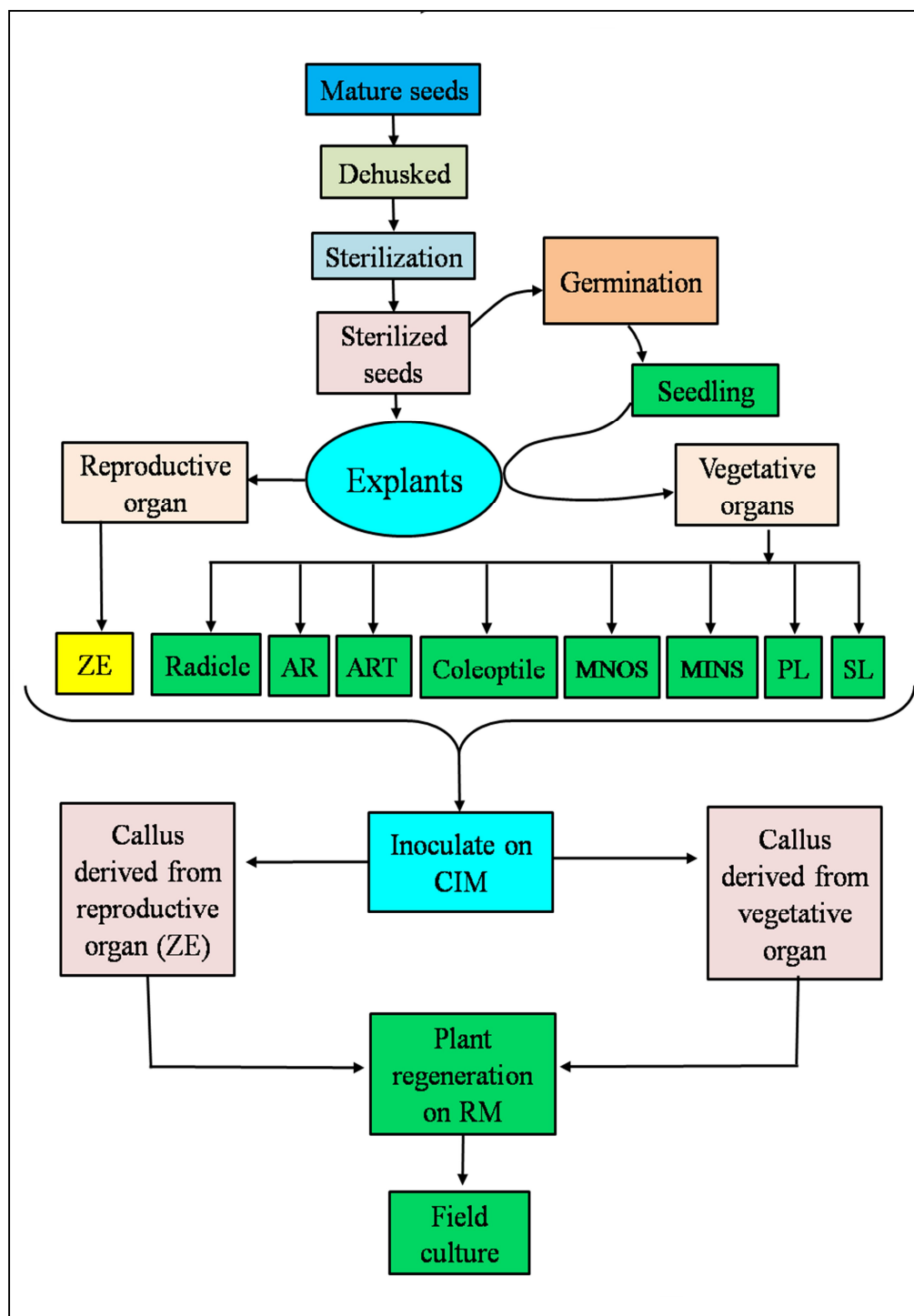


Fig. 16: A schematic representation, showing the comparison on callus induction and subsequent plant regeneration from different explants.

5.2.2.3 Plant regeneration

For plant regeneration, the calli age of four groups (3, 4, 5 and 6 w) were transferred to regeneration medium MS + 2.0 mg/l BAP + 0.5 mg/l NAA + 1.0 mg/l Kin. Regenerated shoots of 3-5 cm were placed on half strength MS + 0.5 mg/l IBA for rooting and then the rooted plantlets were transferred for pot culture. The number of produced shoots per callus and the frequencies of survived plants in natural condition were determined on respect of using callus derived from different explants. The pH of all media was adjusted at 5.8.

5.2.2.4 Determination of RGR and RWC

The calli age of four weeks and approximately uniform size (100 mg) were weighted individually which was known as initial fresh weight (FWi) and cultured into vessel singly in CIM. After four weeks, calli were rinsed with sterile distilled water 4-5 times. Excess water of calli was sacked by blotting paper and final fresh weight (FWf) was recorded. Relative growth rate (RGR) of callus was determined using the standard formula $RGR = (FWf - FWi) / FWi$ followed by Smith and McComb (1981). Targeted calli were incubated at 60°C for 48 hrs and after drying, dry mass of calli was weighted. The relative water content (RWC) of callus was calculated using the formula $(FW - DW) / DW$; where FW = fresh weight, DW = dry weight and percentage values were determined following the method of Al-Khayri and Al-Bahrany (2004).

5.2.2.5 Data recording and statistical analysis

The average or mean values were computed from four replicates with standard error (SE). The experiments were laid out as completely randomized design (CRD). All the experiments were conducted for three times. Analysis of variance (ANOVA) and post hoc Duncan multiple range test (DMRT) were done using SPSS17.0 software.

5.3 Results

5.3.1 Response to callus initiation of the explants

Nine different explants were examined on response to callus induction (CI); and the result showed that the explants zygotic embryo (ZE) responded with highest frequency (93.13%); followed by MNOS (66.11%), MINS (56.50%), ART (35.42%), SL (34.64%), and the lowest was 19.58% in coleoptile (**Table 23**). A wide range of variations were found in the period of callus initiation for different explants. In this case, ZE initiated callus very earlier than other explants. The recorded data showed that ZE initiated callus induction within 2-5 d; and in this period all the responded explants of ZE initiated callus induction. On the other hand, no initiation was found within 2-5 d for any other explants tested, and 18.36% MNOS exhibited calli initiation within 6-9 d after inoculation. The rest explants initiated calli within 10-17 d, whereas till 13 d of inoculation no initiation was found for coleoptile, it began to initiate callusing within 14 -17 days.

5.3.2 Primary and secondary callus induction

Responded explants produced the primary callus with wide range of significant difference; and the primary callus developed secondary callus by proliferation (**Fig. 17**). Data were recorded as 79.06, 63.33 and 53.50% primary callus induction (PCI) for ZE, MNOS and MINS respectively, while the lowest (15.42%) was found for coleoptile (**Table 24**). The highest value of secondary callus induction (SCI) was observed in ZE (70.63%) and the explants coleoptile showed the lowest frequency (12.50%). Lower number of SCI was found in radicle (17.14%), AR (20.36%) and PL (22.86%). On the other hand MNOS (61.11%) and MINS (51.00%) exhibited the maximum callusing among the explants which obtained from vegetative organs. In respect to response on callus induction, production of primary and secondary callus, the performance of the explants were differed significantly at $p \leq 0.01$ (**Table 27**).

After initiation of callusing, all the responded explants didn't produce primary callus. At the same way, no proliferation was found in some primary calli which derived from different explants to produce secondary callus. The frequencies were decreased

considering three parameters i.e. ‘responded explants to PCI (A)’, ‘PCI to SCI (B)’ and ‘responded explants to SCI (C)’ for different explants experimented. The decline rates were determined and the highest values were observed in ZE as 15.11, 10.66 and 24.16% for A, B and C respectively (**Fig. 18**). On the other hand, MNOS exhibited the lowest decline rates as A (2.99%), B (2.81%) and C (5.37%).

Table 23: Efficiency to initiate callus earlier of different explants

Explants	Inoculated explants	Responded explants to induce callus (%)	Frequency of callus initiation in different period (%)			
			2-5 d	6-9 d	10-13 d	14-17 d
ZE	320	298	298	0	0	0
		93.13 ± 2.13a	93.13 ± 2.13			
Radicle	280	74	0	0	27	47
		26.43 ± 1.70f			9.64 ± 0.68	16.79 ± 0.68
AR	280	82	0	0	32	50
		29.29 ± 1.70f			11.43 ± 0.58	17.86 ± 0.41
ART	240	85	0	0	30	55
		35.42 ± 1.85d			12.50 ± 0.83	22.92 ± 0.80
Coleoptile	240	47	0	0	16	31
		19.58 ± 0.80g			6.25 ± 0.42	13.33 ± 0.68
MNOS	180	119	0	27	42	50
		66.11 ± 2.10b		18.36 ± 0.56	19.97 ± 0.64	27.78 ± 0.64
MINS	200	113	0	18	40	55
		56.50 ± 1.26c		10.97 ± 0.58	18.03 ± 0.82	27.50 ± 0.96
PL	280	85	0	0	33	52
		30.36 ± 1.58ef			11.79 ± 0.36	18.57 ± 0.58
SL	280	97	0	0	40	57
		34.64 ± 1.22de			13.21 ± 0.58	20.36 ± 0.36

In the column ‘responded explants to induce callus’ mean values followed by same letter (s) are not significantly different at $p \leq 0.05$ according to DMRT.

Table 24: Induction of primary and secondary callus

Explants	Inoculated explants	Primary callus (%)	Secondary callus (%)
ZE	320	260	226
		79.06 ± 1.39a	70.63 ± 2.13a
Radicle	280	61	48
		21.79 ± 1.22f	17.14 ± 1.01g
AR	280	69	57
		24.64 ± 0.90ef	20.36 ± 1.07fg
ART	240	72	63
		30.00 ± 1.18d	26.25 ± 1.05de
Coleoptile	240	37	30
		15.42 ± 0.80g	12.50 ± 0.48h
MNOS	180	114	110
		63.33 ± 1.92b	61.11 ± 1.92b
MINS	200	107	102
		53.50 ± 2.06c	51.00 ± 1.73c
PL	280	74	64
		26.43 ± 0.71de	22.86 ± 1.84ef
SL	280	85	76
		30.36 ± 1.35d	27.14 ± 1.01d

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

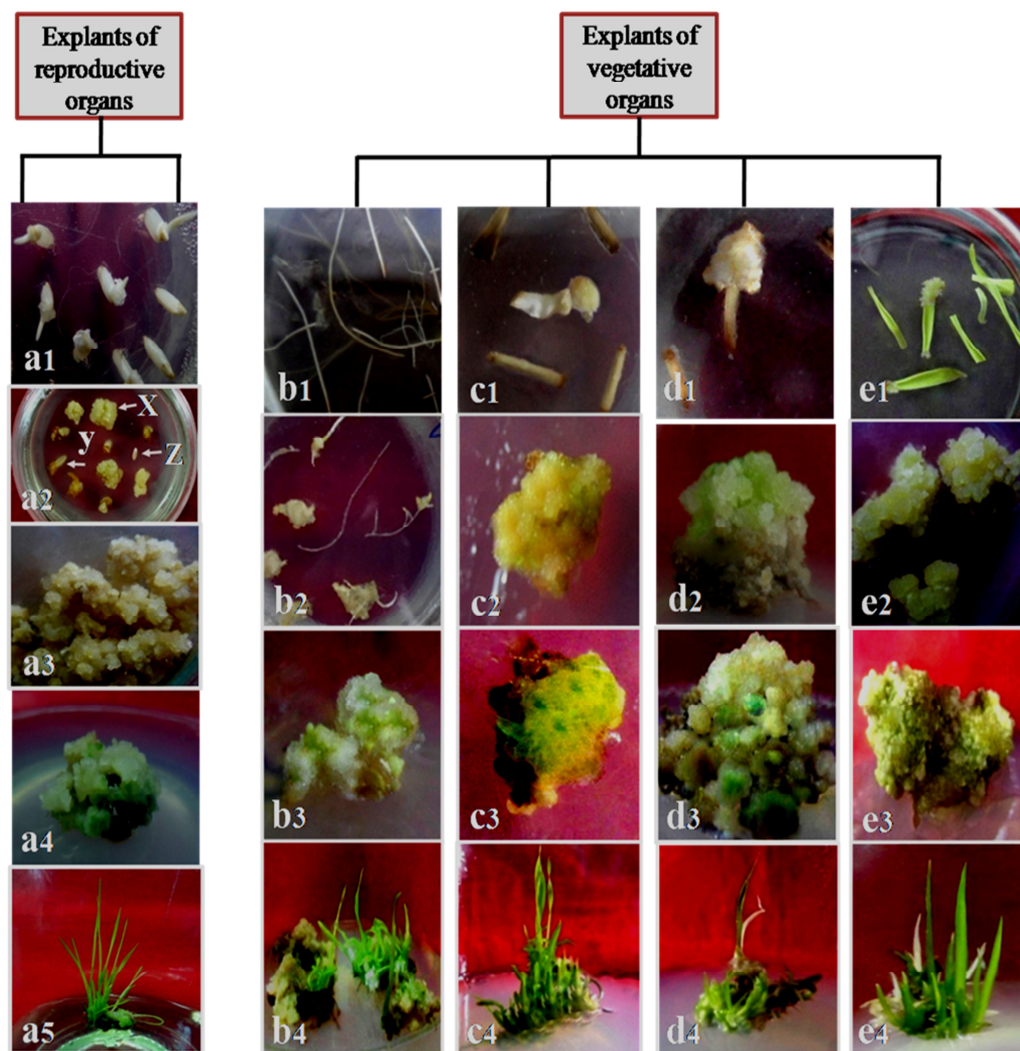


Fig. 17 (a₁-e₄): Callus induction and subsequent plant regeneration using different explants of BRR1 dhan29. a₁) Responded mature seeds (ZE), a₂) ZE derived primary callus, a₃) ZE derived secondary callus age of 3 w, a₄) Green spotted embryogenic callus going to regeneration, a₅) regenerated shoot form ZE embryo derived calli, b₁) inoculated root, b₁) callus initiation from root, b₃-b₄) regeneration from root induced callus, c₁-c₂) callus induction from MNOS, c₃-c₄) regeneration from MNOS induced callus, d₁-d₂) callus induction from MINS, d₃-d₄) regeneration from MINS induced callus, e₁) callus initiation from PL, e₂) secondary callus of PL, e₃) nodular green callus of PL and e₄) regenerated shoot from PL induced callus.

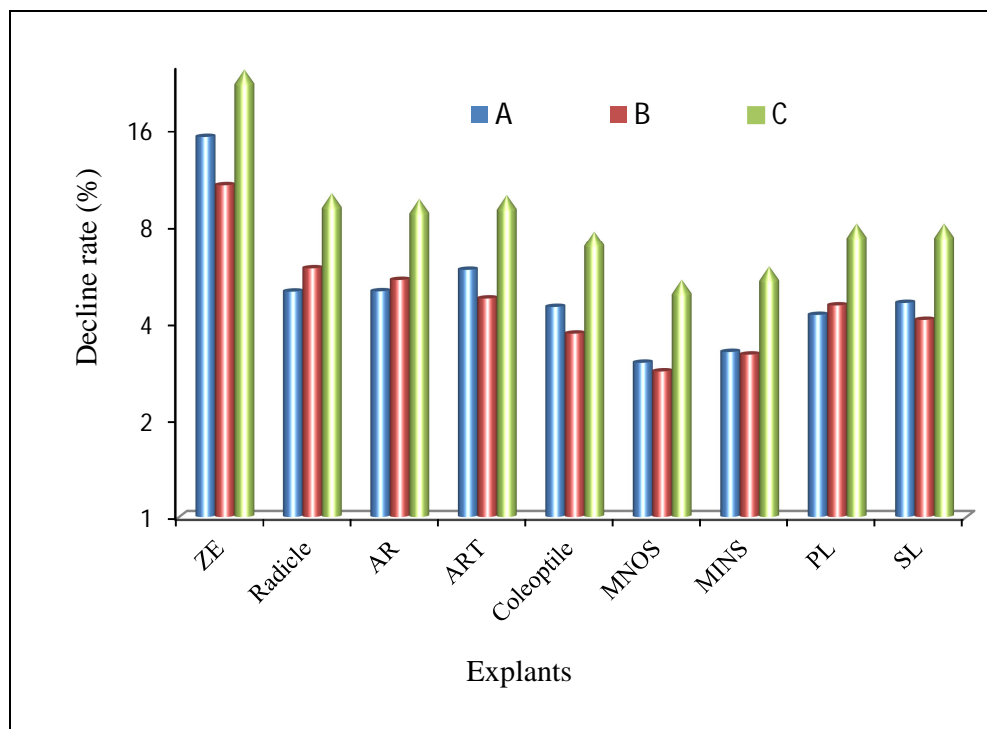


Fig. 18: Decline rate of frequencies in three parameters related to callus induction. Decline rate of A: Responded explants to PCI, B: PCI to SCI and C: Responded explants to SCI.

5.3.3 Relative growth rate and water content of the calli

The calli age of three weeks derived from nine different explants were culture also for three weeks and used to determine relative growth rate. Calli derived from various explants showed significantly different relative growth rate at $p \leq 0.01$. Out of nine types of explants the highest RGR was found in the calli derived from ZE (3.87) and the calli which induced from radicle (1.12) gave the lowest value (**Fig. 19**). Among the explants which obtained from vegetative organs of seedlings, MNOS exhibited the maximum RGR (2.36). On the other hand minimum value of RWC was recorded for the same explants MNOS (0.10) and the maximum in AR (0.18) (**Fig. 20**).

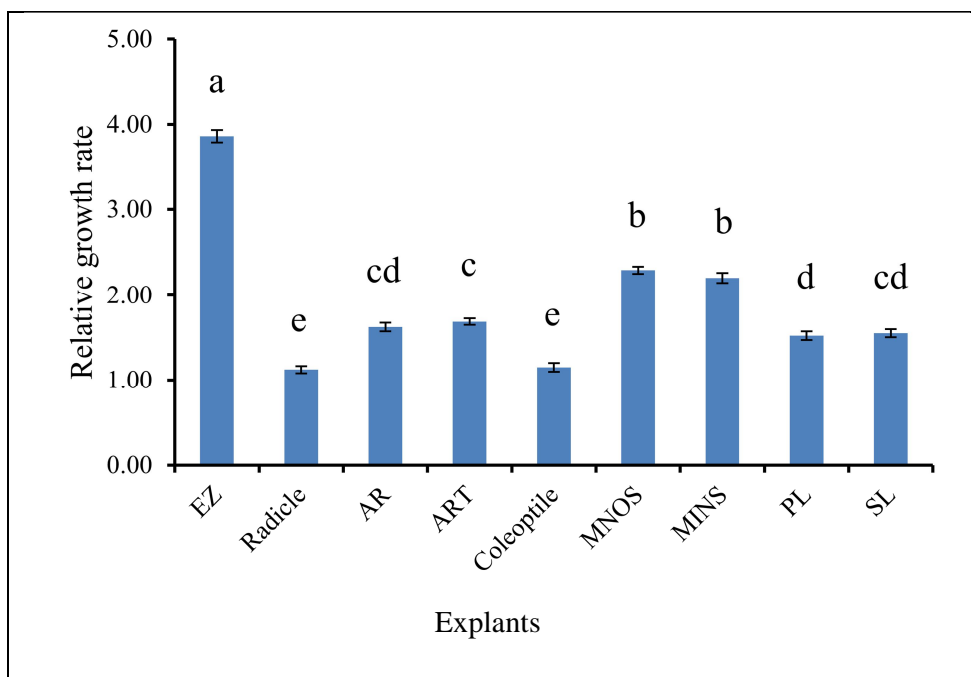


Fig. 19: Relative growth rate of the examined explants.

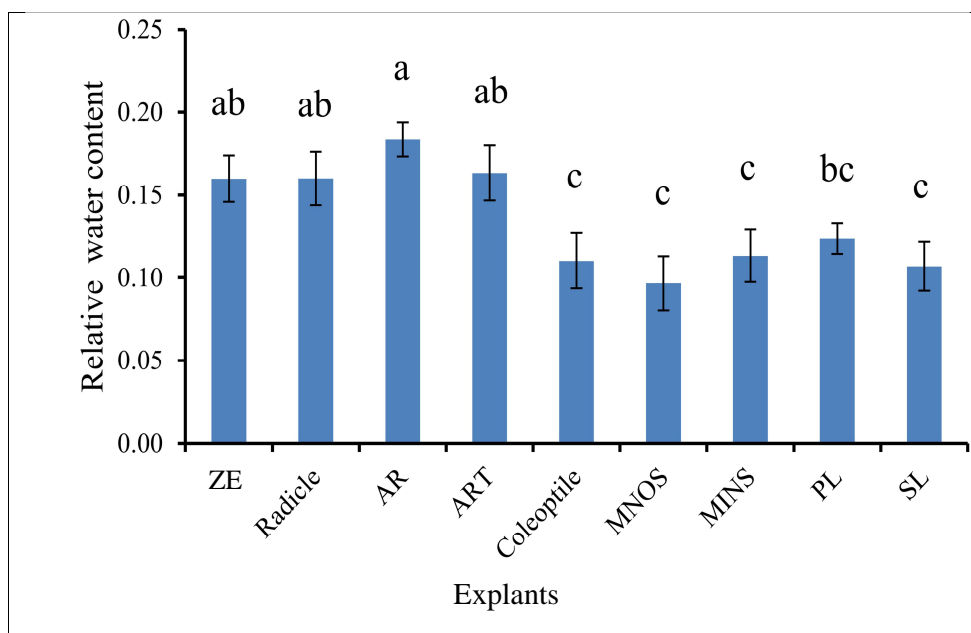


Fig. 20: Relative water content of the tested explants.

5.3.4 Regeneration efficiency of calli

Among nine types of explants, significant difference to plant regeneration was found; and the calli age of 6 w, induced by MNOS regenerated at the highest frequency (90.63%) (**Table 25**). The calli age of 5 w, induced by PL, SL, coleoptile, AR and radicle gave their maximum regeneration as 75.00, 72.92, 71.88, 47.50 and 45.00% respectively. In the cases of ZE, maximum 55.00% regeneration was recorded from the calli age of 4 w. Without consideration of callus age, on an average the calli came from MINS performed with the highest frequency of plant regeneration (83.60%) and the lowest 38.13% in AR (**Fig. 21**). The explants were significantly varied on respect to their regeneration efficiency at $p \leq 0.01$ (**Table 27**). Number of shoots per callus was recorded for the calli of different age groups. On an average, maximum 10.08 shoots were produced by the calli age of 5 weeks which derived from MNOS. The lowest value (2.05) was found for radicle derived calli when 3 weeks old calli were cultured on regeneration medium (**Table 26**).

A significant variation was also found in survival rate of field culture. The highest survival rate (90.63%) was recorded in the plantlets obtained from 6 w old calli of MINS (**Table 26**). The plantlets regenerated from radicle, coleoptile, PL and SL showed approximately similar survival ability. On the other hand the plants developed from the calli age of 6 w by ZE survived at the lowest rate (47.50%) among the explants tested.

Table 25: Regeneration efficiency based on four age groups of calli derived from different explants

Explants	Age of callus (weeks)	No. of inoculated callus	Responded callus to regeneration (%)	Plant regeneration (%)
Zygotic embryo (ZE)	3	160	69.38 ± 2.13hi	51.25 ± 2.60lm
	4	160	78.13 ± 1.88efg	55.00 ± 2.28kl
	5	160	71.25 ± 2.27ghi	48.75 ± 2.17lmn
	6	160	56.88 ± 1.20j	46.88 ± 2.13lmn
Radicle	3	40	42.50 ± 2.50lm	35.00 ± 2.89pq
	4	40	45.00 ± 2.89klm	37.50 ± 2.50opq
	5	40	47.50 ± 2.50kl	45.00 ± 2.89mno
	6	40	45.00 ± 2.89klm	42.50 ± 2.50nop
Adventitious root (AR)	3	40	37.50 ± 2.50m	32.50 ± 2.50q
	4	40	42.50 ± 2.50lm	35.00 ± 2.89pq
	5	40	52.50 ± 2.50jk	47.50 ± 2.50lmn
	6	40	45.00 ± 2.89klm	37.50 ± 2.50opq
Adventitious root tip (ART)	3	48	42.50 ± 2.50lm	35.00 ± 2.89pq
	4	48	45.00 ± 2.89klm	37.50 ± 2.50opq
	5	48	57.50 ± 2.50j	52.50 ± 2.50lm
	6	48	52.50 ± 2.50jk	45.00 ± 2.89mno

Each experiment had four replications. Different letter (s) in a column indicate significant different at $p \leq 0.05$ according to DMRT.

Contd. (Table 25)

Explants	Age of callus (weeks)	No. of inoculated callus	Responded callus to regeneration (%)	Plant regeneration (%)
Coleoptile	3	32	71.88 ± 3.11ghi	65.63 ± 3.13ij
	4	32	78.13 ± 3.11efg	68.75 ± 3.61ghij
	5	32	81.25 ± 3.61def	71.88 ± 3.13fghi
	6	32	71.88 ± 3.13ghi	65.63 ± 3.13ij
Mesocotyl nodal segment (MNOS)	3	64	81.25 ± 2.55def	76.56 ± 1.56defg
	4	64	84.38 ± 1.80cde	79.69 ± 1.56cdef
	5	64	87.50 ± 2.55cd	84.38 ± 3.13abcd
	6	64	96.88 ± 3.13a	90.63 ± 1.80a
Mesocotyl internodal segment (MINS)	3	64	81.25 ± 2.55def	78.13 ± 3.13cdef
	4	64	84.38 ± 1.80cde	81.25 ± 2.55bcde
	5	64	89.06 ± 2.99bc	85.94 ± 2.99abc
	6	64	95.31 ± 2.99ab	89.06 ± 1.56ab
Primary leaf (PL)	3	40	67.50 ± 2.50i	62.50 ± 2.50jk
	4	40	72.50 ± 2.50ghi	65.00 ± 2.89ij
	5	40	77.50 ± 2.50efg	75.00 ± 2.89efgh
	6	40	75.00 ± 2.89fghi	67.50 ± 2.50hij
Secondary leaf (SL)	3	48	68.75 ± 2.08i	60.42 ± 2.08jk
	4	48	72.92 ± 2.08ghi	68.75 ± 2.08ghij
	5	48	77.08 ± 2.08efgh	72.92 ± 2.08fghi
	6	48	70.83 ± 2.41ghi	62.50 ± 2.41jk

Each experiment had four replications. Different letter (s) in a column indicate significant different at $p \leq 0.05$ according to DMRT.

Table 26: Efficiency of shoot formation and survival ability in field culture based on different age groups and explants

Explants	Age of callus (weeks)	No. of inoculated callus	No. of green shoot per callus	Survival rate of field culture (%)
Zygotic embryo (ZE)	3	160	3.02 ± 0.08k	50.63 ± 2.13f
	4	160	4.22 ± 0.11ij	51.88 ± 2.13f
	5	160	3.03 ± 0.09k	49.38 ± 1.57f
	6	160	3.21 ± 0.10k	47.50 ± 1.77f
Radicule	3	40	2.05 ± 0.06l	75.00 ± 2.89de
	4	40	2.23 ± 0.09l	77.50 ± 2.50cde
	5	40	2.13 ± 0.09l	77.50 ± 2.50cde
	6	40	3.10 ± 0.12k	72.50 ± 2.50e
Adventitious root (AR)	3	40	2.03 ± 0.09l	72.50 ± 2.50e
	4	40	2.15 ± 0.06l	75.00 ± 2.89de
	5	40	2.10 ± 0.08l	80.00 ± 4.08bcde
	6	40	3.28 ± 0.13k	75.00 ± 2.89de
Adventitious root tip (ART)	3	48	3.25 ± 0.09k	77.50 ± 2.50cde
	4	48	3.38 ± 0.13k	80.00 ± 4.08bcde
	5	48	3.53 ± 0.17k	80.00 ± 4.08bcde
	6	48	4.08 ± 0.22j	75.00 ± 2.89de
Coleoptile	3	32	3.34 ± 0.17k	81.25 ± 3.61abcde
	4	32	4.00 ± 0.18j	84.38 ± 3.13abcd
	5	32	4.13 ± 0.14j	84.38 ± 3.13abcd
	6	32	4.59 ± 0.19i	81.25 ± 3.61abcde

Each experiment had four replications. Different letter (s) in a column indicate significant different at $p \leq 0.05$ according to DMRT.

Contd. (Table 25)				
Explants	Age of callus (weeks)	No. of inoculated callus	No. of green shoot per callus	Survival rate of field culture (%)
Mesocotyl nodal segment (MNOS)	3	64	7.83 \pm 0.21cd	85.94 \pm 3.93abc
	4	64	8.31 \pm 0.23b	87.50 \pm 2.55abc
	5	64	10.08 \pm 0.26a	84.38 \pm 3.13abcd
	6	64	9.92 \pm 0.20a	89.06 \pm 2.99ab
Mesocotyl internodal segment (MINS)	3	64	7.41 \pm 0.17d	87.50 \pm 4.42abc
	4	64	8.11 \pm 0.22bc	81.25 \pm 2.55abcde
	5	64	9.89 \pm 0.23a	85.94 \pm 1.56abc
	6	64	9.72 \pm 0.23a	90.63 \pm 1.80a
Primary leaf (PL)	3	40	5.43 \pm 0.22gh	80.00 \pm 4.08bcde
	4	40	5.93 \pm 0.21f	77.50 \pm 2.50cde
	5	40	6.90 \pm 0.23e	75.00 \pm 2.89de
	6	40	6.63 \pm 0.20e	72.50 \pm 2.50e
Secondary leaf (SL)	3	48	4.65 \pm 0.18i	77.08 \pm 2.08cde
	4	48	5.10 \pm 0.20h	79.17 \pm 4.17bcde
	5	48	5.75 \pm 0.19fg	81.25 \pm 3.99abcde
	6	48	6.02 \pm 0.21f	83.33 \pm 3.40abcd

Each experiment had four replications. Different letter (s) in a column indicate significant different at $p \leq 0.05$ according to DMRT.

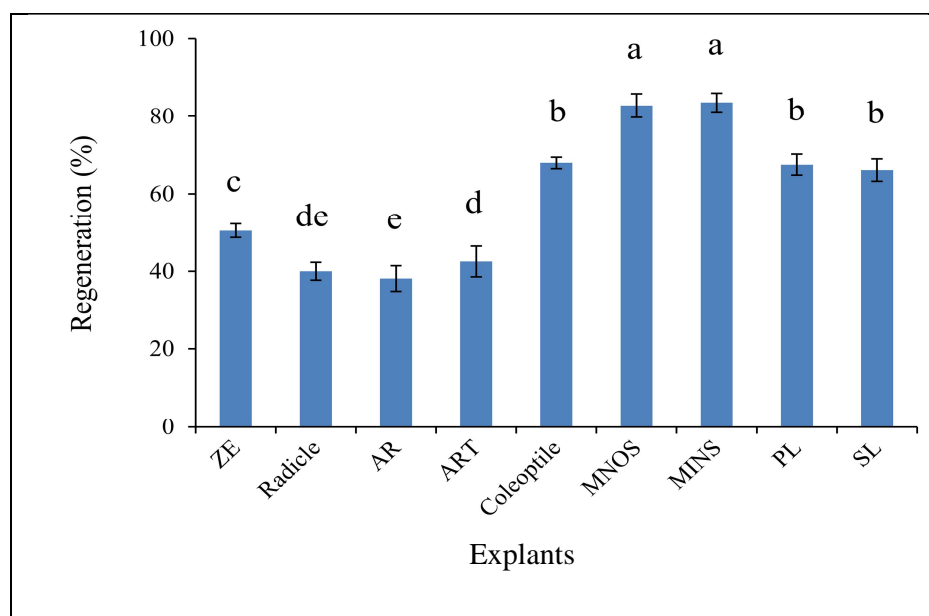


Fig. 21: Frequency of plant regeneration of the calli derived from different explants.

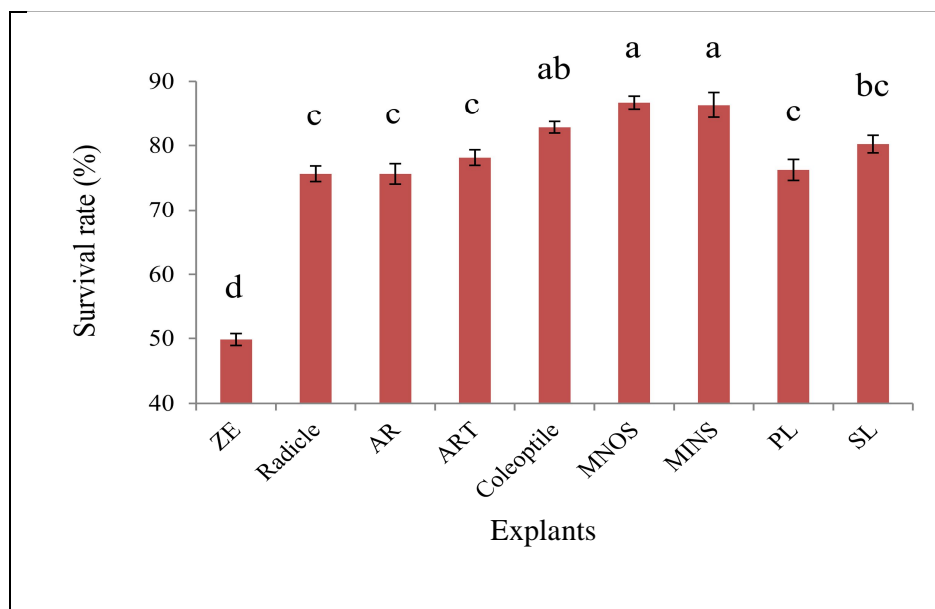


Fig. 22: Survival rate of regenerated plantlets developed from different explants in field culture.

Table 27: Analysis of variance (ANOVA) subjected to callus induction, plant regeneration and related conducted experiments

Subject of ANOVA (Data source)	Source of variation	df	Mean sum of square
Responded explants to callus induction (Table 23)	Explants	8	2262.45**
	Replication	2	24.28 ^{NS}
	Error	16	9.162
Primary callus induction (Table 24)	Explants	8	1887.50**
	Replication	2	6.42 ^{NS}
	Error	16	7.47
Secondary callus induction (Table 24)	Explants	8	1764.13**
	Replication	2	23.15*
	Error	16	6.70
Relative growth rate of callus (Fig. 19)	Explants	8	2.82**
	Replication	2	0.03 ^{NS}
	Error	12	0.01
Relative water content of callus (Fig. 20)	Explants	8	0.004**
	Replication	2	0.0003 ^{NS}
	Error	12	0.001
Responded callus to plant regeneration (Table 25)	Explants	8	4588.63**
	Age of callus	3	463.82**
	Replication	3	168.90**
	Error	129	35.93
Plant regeneration (Table 25)	Explants	8	40162.01**
	Age of callus	3	1758.38**
	Replication	3	60.23 ^{NS}
	Error	129	4343.35
Number of shoot per callus (Table 26)	Explants	8	90.19**
	Age of callus	3	10.48**
	Replication	3	0.58 ^{NS}
	Error	128	1.09
Survival rate in field culture (Table 26)	Explants	8	1936.76
	Age of callus	3	12.77
	Replication	3	49.20
	Error	129	35.68

* = Significant at $p \leq 0.05$, ** = Significant at $p \leq 0.01$ and NS= Non-significant.

5.4 Discussion

A mass of undifferentiated cells that lack of defined histological structure is usually considered as callus. To induce calli root, nodal and internodal part of stem, shoot tip, shoot apical meristem, leaf, auxillary bud, flower buds, anther, pollen, immature and mature seed, cotyledons, hypocotyl and epicotyl of young seedlings etc. are used as the typical explants (Jha and Ghosh 2009).

In this study nine different types of explants were considered which were in two groups. One was mature seeds (ZE) that directly related to the organ of reproduction; and the other group had eight types of explants i.e. radicle, AR, ART, Coleoptile, MNOS, MINS, PL and SL were obtained from *in vitro* grown seedlings that related to vegetative organ. Among the explants ZE performed the best with high frequency of CI (70.63); and its callusing was initiated very earlier than other explants. Out of the rest eight different explants obtained from seedlings, MNOS gave the maximum callus induction (61.11%), while callus initiation was found later than ZE (**Table 23, 24**). Moreover, all the explants related to vegetative organ did delay to initiate callus comparison to ZE. The result indicated that the source of explants affected CI, and a clear indication was observed that ZE is the best explants considering the highest efficiency and earlier initiation of CI. Radhesh et al. (2013) reported on the efficiency to CI of root, coleoptile, leaf base and mature seed for indica rice cultivar IR36, and recorded 39.6, 53.6, 47.3, 99% callusing respectively, while no CI was found in leaf blade. Sahrawat and Chand (2001) obtained 72.8% embryogenic callus from coleoptile segments of 4 d old seedlings in indica rice cultivar Kasturi.

Under this study, on a constant culture condition, hormonal combination and media composition, significant differences were found among the explants on respect to CI. It might be created due to internal physiological and especially for control of genes expression which regulate to cells totipotency. The capacity of cultured plants for callusogenesis, organ formation, and somatic embryogenesis depends on the activity

of genes that determine and maintain the meristematic state of cells, level of hormones in the cells, and sensitivity to hormones, as well as on the activity of other genes that control different stages of plant morphogenesis (Ezhova 2003). In 2007 Verdeil et al. mentioned the mechanisms to induce dedifferentiation of somatic cell into a totipotent embryogenic cell that can either be regenerated or develop an embryo and subsequent entire plant. In most species it has been examined that expression of *SERK1* (SOMATIC EMBRYO RECEPTOR KINASE1) gene is associated with SE and marks embryogenic competence in some species along with SE can be enhanced in *Arabidopsis* by over expression of *SERK1* (Hecht et al. 2001, Karami et al. 2009). Nolan et al. (2009) reported on the expression of *SERK1* gene which generally associated with developmental change. Importance of totipotency to regenerate *in vitro* plant has been investigated earlier. Haberlandt (1902) visualized it in any type of plant cell. Though each cell of a plant body carries the potentiality to regenerate plants, yet the meristematic cells are the best one to prove the presence of totipotency of a cell (Hossain 2010). In 1838 Schwann and Scheilden reported on totipotency theory as cells are autonomic, and in principle, are capable of regenerating to give a complete plant.

A remarkable difference was investigated in the periods of callus initiation, and it could be noticed as the explants ZE is very efficient to initiate callusing earlier. Cent percent response to CI within 2-5 d pronounced the explant ZE on the rest eight (**Table 23**). On the contrary, a large number of responded explant of ZE failed to form primary and as well as secondary callus. Whereas, all the explants related to vegetative organs produced primary and secondary callus with high frequency considering responded explants, though less number of explants responded to callus initiation. Furthermore, all the eight types of explants which came from vegetative organs initiate callusing later than ZE, even in case of coleoptile callus was initiated after passing 14 d of inoculation. Sahrawat and Chand (2001) reported that callusing was initiated at the cut ends and next entire the length of the coleoptile segments.

They also noticed that the explant age of 7 d produced lower CI than age of 3 and 4 d. In our study seedlings age of 10 d were used and coleoptile performed the lowest CI. A study by Guzzo et al. (1994) on the origin of totipotent cells in carrot cell-suspension cell cultures, derived from hypocotyl explants, showed that procambial cells gave rise to the embryogenic cell lines. Meristematic cells, mesophyll cells of the leaf or the parenchyma of root cortex present in the explant are stimulated first to grow and divide. The growth and division of quiescent (differentiated) or non-dividing cells began first at the cut surface of the explant and continued proliferation produce a mass of tissue which spreads over the surface of the explant (Jha and Ghosh 2009). Explants are consisted of various types of cells. When these are treated with suitable growth regulators, then mRNA of the cells are stimulated which gives the indication to the protein-enzymes related to cell division (Hossain 2010). However, capability of callus initiation might be depend on genetic consistent of the cells of explants in which the specific genetic materials are stimulated by the internal physiological factors.

All the responded explants didn't produce primary callus; and at the same way no proliferation was happened in some primary callus for the explants experimented. As a result, the frequencies of PCI were decreased than responded explants and also the declined values were found in SCI than PCI. The highest declined rates were observed in ZE and lowest in MNOS the parameters 'responded explants to PCI' and 'PCI to SCI' (**Fig. 18**). However, higher rate of decline expressed more disability to be existent of the calli, derived from specific explant. Ultimately the explant would be considered as less importance for the purpose of in vitro culture. Based on the view, the explant MNOS is the best one carrying its lowest decline rate, but considering the highest capacity to callus induction ZE is the most suitable explant for *in vitro* culture among the explants examined.

The calli derived from different explants were cultured and the results indicated clear and significant difference among them. Vigorous growth was found in the calli of ZE, whereas, the calli which came from other explants showed lower rate of growth in same culture condition. The lowest growth was observed in the calli developed from radicle, AR and ART which are basically the root related parts of seedlings (**Fig. 19**). The MNOS, MINS, PL and SL produced calli perform with moderate growth. Coleoptile also showed lower growth as shown in radicle, AR and AT. The results indicated that the explants which came from shoot of the seedlings are better than that of the explants related to roots. The cells of the calli developed from AR or ART or radicle contain large amount of water in their cell sap which was experimented and the highest RWC was recorded in AR (0.18) and the lowest in MNOS (0.10) (**Fig. 20**). The explant ZE performed at the highest RGR (3.87) and radicle gave lowest (1.12) (**Fig. 19**). A much amount of water contained in the protoplast of the cells of calli developed from radicle, AR and ART that might be a physiological factor to inhibit the growth of callus.

For plant regeneration two factors were experimented such as age and source of calli that is from which explants the calli were produced. Considering the both factors results showed significant difference to regeneration among the explants along with the ages of the calli at $p \leq 0.01$. Sahrawat and Chand (2001) used 13 w old (after culture initiation) calli for plant regeneration and reported that the calli derived from 3, 4 and 5 d old coleoptile segments regenerate with significantly higher frequencies. In their investigation age of explants were examined and the highest frequency was obtained from the calli which produced by 4 d old coleoptile. On the other hand our experiments were worked out relating on age of calli derived from various explants which obtained from 10 d old seedlings. Out of nine different explants, MNOS performed the best with the frequency of 90.63% regeneration when 6 w old calli was transferred to regeneration medium (**Table 25**). MINS exhibited approximately similar performance to MNOS, and our observation was that the calli developed from

the explant which came from vegetative organs of the seedlings showed remarkably better regeneration than ZE except radicle, AR and ART. Hence it could be suggested that the part of seedlings which move to light (= shoot) such as MNOS, MINS, coleoptile, PL and SL are very efficient for plant regeneration. In contrast, the organs those moves to gravitation force (= root) i.e. radicle, AR and ART are not better to *in vitro* culture. However, 80% regeneration was recorded in indica rice cultivar IR36 by using scutellar derived calli (Radhesh et. al. 2013). In this study 32.50 - 90.63% regeneration was recorded from ZE for Bangladeshi indica rice genotypes BRRI dhan29 (**Table 25**). In the same media and hormonal combination the explant MNOS performed with one and half fold higher regeneration than ZE based on their highest efficiency. Hence, it could be suggested that the explant MNOS is the most suitable explant to regenerate rice plant *in vitro*, although its efficiency to CI is not so satisfactory as seen in ZE. Furthermore, it could be stated that MNOS would be able to give the highest performance in callus induction and regeneration both.

In the case of ZE comparatively lower aged calli gave better results on regeneration, whereas, the calli derived from all of the rest explants need to higher age to be regenerated with maximum frequency. This is also a prominent feature as well as dissimilarity of the explants obtained from vegetative organs on comparison to ZE the reproductive organ.

5.5 Conclusion

The various vegetative parts of rice seedling used as explants, and they responded differently in callus induction as well as plant regeneration. Mesocotyl, especially nodal segment of mesocotyl is the most efficient explant because of its high capability to re-differentiation; yet it produce in lower rate of callusing than the zygotic embryos. An explant which can perform better to induce callus, might not to be suitable for regeneration. In contrast, the calli derived from the explant of lower efficiency, remarkable re-differentiated and produce progeny *in vitro* at high rate; and among the explants tested, mesocotyl and nodal part (node) are so ones.

**Chapter
VI**

**Genetic
Transformation**



6. Genetic Transformation

6.1 Introduction

The normal growth and development of rice (*Oryza sativa* L.) are affected by various abiotic stresses. In particular, it is predicted that water deficit will continue to be a major abiotic stress factor affecting global crop yields (Hasegawa et al. 2000). Now a days to produce high yields including expected agronomical traits that give rise to strong and healthy plants along with resistant to disease and can resist adverse environmental conditions like high salinity and drought is one of the main concerns to plant scientist while three billion people consider rice as their staple food, accounting for 50-80% of their daily calorie intake.

Rice is a salt sensitive monocot (Singh and Sengar 2014) while salinity affects about 400 million hectares of land in the world, of which 54 million are found in South and South-east Asia. It is sensitive above a soil conductivity of 3 dS/m equivalent to 30 mM. Within Bangladesh, greater than 1 million hectares of coastal areas are affected by varying degrees of 4-20 dS/m in coastal areas depending upon the season and distance from the rivers. Production of rice is therefore under pressure: salinity may cause plant demise as well as reduction in growth and development, resulting in up to 50% of a reduced yield (Nozulaidi et al. 2015). This increase of the environmental stress poses serious threats to global agricultural production and food security. It has been estimated that two-thirds of the yield potential of major crops are routinely lost due to unfavorable growing environments.

During salinity stress, several classes of Na⁺ transporters have been shown to play central roles in Na⁺ homeostasis. Three major Na⁺ transporters are involved in plant Na⁺ tolerance: namely, the *NHX*, *SOS1* and *HKT* transporters (Yamaguchi et al. 2013). Genes encoding Na⁺ transporters integrating to rice through genetic engineering can play a crucial role in conferring salinity tolerance (Singh and Sengar 2014). Ji et al. (2013) reported that high complexity of the regulatory networks involved in plant response to salinity. Several reports have been described the physiological phenomena on plants response to salinity stresses (Tuteja 2007). High

salinity stress causes an imbalance in sodium ion (Na⁺) homeostasis, which is normally maintained by the coordinated action of various pumps, ions, constitutive overexpression of the plasma membrane Ca²⁺ sensors, and its downstream interacting partners, which ultimately results in the efflux of excess Na⁺ ions (Tuteja 2007, Ma et al. 2014, Razzaque et al. 2014). Moreover, drought is another major environmental factor which inhibits the growth, productivity and distribution of plants. It is estimated to be one of the most serious yield reducing stresses in the agriculture.

Chaves and Oliveira (2004) reported that water deficit is a main problem for crop production specially rice worldwide, limiting the growth and productivity of many crop species particularly in rain-fed agricultural areas. Rice (*Oryza sativa* L.) is subjected to a range of abiotic stresses that affect their growth and development. In particular, it is predicted that water deficit will continue to be a major abiotic stress factor affecting global crop yields (Sharma and Lavanya 2002). Haque et al. (2013) reported that increased level of mannitol stress reduced the ability to callus induction and regeneration. However, to improve the ability to survive in adverse environmental condition of agronomical important plants biotechnological tools have been used, while it has been reported that tissue culture of monocots is difficult compared to dicots (Morel and Wetmore 1951). Jain (1997) mentioned that difficulty of embryogenic cell cultures and plant regeneration especially for indica subspecies. Transgenic plants tolerant to abiotic stresses were reported in cereals by some author (Nguyen and Sticklen 2013, Banu et al. 2014). Several reports have shown that most of the indica lines are less responsive to *in vitro* culture compared to japonica lines (Abe and Fursuhara 1984, Reddy et al. 1994, Mikami and Kinoshita 1988). Potentiality for callus induction and regeneration depends on some factors like genotype, physiological and biochemical status of the explants, composition and concentration of different ingredients of culture medium (Abe and Futsuhara 1986, Rueb et al. 1994, Ge et al. 2006). However, biotechnology as well as genetic transformation methods are been applied to produce transgenic plants having targeted genes, in particular related to heat, cold, drought, salinity and insect resistant lines. *Agrobacterium*-mediated genetic transformation is widely used method for improving agronomic important plants.

Raineri et al. (1990) need to keep obtained transformed rice cells that expressed neomycin-phosphotransferase (NPT) and β -glucuronidase (GUS) activities and they suggested that T-DNA had been transferred to, integrated in, and expressed in, rice cells. Gould et al. (1991) described the transfer of genes for NPT and GUS into shoot apices of corn, subsequent regeneration of plants, and detection of the transferred genes in the F1 progeny by Southern hybridization. Aananthi et al. (2010) highly regenerating indica rice cultivar Pusa Basmati 1 was subjected to genetic transformation mediated by *Agrobacterium tumifaciens* EHA 105 harbouring the virulent plasmid pCAMBIA 1305.1. Ge et al. (2006) reported that indica sub-species are more specific than japonica for *Agrobacterium*-mediated transformation. While transgenic rice has also been produced using *Agrobacterium tumifaciens* strains by some authors (Raineri et al. 1990, Chan et al. 1992, Li et al. 1992, Chan et al. 1993, Hiei et al. 1994, Saika and Toki 2010, Puhan et al. 2012, Nishimura et al. 2007, Rao and Rao 2007, Yi et al. 2001, Zou et al. 2008, Datta et al. 2000, Dong et al. 1996, Hiei and Komari 2008, Kumar et al. 2005, Labra et al. 2001, Rashid et al. 1996, Sahoo et al. 2011, Sahoo and Tuteja 2014, Amin et al. 2016). Plant transformation mediated by *Agrobacterium* has been done for various plants species viz. grasspea (Barik et al. 2005), mulberry (Bhatnagar and Khurana 2003), wheat (Cheng et al. 1997), maize (Gould et al. 1991), sorghum (Jeoung 2001), tobacco (Bakhsh et al. 2014) and cotton (Bajwa et al. 2014, Awan et al. 2015).

In tolerant plants, there are many defense mechanisms to resist adverse environmental conditions, such as osmoregulation, ion homeostasis, antioxidant and hormonal systems, helping plants to stay alive and development prior to their reproductive stages (Hasegawa et al. 2000). Biochemical and physiological changes in plants growing under salt or water-deficit conditions have been broadly investigated in rice (Ashraf and Harris 2004, Castillo et al. 2007, Ashraf 2010). However, the existing protocols for transformation and regeneration of indica rice are tedious, lengthy, and highly genotype-specific with low efficiency of transformation (Hiei and Komari 2008, Yookongkaew et al. 2007, Hiei et al. 1994, Khanna and Raina 1999, Arockiasamy and Ignacimuthu 2007, Hiei et al. 1997, Kumar et al. 2005). Considering the significance of genetic transformation in functional genome, an easy,

rapid, reproducible, widely applicable and highly efficient transformation and regeneration protocol is needed to develop for indica rice genotypes. Therefore, researchers have used either different explants (Hiei and Komari 2008, A Idemita and Hodges 1996, Hiei and Komari 2006), or changed ratios of different components of the culture medium (Yookongkaew et al. 2007), used different gelling agents (Kumar et al. 2005, Jain et al. 1996) and different *Agrobacterium* strains (Kumar et al. 2005).

Bangladeshi indica rice cultivar, BRRI dhan29 is an early-maturing, high-yielding cultivated variety which is popular to the farmers in coastal areas; while the farmers cannot grow this rice where soil salinities are greater than 4 dS/m (40 mM). To improve tolerance ability against salinity and drought, the rice cultivar would be great benefited to the farmers of the coastal region in Bangladesh. Hence present study was under taken on following objectives.

- *In silico* analysis of targeted gene and cloning to related vectors for transformation.
- Confirmation of cloning by PCR, restriction enzyme (RE) analysis.
- To develop an efficient and reproducible *Agrobacterium tumefaciens*-mediated transformation protocol for Bangladeshi indica rice variety of BRRI dhan29.
- Development of transgenic rice plants along with conferring tolerance to salt and drought through overexpression of *PDH47* gene (pea DNA helicase 47).
- Analysis of transgenics for different abiotic stress especially salinity and drought.

6.2 *In silico* analysis, cloning of targeted gene to related vectors

6.2.1 *In silico* analysis

Pisum sativum DEAD box RNA helicase mRNA, complete cds

GenBank: AY167670.1

FASTA Graphics

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DEFINITION *Pisum sativum* DEAD box RNA helicase mRNA, complete cds.

ACCESSION AY167670

VERSION AY167670.1 GI:25809053

KEYWORDS.

SOURCE *Pisum sativum* (pea)

ORGANISM *Pisum sativum*

Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;

Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;

rosids; fabids; Fabales; Fabaceae; Papilionoideae; Fabeae; Pisum.

REFERENCE 1 (bases 1 to 1646)

AUTHORS Vashisht, A. A. and Tuteja, N.

TITLE Cold stress-induced pea DNA helicase 47 is homologous to eIF4A and inhibited by DNA-interacting ligands

JOURNAL **Arch. Biochem. Biophys.** **440** (1), 79-90 (2005)

PUBMED 16009326

REFERENCE 2 (bases 1 to 1646)

AUTHORS **Vashisht, A.A. and Tuteja, N.**

TITLE Direct Submission

JOURNAL Submitted (22-OCT-2002) Plant Molecular Biology Lab, International Center for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi, Delhi 110067, India

FEATURES Location/Qualifiers

source 1..1646

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CDS 148..1389

/note="translation initiation factor; upregulated by cold stress"

/codon_start=1

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/protein_id="AAN74635.1"

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REDQRILSSGVHV VVGTPGRVFDMLRRQSLRPDYIKMFVLDEADEMLSRGFK
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ORIGIN

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1561 tgataagtac ttacaacgtg gaatccatta ttgtctgaat tgggtattg atggtgttt
1621 caccttcaa aaaaaaaaaa aaaaaa

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6.2.2 Enzymes that don't cut CDS of *PDH47* gene

AatII (G_ACGT^C)	BspEI (T^CCGG_A)	NciI (CC^S_GG)
AccI (GT^MK_AC)	BspMI	NcoI (C^CATG_G)
AcII (AA^CG_TT)	(ACCTGCNNNN^NNNN_)	NgoMIV (G^CCGG_C)
AfeI (AGC GCT)	BspQI (GCTCTTCN^NNN_)	NheI (G^CTAG_C)
AflIII (C^TTAA_G)	BsrBI (CCG CTC)	NmeAIII
AgeI (A^CCGG_T)	BsrDI (GCAATG_NN^)	(GCCGAGNNNNNNNNNN
AhdI (GACNN_N^NNGTC)	BsrFI (R^CCGG_Y)	NNNNNNNNNN_NN^)
AleI (CACNN NNGTG)	BsrGI (T^GTAC_A)	NotI (GC^GGCC_GC)
AlwNI (CAG_NNN^CTG)	BssHII (G^CGCG_C)	NruI (TCG CGA)
ApaI (G_GGCC^C)	BstBI (TT^CG_AA)	PacI (TTA_AT^TAA)
ApaLI (G^TGCA_C)	BstEII (G^GTNAC_C)	PaeR7I (C^TCGA_G)
AscI (GG^CGCG_CC)	BstUI (CG CG)	PciI (A^CATG_T)
AseI (AT^TA_AT)	BstZ17I (GTA TAC)	PfiFI (GACN^N_NGTC)
AsiSI (GCG_AT^CGC)	BtgI (C^CRYG_G)	PleI (GAGTCNNNN^N_)
AvaI (C^YCGR_G)	BtgZI	PluTI (G_GCGC^C)
AvrII (C^CTAG_G)	(GCGATGNNNNNNNNNN^N	PmeI (GTTT AAAC)
BaeI	NNN_)	PpuMI (RG^GWC_CY)
(_NNNNN^NNNNNNNNNN	BtsI (GCAGTG_NN^)	PshAI (GACNN NNGTC)
NACNNNGTAYCNNNN	ClaI (AT^CG_AT)	PspOMI (G^GGCC_C)
NNN_NNNNN^)	CspCI	PspXI (VC^TCGA_GB)
BamHI (G^GATC_C)	(_NN^NNNNNNNNNNNCAA	PvuI (CG_AT^CG)
BanII (G_RGCY^C)	NNNNNGTGGNNNNNNNNN	RsrII (CG^GWC_CG)
BbsI	N_NN^)	SacI (G_AGCT^C)
(GAAGACNN^NNNN_)	DraIII (CAC_NNN^GTG)	SacII (CC_GC^GG)
BbvCI (CC^TCA_GC)	EaeI (Y^GGCC_R)	SalI (G^TCGA_C)
BcgI	EagI (C^GGCC_G)	SapI
(_NN^NNNNNNNNNNCG	EarI (CTCTTCN^NNN_)	(GCTCTTCN^NNN_)
ANNNNNNTGCNNNNNN	EciI	SbfI (CC_TGCA^GG)
NNNN_NN^)	(GGCGGANNNNNNNNN_N	ScaI (AGT ACT)
BciVI	N^)	SexAI (A^CCWGG_T)
(GTATCCNNNNN_N^)	Eco53kI (GAG CTC)	SfiI
BclI (T^GATC_A)	EcoNI (CCTNN^N_NNAGG)	(GGCCN_NNN^NGGCC)
BfuAI	EcoO109I (RG^GNC_CY)	SfoI (GGC GCC)
(ACCTGCNNNN^NNNN_)	EcoRV (GAT ATC)	SgrAI (CR^CCGG_YG)
BglI (GCCN_NNN^NGGC)	FseI (GG_CCGG^CC)	SmaI (CCC GGG)
BmgBI (CAC GTC)	HgaI	SnaBI (TAC GTA)
BmrI	(GACGCNNNNN^NNNNN_)	SpeI (A^CTAG_T)
(ACTGGGNNNN_N^)	HincII (GTY RAC)	SspI (AAT ATT)
BmtI (G_CTAG^C)	HpaI (GTT AAC)	Swal (ATTT AAAT)
BpmI	HpaII (C^CG_G)	TaqI (T^CG_A)
(CTGGAGNNNNNNNNNN	Hpy99I (_CGWCG^)	TspMI (C^CCGG_G)
NNNNN_NN^)	KasI (G^GCGC_C)	Tth111I
BsaBI (GATNN NNATC)	MfeI (C^AATT_G)	(GACN^N_NGTC)
BsaHI (GR^CG_YC)	MluI (A^CGCG_T)	XbaI (T^CTAG_A)
BsaI (GGTCTCN^NNNN_)	MlyI (GAGTCNNNNN)	XcmI
BsaWI (W^CCGG_W)	MmeI	(CCANNNN_N^NNNNNT
BsiEI (CG_RY^CG)	(TCCRACNNNNNNNNNNNN	GG)
BsiHKAI (G_WGCW^C)	NNNNNNN_NN^)	XhoI (C^TCGA_G)
BsiWI (C^GTAC_G)	MscI (TGG CCA)	XmaI (C^CCGG_G)
BsmBI	MspI (C^CG_G)	XmnI (GAANN NNTTC)
(CGTCTCN^NNNN_)	NaeI (GCC GGC)	ZraI (GAC GTC)
BsoBI (C^YCGR_G)	NarI (GG^CG_CC)	
BspDI (AT^CG_AT)		

6.3 Materials and Methods

6.3.1 Plant materials

Mature seeds of BRRI dhan29 were used for this study as a popular indica rice variety in Bangladesh. It was collected from Bangladesh Rice Research Institute (BRRI), Regional Centre, Rajshahi, Bangladesh.

6.3.2 Methods

6.3.2.1 Seed sterilization and embryogenic callus induction

Healthy seeds were surface-sterilized with 70% ethanol (v/v) for 1 min, followed by 30 min in 50% (v/v) commercial bleach with shaking at 180 rpm. Thereafter the seeds were washed 6-7 times with sterile distilled water and shocked excess water with autoclaved Whatman 1 filter paper. Ten seeds were inoculated per petri dishes on callus induction medium (CIM) to induce callus and incubated at $27 \pm 1^\circ\text{C}$ in dark. The CIM was prepared using basal MS medium supplemented with 2.5 mg/l 2,4-D, 0.5 mg/l BAP, 30 g/l maltose, 0.3 g/l casein hydrolysate, 0.4 g/l L-proline, gelled with 3.0 g/l phytigel and before autoclaving pH was adjusted to 5.8. After 14 days cultured in dark, embryogenic calli were selected discarding non-embryogenic calli (compact, non-friable calli that developed root like structures). These embryogenic calli were cut into approximately 3 equal halves and sub-cultured again onto fresh CIM and before transformation with *Agrobacterium tumefaciens* it was kept in dark condition at $27 \pm 1^\circ\text{C}$ for 4 days.

6.3.2.2 Preparation of media used at various stages

Callus induction medium (CIM)

For callus induction the MS basal salts and vitamins supplemented with 2.5 mg/l 2, 4-D, 30 g/l sucrose, 0.3 g/l casein hydrolysate, 0.4 g/l L-proline, pH 5.8 and 3 g/l phytigel were used.

Re-suspension medium (RSM)

MS basal medium supplemented with 68 g/l sucrose, 36 g/l glucose, 3 g/l KCl, 4 g/l MgCl₂, pH 5.2 and 150 µM acetosyringone (freshly prepared at a concentration of 1 M in 100% Dimethyl sulfoxide).

Co-cultivation medium (CCM)

As co-cultivation medium MS + 30 g/l maltose, 0.3 g/l casein hydrolysate, 0.6 g/l L-proline, 10 g/l glucose, 3 mg/l 2, 4-D, 0.25 mg/l BAP, pH 5.2, 3 g/l phytigel were used and 150 µM acetosyringone added after autoclaving.

Selection medium (SM)

MS salts supplemented with 30 g/l maltose, 0.3 g/l casein hydrolysate, 0.6 g/l L-proline, 3 mg/l 2,4-D, 0.25 mg/l BAP, pH 5.8, 3 g/l phytigel; 250 mg/l cefotaxime and 50 mg/l hygromycin added after autoclaving.

First regeneration medium (RM-I)

MS salts supplemented with 30 g/l sucrose, 2 mg/l BAP, 1.0 mg/l Kin, 1.0 mg/l NAA, pH 5.8, 10 g/l agarose; 250 mg/l cefotaxime and 30 mg/l hygromycin was added after autoclaving.

Second regeneration medium (RM-II)

MS salts supplemented with 30 g/l maltose, 2 mg/l BAP, 1.0 mg/l Kin, 1.0 mg/l NAA, pH 5.8, 8 g/l agarose; 250 mg/l cefotaxime and 30 mg/l hygromycin added after autoclaving.

YEP medium

10 g/l bactopectone, 10 g/l yeast extract, 5 g/l sodium chloride, pH 7.0.

Root formation medium (RFM)

Half strength MS salts + 0.5 mg/l IBA + 30 g/l sucrose + 3 g/l phytigel and pH adjusted at 5.8. After autoclaving 250 mg/l cefotaxime and 30 mg/l hygromycin were added.

6.3.2.3 Cloning and restriction digestions

A single colony of DH5-alpha was picked up and inoculated into 5 ml LB medium (Luria-Broth) and grown overnight at 37°C (detailed in general materials and methods, Chapter II). After restriction enzyme digestion of plasmid, the digested product was electrophoresis on an agarose gel. By using standard molecular wt. marker (1 Kb ladder) the desired fragment was identified, cut and purified by one of the following method of PCR product from the gene legated with the pGEM-T vector (**Fig. 23**). First, it was made copies of the targeted gene that inserted into plasmids, using PCR. Then ligated the PCR product with the pGEM-T vector, which has 3'T overhangs that bond with the 5'A overhangs that get added to the ends by thermostable polymerases and transform this ligation product into bacteria, which was plated on selective media.

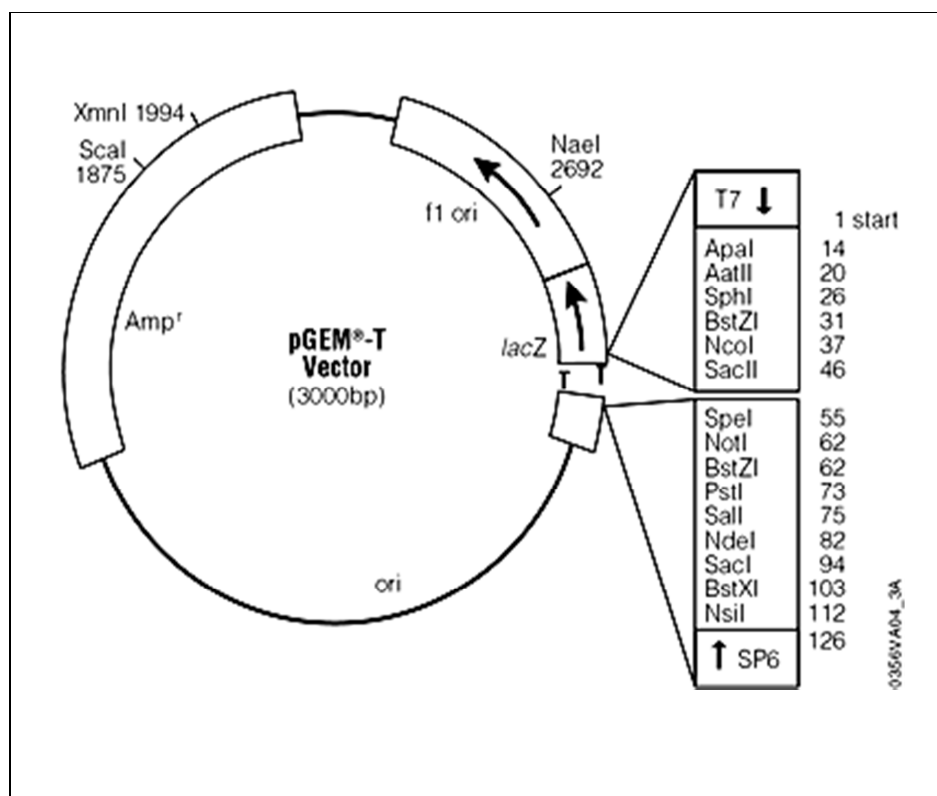


Fig. 23: Empty vector of pGEM-T and its restriction sites.

6.3.2.4 *Agrobacterium*-strains and construct for transformation

Agrobacterium strain (LBA4404) containing DNA helicase gene *PDH47* construct was cultured and prepared for transformation following the standard protocol described by Sahoo et al. (2011) with some modifications (**Fig. 24**). The *PDH47* construct (Accession No. AY167670) was obtained from Dr. Narendra Tuteja of International Center for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India. The gene was cloned in plant transformation vector pCAMBIA121 at XbaI and BamHI restriction sites (**Fig. 25, 26**). The vector pCAMBIA121 contains nptII (neomycin phosphotransferase) and hptII (hygromycin phosphotransferase) genes as the selectable markers for bacteria and plants respectively. It has both uidA (for GUS) and green fluorescent protein (GFP) as the reporter genes. Thereafter, the gene construct was transformed to commonly used *Agrobacterium tumefaciens* strain of LBA4404.

6.3.2.5 Preparation of *Agrobacterium* culture

To prepare primary culture of *Agrobacterium*, single colony from a freshly streaked plate was inoculated in 5 ml of autoclaved liquid YEP medium. The medium was supplemented with the antibiotics of 50 mg/l Kanamycin, 10 mg/l Rifampicin and 25 mg/l Streptomycin. Then the culture was incubated at 28°C on a rotatory incubator shaker at 180 - 200 rpm in dark for 16 - 20 hrs. The secondary culture was prepared by addition of 0.4% primary culture in a 500 ml of conical flask, containing 100 ml YEP medium supplemented with the same antibiotics as used for primary culture and grown under similar conditions. *Agrobacterium* cells were pelleted by centrifugation at $8000 \times g$ for 15 min at 4°C when the OD₆₀₀ reached ~1.0. The cells were re-suspended in MS re-suspension medium.

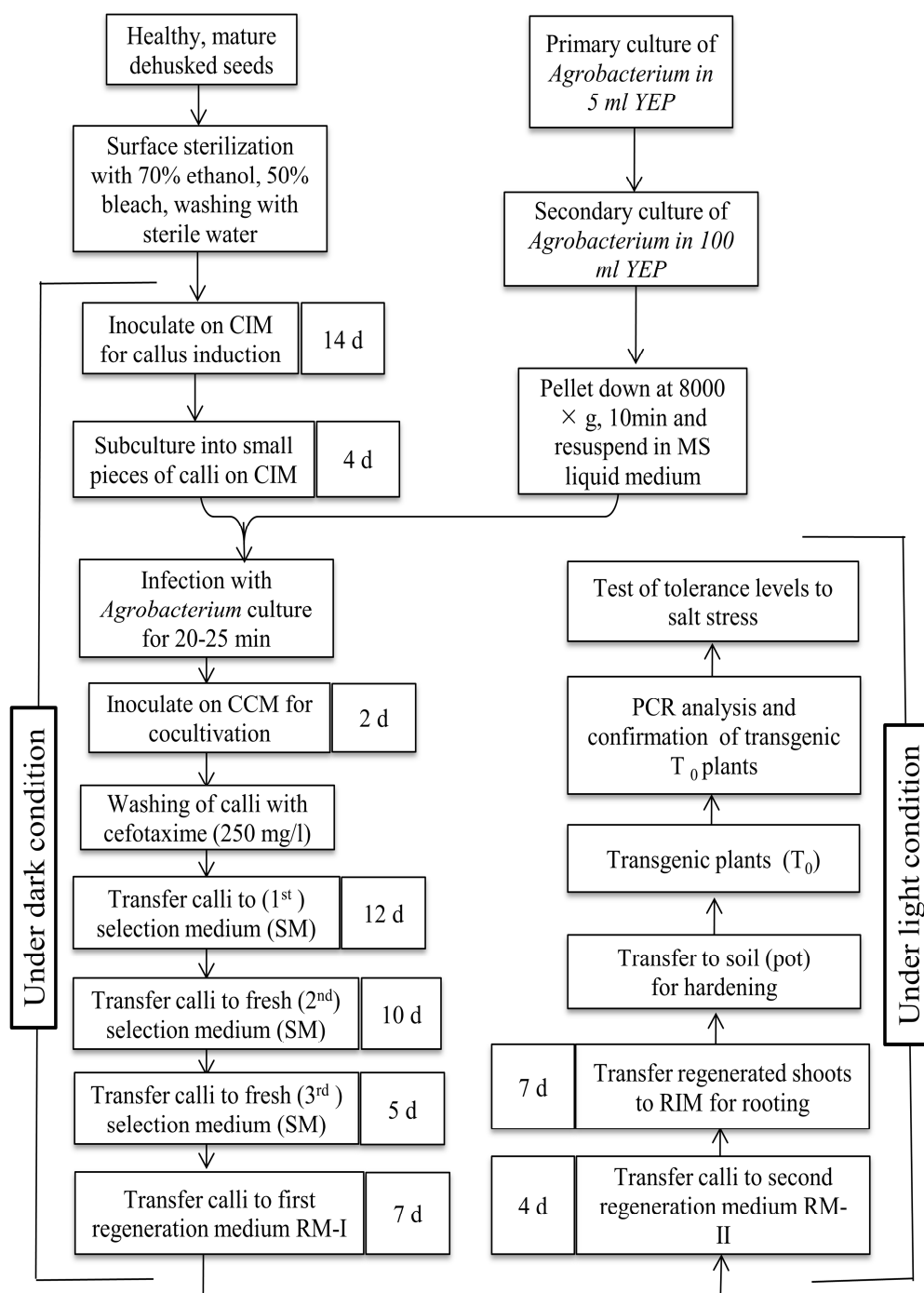


Fig. 24: Schematic representation shows the essential steps of following protocol for plant transformation. The composition of different media was described in methods (Section 6.3.2.2).

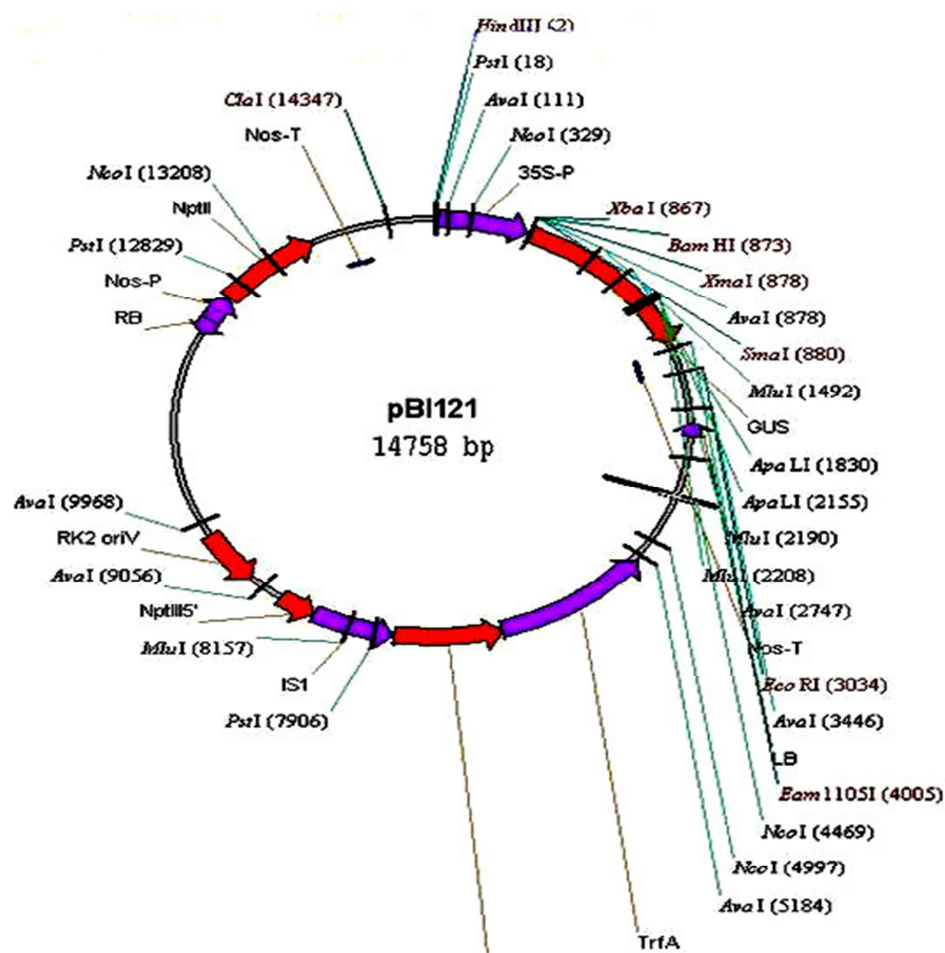


Fig. 25: pBI121 vector map showing its restriction sites.

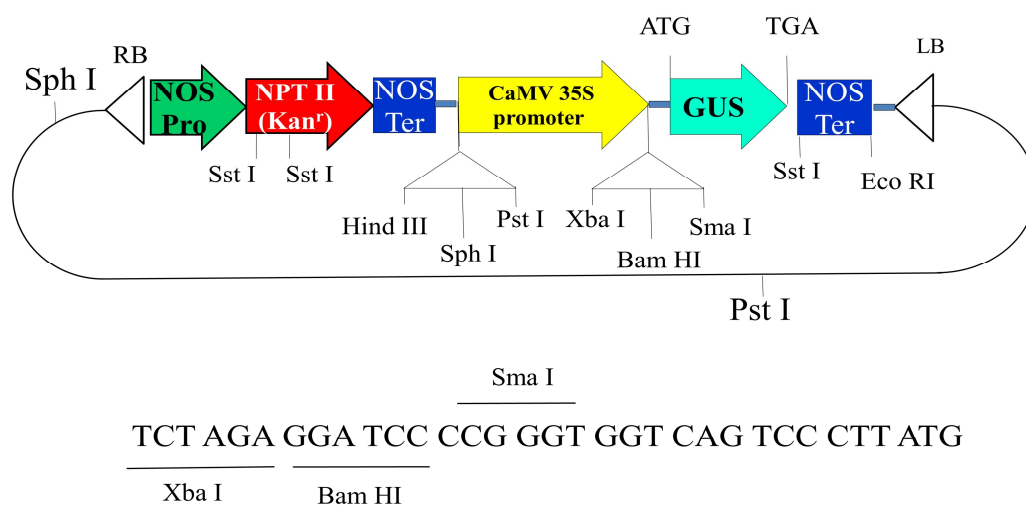


Fig. 26: Cassette of gene constructs showing the restriction sites in pBI121 vector.

6.3.2.6 Infection, co-cultivation and selection of transformed calli

The embryogenic calli which sub-cultured for 4 d were collected; and immersed in *Agrobacterium* culture (LBA4404) for 20-25 min with gentle shaking at 50 rpm for *Agrobacterium*-infection. The *Agrobacterium*-infected calli were taken out and placed on sterile Whatman filter paper No. 1 for 5 min. Then the calli were transferred to co-cultivation medium (CCM) and incubated in the dark at $27 \pm 1^\circ\text{C}$ for around 48 hrs (Ali et al. 2007). After appearing slight growth of *Agrobacterium* around most of the calli, they were taken out and rinsed 8-10 times with 250 mg/l cefotaxime in sterile distilled water. Then the calli were placed on sterile Whatman filter paper No. 1 and transferred onto selection medium (SM) for 1st selection and incubated in dark at $27 \pm 1^\circ\text{C}$ for 12 d. After the first selection the calli of black or brown color were removed from the culture; and the healthy calli with cream color were transferred to fresh SM for 2nd selection and kept for 10 d in same culture condition as described for first selection. Finally the calli were transferred to fresh SM medium for 3rd selection and maintained dark condition at $27 \pm 1^\circ\text{C}$ for 5 d to be proliferated (**Fig. 24**).

6.3.2.7 Regeneration of transformed calli

Two types of regeneration media were used by transformed calli; such as, RM-I and RM-II. For regeneration, two phases were followed; such as first regeneration phase in dark and second phase in light. After third selection of transformed calli color of black or brown were discarded; and only the granular calli were transferred to first regeneration phase and cultured for 7 days in dark at $27 \pm 1^\circ\text{C}$. After that the calli were transferred to second regeneration phase in light at $27 \pm 1^\circ\text{C}$ till the shoots were regenerated. The regeneration frequency was calculated as per the formula given below (Zaidi et al. 2006).

$$\text{Regeneration frequency (\%)} = \frac{\text{Number of microcalli regenerating shoots}}{\text{Number of microcalli incubated}} \times 100$$

Regenerated shoots were shifted onto root formation medium (RFM) and the culture was kept at $27 \pm 1^\circ\text{C}$ in light till sufficient root production.

6.3.2.8 Molecular confirmation of putative transgenic plants

Histochemical staining of GUS expression in leaf samples was performed as described by Jefferson et al. (1987). The transformation efficiency was calculated as per the formula given below (Zaidi et al. 2006).

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

GUS positive plants together with the wild type (WT) plants were analyzed by PCR for confirmation of transgenics. Total genomic DNA was extracted from transgenic plants as described by Dellaporta et al. (1983). In this case, the specific primers (forward: 5'-CGTCTAGAATGGCAGGAGTTGCAC-3' and reverse: 5'-CGGGATCCTCACAGAAGTTCAGCCACATTG-3') were used for PCR analysis. The PCR products were analyzed by 1% agarose gel, containing ethidium bromide and visualized under gel documentation unit. Southern blot analysis was done to analyze the PCR-positive plants for confirmation of *PDH47* gene integration in the transgenic rice genome. For this purpose, 20 µg genomic DNA was isolated from wild type plants along with GUS and PCR positive plants; and restriction digestion was worked out with *Xba*I, a single cutter of T-DNA. Thereafter, the products of the digestion were resolved on 0.8% agarose gel; and subsequently blotted onto nylon membrane and cross-linked by ultra-violet (UV) ray. Using the standard protocol the membrane was probed with, *PDH47* gene was radiolabeled and visualized in phosphor imager. That work has been done under the research group of Dr. Narendra Tuteja at ICGEB, Plant Molecular Biology, New Delhi, India on February 2015 with kind help of my research supervisor during his visit to ICGEB.

6.4 Results and Discussion

6.4.1 Cloning and restriction digestions

PDH47 gene was cloned to pGEM-T vector of DH5-alpha and confirmed by colony PCR using by gene specific primer. From the colony PCR, in ten (10) cases out of ten positive results were shown (**Fig. 27, 28**). Thereafter, restriction digestion of the targeted gene was done with *Bam*HI / *Xba*I (insert) and *Hind*III internal cutter of gene and vector. In this case, two positive results were found out of two (**Fig. 29**), and hence the presence of targeted gene was confirmed in vector pBI121 which was used for the infection made by *Agrobacterium tumifaciens* (LBA4404).

6.4.2 Callus induction and preparation for plant transformation

Among the seventeen rice cultivars tested in the present study however, BRRI dhan29 showed significantly highest regenerating ability (described detail in Chapter IV), and hence the cultivar was selected subjected to plant transformation. In order to use in the experiments on plant transformation, the calli were induced from mature seeds of cultivar BRRI dhan29 using the CIM prepared as MS supplemented with 2.5 mg/l 2,4-D, 0.5 mg/l BAP, 30 g/l maltose, 0.3 g/l casein hydrolysate, 0.4 g/l L-proline, gelled with 3.0 g/l phytigel (**Fig. 30 a, b, c**). In this case, sufficient amount of embryogenic calli were produced that used for bacterial infection and subsequent co-cultivation. Hiei et al. (1994) examined the efficiency of various tissues in rice namely, shoot apices and segments of roots from young seedlings, scutella and immature embryos; and mentioned that the calli derived from matured seed as well as scutella were been most amenable for transformation. The potential for callus formation, regeneration and successful transformation in rice is a varietal characteristics and indica rice reported to be inferior to japonica in this respect (Maggioni et al.1989, Tyagi et al. 2007).

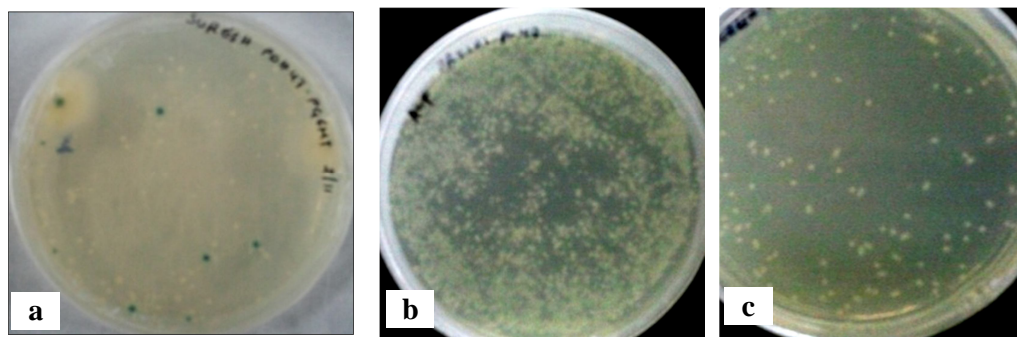


Fig. 27 (a-c): Cloning of *PDH47* gene. a) blue-white screening, b) cloned to pGEM-T vector using DH5-alpha and c) colonies grown after overnight incubation at 37°C.

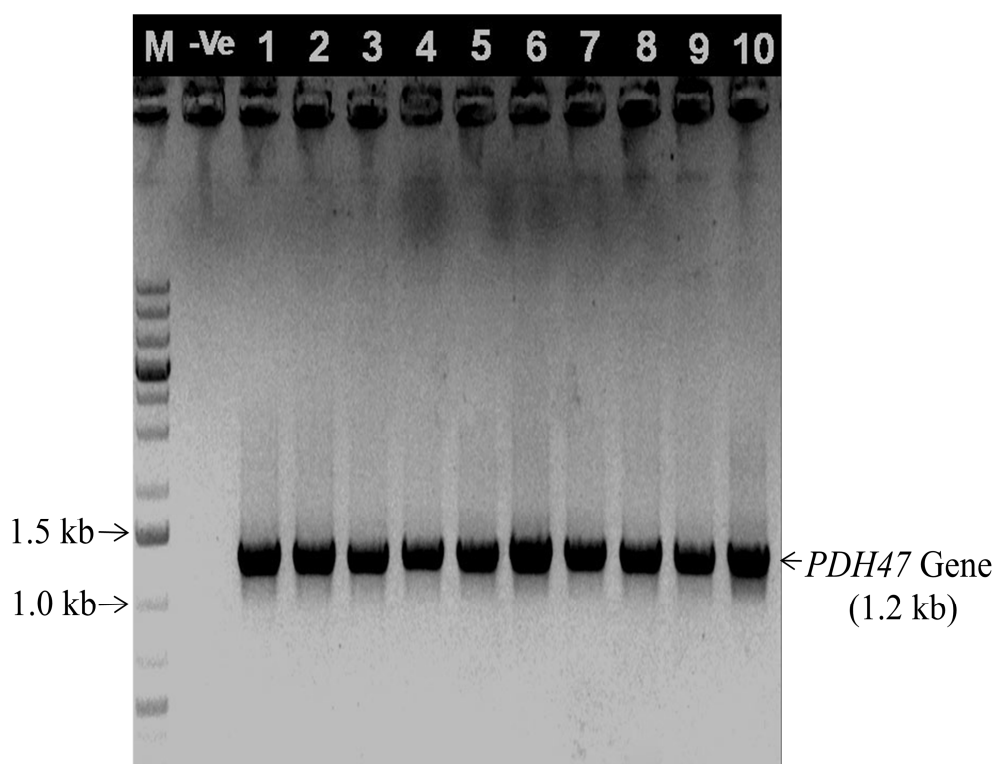


Fig. 28: Colony PCR of *PDH47* gene in pGEM-T vector. Here all ten (1-10) colonies showed positive results. The colonies checked by PCR with gene specific primers (M = Marker, -Ve = Negative control).

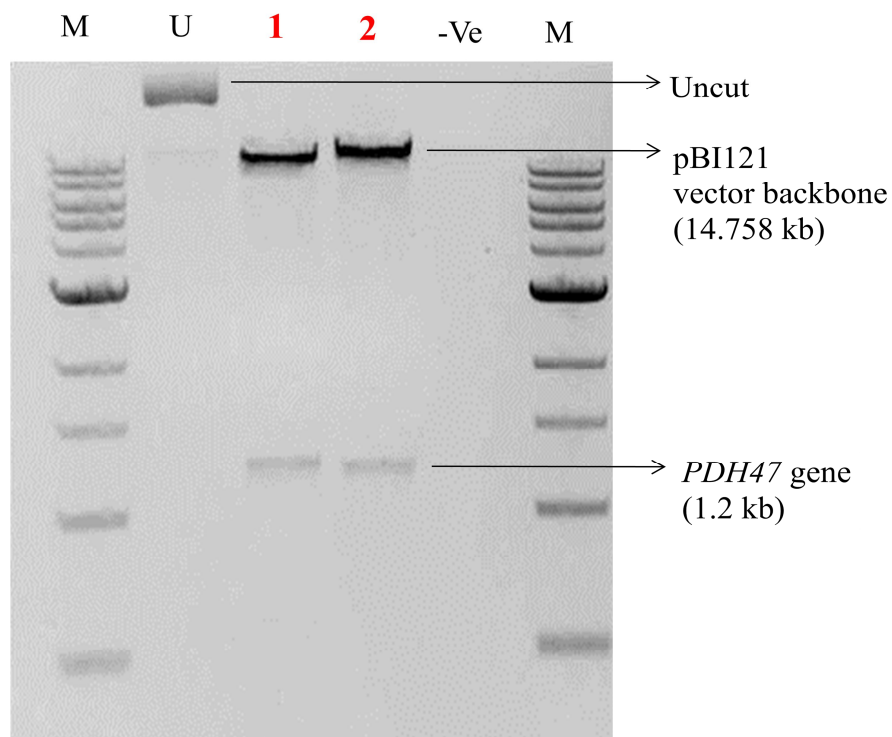


Fig. 29: Restriction digestion of *PDH47* in pBI121 with *Bam*HI / *Xba*I (insert) and *Hind*III (internal cutter of gene and vector). Lane 1 and 2 both showed positive results. M = Marker, U = Uncut, -Ve = Negative control.

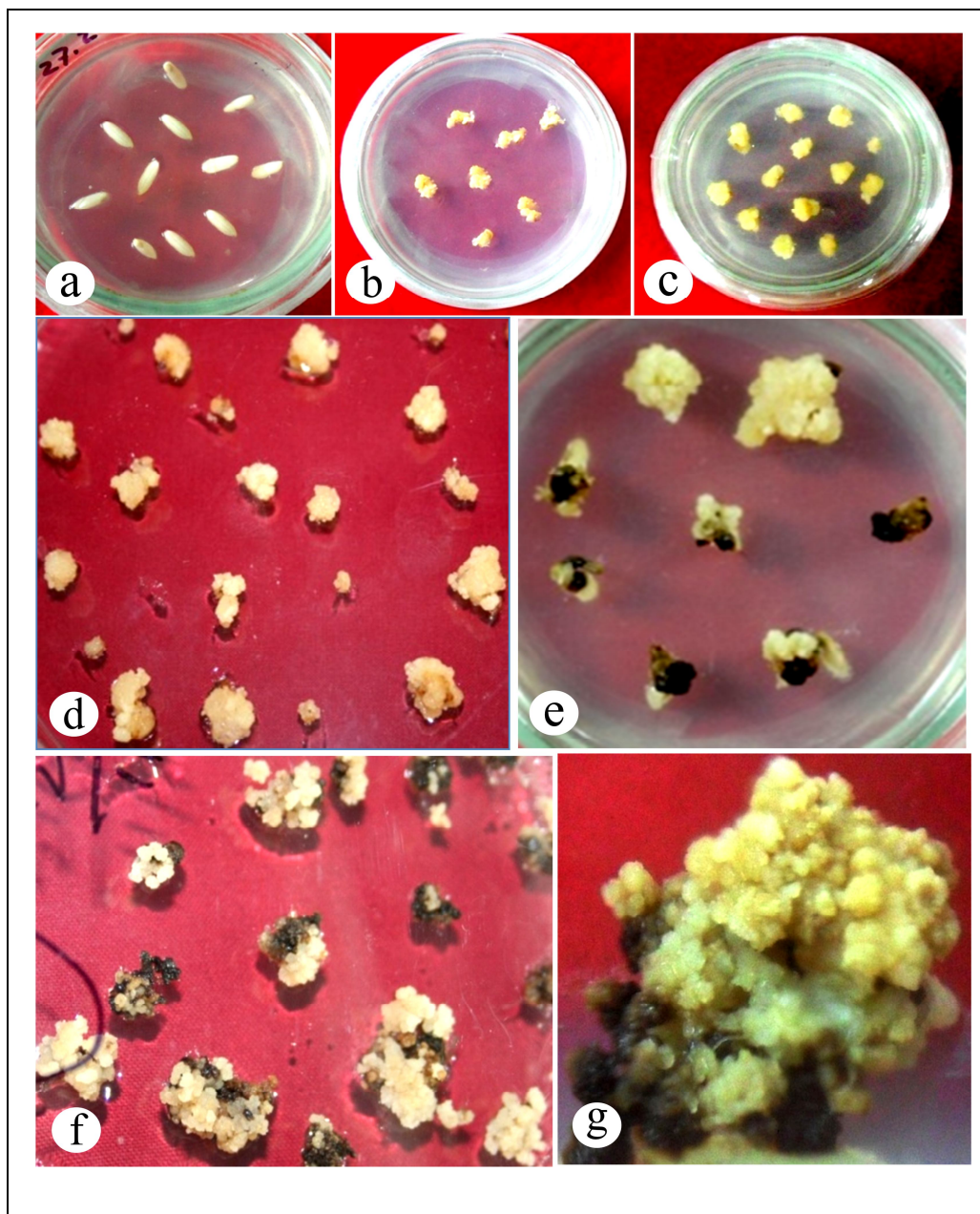


Fig. 30 (a-g): Callus induction, bacterial infection for transformation and selection of infected calli: a) inoculated rice seeds, b) calli age of seven days, c) sub-cultured calli age of 3-4 weeks, d) co-cultivation of infected calli, e) calli cultured on first selection medium, f) calli after third selection and g) infected embryogenic calli.

6.4.3 *Agrobacterium*-mediated transformation and confirmation of transgene integration

The calli after sub-cultured were immersed in *Agrobacterium* culture for bacterial infection (**Fig. 30 d, e, f**) and subsequently regenerated plants from infected calli (**Fig. 31**). Genetic transformation, mediated by *A. tumefaciens* LBA4404 harbouring the virulent plasmid pBI121 construct of targeted gene *PDH47* (1.2 kb) was performed and 23.33% and 18.33% transformants were obtained by the calli derived from MNOS and ZE. After obtaining large number of regenerated shoots, these were subsequently shifted to the root formation medium (RFM) which led to development of normal roots. The regenerated plants (T_0) exhibited normal morphology and growth at plantlet stage. The transgenic plant (T_0) analyzed for their transgenic status by screening for the expression of reporter-GUS. Thereafter, the plants which showed the expression of GUS were further confirmed for transgene integration by PCR analysis.

The putatively transformed plants which regenerated by ZE and MNOS derived calli were tested separately for the presence of the targeted gene *PDH47* (1.2 kb). The successful integration of transgenics into whole genome was confirmed by PCR and southern hybridization. For this purpose PCR analysis was performed using gene-specific primers as mentioned in methods (section 6.3.2.8). In case of putatively transgenic of ZE derived calli, the results showed that out of eleven putatively transgenic (Pt1 - Pt11), PCR positive was found in four cases (Pt4, Pt6, Pt7 and Pt9) (**Fig. 32**). Therefore, it could be confirmed preliminary that the targeted gene *PDH47* was integrated into transgenic plants which regenerated from the *Agrobacterium* infected calli. On the other hand non-transformed BRRI dhan29 was used as control (-Ve Cont.) where no band was found. However, for further analysis southern hybridization was performed for the PCR positive plants such as Pt4, Pt6, Pt7 and Pt9. In this case, positive results were gained for all the tested plants regarding PCR positive analysis which confirmed the targeted gene *PDH47* integration successfully (**Fig. 33 b**). However, in the previous reports, Chan *et al.* (1993) obtained a few transgenic rice plants by inoculating immature embryos with a strain of *A.*

tumefaciens and proved the inheritance of the transferred DNA to the progeny by southern hybridization. Raineri et al. (1990) obtained transformed rice cells that expressed neomycin-phosphotransferase (NPT) and β -glucuronidase (GUS) activities and they suggested that T-DNA had been transferred to, integrated in, and expressed in, rice cells. Gould et al. (1991) described the transfer of genes for NPT and GUS into shoot apices of corn, subsequent regeneration of plants, and detection of the transferred genes in the F₁ progeny by southern hybridization. In the present study, however the southern analysis of the transformed plants with single cutter enzyme (*Xba*I) between left and the right border of T-DNA confirmed them to be either single or two copy integrations. From the transformation, positive transformants were selected and desired size bands of *PDH47* (1.2 kb) indicated positively transformed rice plant of BRRI dhan29 for the transgenic plants T₀ (**Fig. 32, 33**).

Lin et al. (2009) reported about 6.0% transformation efficiency for indica rice varieties. In the present investigation approximately similar frequency on transformation was recorded for BRRI dhan29 (6.67%) through described techniques. Lin et al. (2009) used the half strength MS, while MS basal was used in the present study along with the modifications were done in the use of acetosyringone in the bacterial culture, and the use of separate infection as well as co-cultivation media. Liquid culture medium was also used to grow the bacteria.

Aananthi et al. (2010) reported that in rice, efficient transformation and subsequent regeneration using *Agrobacterium*-mediated methods are dependent on several factors. These included choice of the explants, hormonal composition of the medium used, nutritional supplements, temperature and duration of co-cultivation, virulence of the *Agrobacterium* strain and concentration of antibiotic selection marker. The similar reports have been published by several authors (Saharan et al. 2004, Katiyar et al. 1999, Tyagi et al. 2007). Bakhsh et al. (2014) examined the effectiveness of *Agrobacterium* strains in tobacco, and reported that LBA4404 performed the maximum number of transgenic shoots followed by GV2260, EHA105, C58C and

AGL1. The presence of the transgene and its effect was tested by the experiment on leaf disk senescence (LDS) in 100 mM and 200 mM salt stress (**Fig. 35**). During the assay, the pieces leaf disc of wild type (WT) BRRI dhan29 plant (control) showed dark brown stripes with necrotic tissues after 7 days immersed in 200 mM NaCl level. On the other hand the leaf of the transgenic plants remained largely green and healthy. Therefore, the transgenic plants might be carried the targeted gene while they were been able to hold the chlorophyll or other pigments into their mesophyll tissues and showed tolerance to salt stress.

6.4.4 Efficiency of explants response to transformation

In this study, two types of calli derived from different explants i.e. zygotic embryos (ZE) and mosocotyl nodal segment (MNOS) were tested for the experiments on transformation. The results showed that 23.33% and 18.33% putatively transformed plants were regenerated from the infected calli of MNOS and ZE respectively (**Fig. 34**). While in PCR analysis, greater number of PCR positive plants was gained from the putatively transformed plants produced by ZE (6.67%) than MNOS (3.33%). Hence, it could be claimed that ZE derived calli were more suitable than the calli of MNOS to be infected by *Agrobacterium tumefaciens* LBA4404. The frequency of gene integration in genetic transformation significantly depends on the calli source as well as the types of explants. Hiei et al. (1994) reported similar results testing eight types of explants, and gained maximum gene expression by scutellum callus using *A. tumefaciens* EHA101 (pIG121Hm).

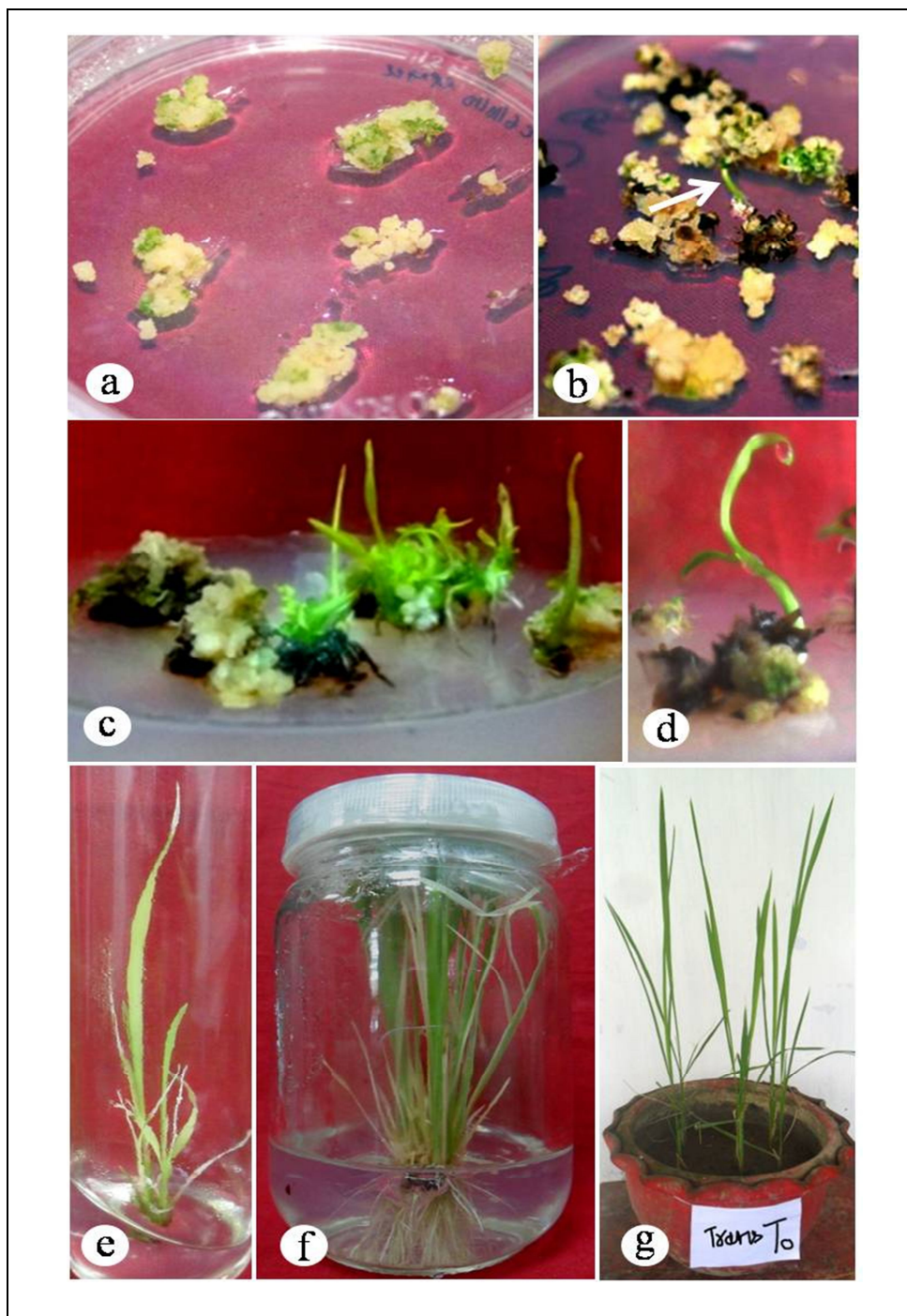


Fig. 31 (a-g): Regeneration from transformed calli. a) green spots produced on *Agrobacterium* infected calli in selection medium (Hygro-H) after four weeks of sub-cultured showing bacterial infection for transformation, b) shoot regeneration initiation (arrow marked), c-d) regenerated shoots, e) shoots transferred to root formation medium, f) regenerated well rooted plantlets and g) transformants (T_0).

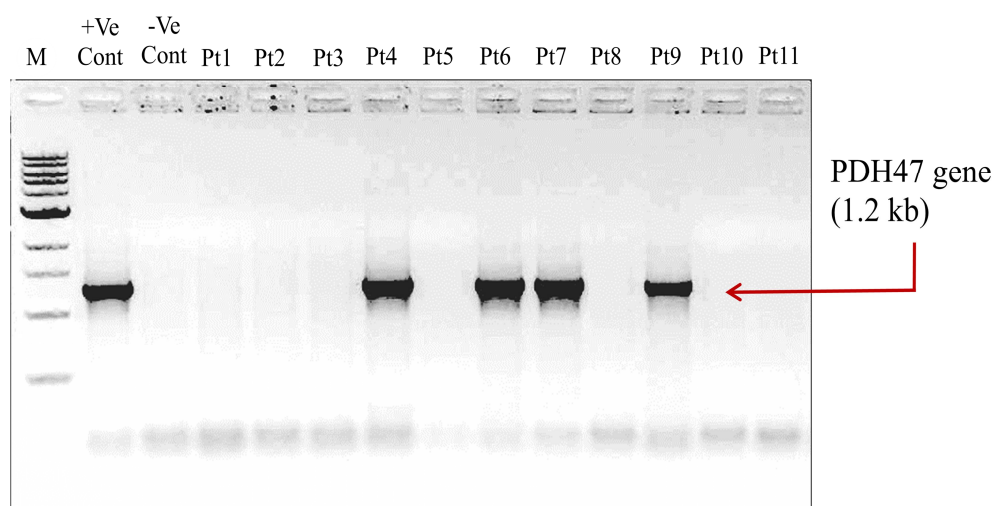


Fig. 32: PCR analysis of putatively transformed plants (T_0) for confirmation of integration the targeted gene *PDH47* (1.2 kb) in the regenerated plants from infected calli. The transgenic plants Pt4, Pt6, Pt7 and Pt9 showed positive results. In case of Pt1, Pt2, Pt3, Pt5, Pt8, Pt10 and Pt11 band of targeted gene was not formed. M = Marker, Cont = Control and Pt = Plant (T_0).

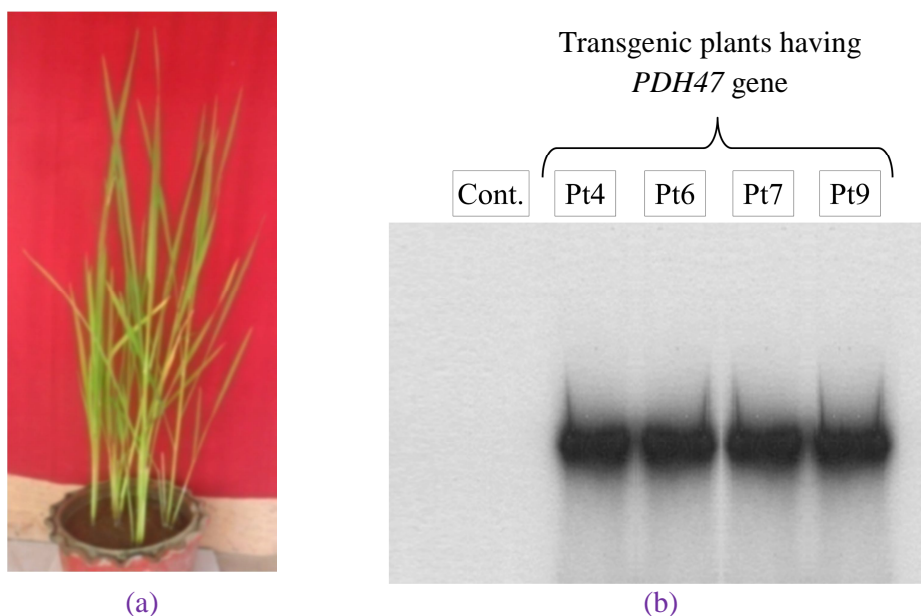


Fig. 33 (a-b): Confirmation of transgenic plants overexpressing of *PDH47* gene. a) Transgenic plants produced *in vitro* from *Agrobacterium* infected calli and b) Southern analysis of the transgenic plants confirming targeted gene insertion.

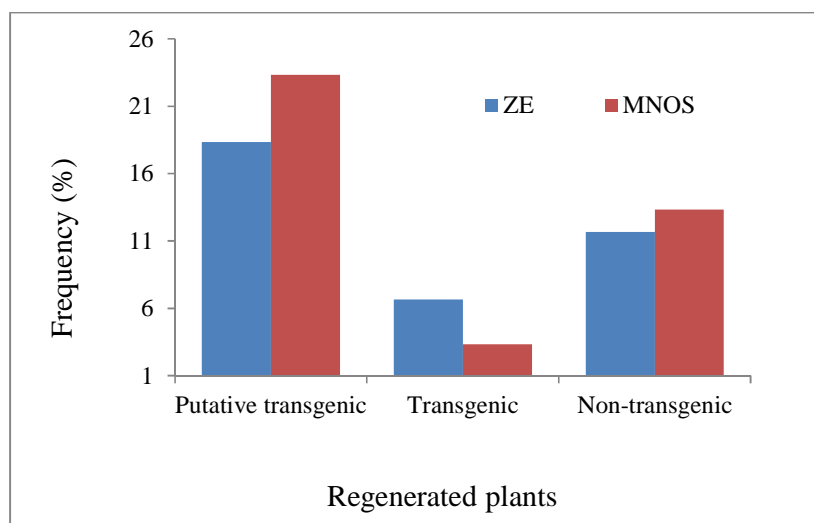


Fig. 34: Results on different experiments conducted for transgenic plants (T_0) analysis. LDSA = Leaf disk senescence assay, SBA = Southern blot analysis.

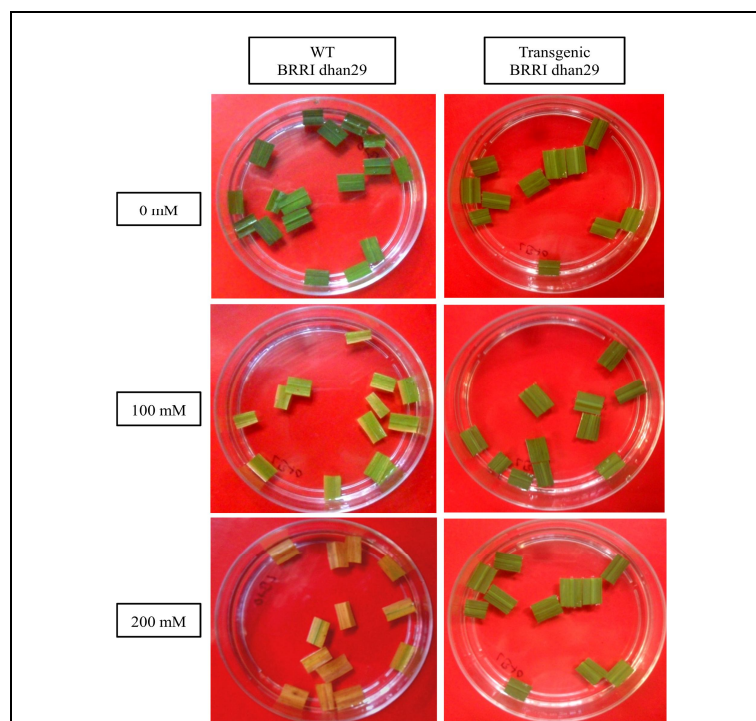
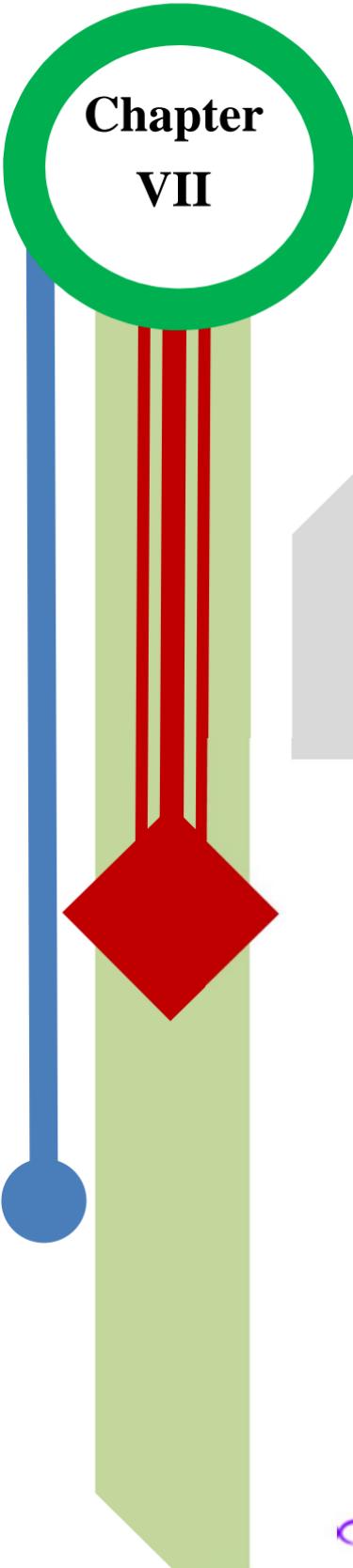


Fig. 35: Leaf disk senescence assay of T_0 transformants response to NaCl induced stress. The leaf of transgenic BRR1 dhan29 showed healthier and greener appearance than WT (control).

6.5 Conclusion

Plant transformation has been done using the cultivar BRRI dhan29 the significantly highest efficient regenerating ability *in vitro* by *Agrobacterium*-mediated LBA4404 strain and 23.33% and 18.33% putatively transgenic plants were regenerated which analyzed by PCR. In this cases 6.67% and 3.33% PCR positive plants were obtained and all the PCR positives showed confirmation of the targeted gene *PDH47* (1.2 kb) into the transgenics were proved by southern hybridization. The transgenics (T₀) showed tolerance to NaCl stress at 200 mM level when LDS was tested. Hence, the plants would be considered for further molecular analysis related to gene expression. However, the standardized protocol of plant transformation for BRRI dhan29 may helpful to advance biotechnological research to develop highly efficient transgenics characterized of expected agronomical traits. Because of time limitation, the advance related experiments couldn't be conducted in this study. Further works however, would be taken based on the present investigations.



**Chapter
VII**



**General
Discussion**



7. General Discussion

Experiments on somatic embryogenesis were conducted testing the effect of plant growth regulators (PGRs) for seventeen Bangladeshi indica rice varieties. For this purpose, fifteen types of hormonal concentration and combination (T_1 - T_{15}) were used; and considering the efficiency to callus induction and subsequent plant regeneration the genotypes were screened (Chapter IV, **Table 10**). Among the varieties BRRI dhan29 (78.67%) performed the best to CI in T_5 (2.5 mg/l 2,4-D) and the lowest ability was observed for both the varieties BR11 and BRRI dhan27 recording 33.33% of callusing in T_1 (0.5 mg/l 2,4-D). Therefore, it was found that the range (33.33 - 78.44%) of callusing ability was highly varied among the genotypes tested in this study. The variety BRRI dhan29 produced 2.35 fold higher frequency of callus induction (CI) than BR11 and BRRI dhan27. Analysis of variance showed that the varieties were significantly differed at $p \leq 0.001$.

Hoque et al. (2007) described the significant variation for six Bangladeshi indica rice genotypes at $p = 0.001$; and callus induction frequency was ranged as 60.5 - 76.3%. They measured the highest callusing for BRRI dhan38 on MS basal medium supplemented with 2.0 mg/l 2,4-D, 20.0 g/l sucrose and gelled with 8.0 g/l agar. In this case highest 78.67% callusing was measured for BRRI dhan29 using MS + 2.5 mg/l 2,4-D with 30.0 g/l sucrose. However, the results were near to similar on callusing efficiency for different variety, different concentration of 2,4-D and sucrose. Alam et al. (2012) reported that on callus induction for four indica rice varieties *viz.* BRRI dhan28, BRRI dhan29, BRRI dhan47 and Bina dhan-7 and recorded highest frequency for BRRI dhan47 (86.00%) in MS +500 mg/l L- Proline + 2.0 mg/l 2, 4-D + 0.8 mg/l BAP. Zafar et al. (1992) obtained highest callus formation in basmati rice cv.370 on MS with 2 mg/l of 2,4-D. Somatic embryogenesis was achieved on either N6 or MS medium with 2.0 mg/l each of 2,4-D and Kinetin. The present investigations argued with the previous reports and claimed that the difference might be occurred due to effect of different rice genotype and different growth regulators used in the experiments. Such genotypic effect of indica rice was described by some authors previously (Hoque and Mansfield 2004, Vega et al. 2009, Ramesh et al. 2009,

Haque et al. 2013, Joyia and Khan 2013, Mannan et al. 2013, Puhan et al. 2012, Ghobeishavi et al. 2014). Furthermore, another report mentioned that potentiality for callus induction and plantlets regeneration in culture condition depends on a number of factors, like genotype of the donor plants, physiological and biochemical status of the explants, composition and concentration of different ingredients of culture medium etc. Upadhyaya et al. (2015) used different concentrations of 2,4-D (1.0, 1.5, 2.0, 2.5 mg/l) for callus induction from mature embryos of three japonica rice cultivars viz. Sita, Rupali and Masuri. They recorded 63.36 - 92.23% callus induction and mentioned that all the varieties produced calli at highest rate in 2.0 mg/l 2,4-D concentration. In the present study, MS medium supplemented with seven different concentrations of 2,4-D (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mg/l) singly and/ or combined with 0.5 mg/l Kinetin, have been tested and the results were ranged as 33.33 - 78.67% where the highest callusing was measured from 2.5 mg/l 2,4-D (T₅) for BRRI dhan29 (**Table 10**). However, Mannan et al. (2013) reported that about the effect of 2,4-D (2.0 mg/l) singly and combined with Kin (0.25, 0.5, 1.0, 1.5 mg/l) for callusing. They recorded the highest values in 2.0 mg/l 2,4-D (singly) and in 2.0 mg/l 2,4-D + 0.5 mg/l Kinetin (combined) for the rice varieties Kalijira (97.22%) and Chinigura (94.44%); while they reported that callus initiated earlier at the combined condition. The present investigation agrees with their reports as 2,4-D produce calli with higher rate than combined with Kinetin. Therefore, this study suggests that 2.5 mg/l 2,4-D individually influenced better than combined with Kinetin efficient callusing.

Four different basal media which commonly used in tissue culture i.e. MS, N6, LS and SK1 were examined to investigate their effect on callusing for the studied varieties. In this case, an effective and optimal concentration of 2,4-D (2.5 mg/l) was added as constant with all the basal media individually. Here, MS medium showed its supremacy than others, because all the varieties produced maximum callus in MS. The variety BRRI dhan29 gave the maximum callusing (77.33 %) in MS; while the same variety produced 61.33% in SK1 (**Table 12**). The range of callus induction was 36.00-77.33%; hence wide range of variation was found in the efficiency of the media tested. Aananthi et al. (2010) examined the effect of MS and N6 for callusing and MS

had been considered as better than N6. The results of this study agreed well with their reports and state that the nutrient composition of MS is suitable for *in vitro* rice culture.

All the studied varieties were investigated to find out their efficiency on plant regeneration using fifteen types of hormonal combinations ($H_1 - H_{15}$) as shown in Chapter IV (**Table 9, 14**). Among the varieties BRRI dhan29 performed the best to plant regeneration (70.67%) in H_{11} (2.0 mg/l BAP + 0.1 mg/l Kin + 0.5 NAA) and the lowest ability was observed for the variety BRRI dhan32 (22.67%) in H_{15} (4.0 mg/l BAP + 0.5 mg/l Kin + 0.5 mg/l NAA). Therefore, it was found that the range (22.67 - 70.67%) of regeneration ability was highly varied among the genotypes examined in this study. The variety BRRI dhan29 produced 3.12 fold higher frequency of regeneration than BRRI dhan32. Analysis of variance showed that the varieties were significantly differed at $p \leq 0.001$. A remarkable result was found for BRRI dhan32 considering the features on callus induction and regeneration. In this case, although BRRI dhan32 showed the lowest ability to regeneration (27.67%) it was more efficient to induce callus (77.33%) ranked as the 2nd highest among the seventeen rice genotype tested in this study. Hence, it could be suggested that further research is needed to increase the efficiency on plant regeneration for this variety.

Hoque et al. (2007) reported that on plant regeneration for Bangladeshi indica rice varieties *viz.* BR14, BRRI dhan28, BRRI dhan38, BRRI dhan39 and BRRI dhan40 and mentioned highest 44.30% regeneration for BRRI dhan38 using MS medium supplemented with 1.0 mg/l Kin + 1.0 NAA mg/l. Alam et al. (2012) examined four rice varieties and recorded highest regeneration for BRRI dhan29 (84.33%). By using MS + 2.0 mg/l BAP + 0.5 mg/l NAA + 1.0 mg/l Kin maximum 31.75, 38.10 and 30.16 % regeneration was recorded for BR10, BRRI dhan32 and BRRI dhan47 respectively (Siddique et al. 2014). Haque et al. (2013) added 1.0 mg/l Kin and 1.0 mg/l NAA to four concentrations of BA (1.0, 2.0, 3.0 and 4.0 mg/l) as supplements of MS medium for plant regeneration from BRRI dhan53; and recorded highest regeneration 75% in 3.0 mg/l BA. For the varieties Kalijira and Chinigura Mannan et al. (2013) measured 83.33 and 91.67% regeneration in 0.5 mg/l BAP + 0.1 mg/l IBA respectively. The present study on plant regeneration showed dissimilarities with the

previous reports particularly for the regeneration frequency. Such dissimilarities might be happened due to different genotype along with the plant growth regulators (PGRs). However, it was expressed that embryogenic and non-embryogenic callus formation as well as plant regeneration is reported to be genetically determined as described for different cereals including rice (Hoque and Mansfield 2004, Khalequzzaman et al. 2005). Genotypic effect on regeneration has also been described by several authors in indica rice (Khatun et al. 2010, Islam et al. 2010b, Alam et al. 2012, Sah et al. 2014, Puhan et al. 2012, Joyia and Khan 2013). Abe and Futsuhara (1986) reported that many Japonica rice cultivars respond better than the indica varieties. In other cereals *viz.* maize (Morshed et al. 2014), barley (Serhantova et al. 2004, Haque and Islam 2014) and wheat (Saha et al. 2015) were reported the effect of genotype to regenerate plant *in vitro*. The yield of callus and somatic embryos depends on the composition of the medium (Fennel et al. 1996). Therefore, BRRI dhan29 would be considered as suitable genotypes along with suitable growth regulators (2.0 mg/l BAP + 0.1 mg/l Kin + 0.5 NAA) for its better regeneration.

Abiotic stresses such as heat, cold, nutrient, wind, water, drought, salt etc. affect negatively on growth and development of plants. Out of them drought and high salinity show adverse effect on the growth, development and productivity. While some physical (desiccation, heat, cold) and chemical (AgNO_3) stress pre-treatment factors influence positively to improve plants through *in vitro* culture as well as callus induction and subsequent plant regeneration. By treating with the suitable stress factors plant cell and callus might be stimulated resulting would be able to survive in adverse or stress condition.

In the present study, calli were cultured up to 4 weeks onto the four different salt (NaCl) induced stress condition (50, 100, 150, 200 mM) for testing the survival capability of cell or calli. The results showed significant difference among the tested rice varieties *viz.* BR10, BRRI dhan32 and BRRI dhan47. Among the varieties, the calli of BRRI dhan47 exhibited the highest viability (45.33%) after four weeks cultured in 200 mM (~20 dS/m) NaCl (**Table 19**). On the other hand, no calli of BRRI dhan32 could be existed in 200 mM when they were cultured up to four weeks, while BR10 showed poor ability to be alive in salt stress with 10.67% viable callus.

Hence, it could be claimed that BRRI dhan32 was the salt sensitive; while the variety BRRI dhan47 tolerant in nature and BR10 might be considered as moderate genotype showing some ability to survive in NaCl induced *in vitro* stress.

BRRI (2011) mentioned that the variety BRRI dhan47 carried 6 dS/m salt tolerance in their whole life and at plantlet phase they were able to survive at 12-14 dS/m (equivalent to 120-140 mM). Soheilikhah et al. (2013) recorded 47 - 64% decreased cell viability in NaCl induced stress for Safflower (*Carthamus tinctorius* L.) varieties, and mentioned that the accumulation of Na⁺ ions and osmolytes could play an important role in osmotic adjustment in cells under saline stress. In this study 58.67% viable calli were recorded for BRRI dhan47 when the calli were cultured in 150 mM (14 dS/m) up to four weeks. Besides, according to USDA salinity laboratory, the soil having an electrical conductivity (EC_e) of 40 mM (~ 4 dS/m) NaCl or more is considered as saline soil. Therefore, the present investigation shows the agreement with the previous reports and seems that BRRI dhan47 may carries genes in their genomes which related to functions on abiotic stress tolerance. Allen et al. (1994) reported that several genes are involved to control salinity tolerance in different species. For some species, salt sensitivity may be greatest at germination, whereas for other species, sensitivity may increase during reproduction (Howat 2000).

After culturing the calli in different salt concentrations (50, 100, 150 and 200 mM) for four weeks, relative growth rate (RGR), tolerance index (TI) and relative water content (RWC) were determined. In the top level of NaCl stress (200 mM) the range of recorded RGR values were 0.11 - 1.03, TI were 0.02 - 0.20, and WRC were 7.03 - 10.23% respectively (**Fig. 10, 11, 12**). The maximum value of RGR (1.03) for BRRI dhan32 expressed that the variety was able to grow in salt stress condition. Moreover, the variety BRRI dhan47 showed its tolerance ability to survive in stress condition carrying the highest TI value (0.20) than other two varieties. The values of RGR and TI were decreased in higher salt levels than the lower concentrations. The phenomena might be occurred due to interference of Na⁺ and Cl⁻ ions on uptake and translocation processes, nutritional imbalance might be created; and resulting growth of callus is inhibited (Errabi et al. 2007). However, calli of BRRI dhan47 was least affected by the highest dose of salt stress (200 mM) and exhibited high ability grow compared to

BR10 and BRRI dhan32. The calli of BRRI dhan47 might accumulate less Na⁺ ion than salt BR10 and BRRI dhan32 and the phenomena were related to osmotic potential (OP) of the cells. In several species K⁺ is a major cation and contributor to adjust OP under stress condition (Santos-Diaz and Ochoa-Alejo 1994, Bajji et al. 2000). In salt stress Na⁺ concentration is increased which lead to decrease concentration of K⁺ among rice genotypes. As a result essential ions are to be imbalanced and cells of salt sensitive varieties could not be survived. Such reports were mentioned previously in rice (Lutts et al. 1996, Basu et al. 2002), sugarcane (Errabii et al. 2007, Patade et al. 2008), *Carthamus tinctorius* (Soheilikhah et al. 2013) and *Cynara cardunculus* (Benlloch-González et al. 2005).

Partial desiccation, a physical stress factor influenced positively to regenerate rice plants *in vitro* with high frequency (Saharan et al. 2004, Haq et al. 2009). This factor has also been reported to be beneficial for regeneration of indica rice varieties (Jain et al. 1996, Diah and Bhalla 2000, Sahrawat and Chand 2001, Alam et al. 2012, Makerly et al. 2012) and in japonica rice (Tsukahara and Hirosawa 1992). Rance et al. (1994) reported that 2-4 folds higher regeneration from 3 hrs desiccated calli than the control in rice genotypes viz. PN1, IR72 and IR64. Compare to undesiccated calli, 2 and 5 folds higher regeneration was recorded from 48 and 72 hrs desiccation in MR220 and MR232 respectively (Makerly et al. 2012). Under this study the data were recorded around 2–3 folds higher regeneration from desiccated calli in BR10, BRRI dhan32 and BRRI dhan47 (**Table 20, Fig. 14**). The obtained results are in agreement with previous findings and states that an optimal desiccation can improve the efficiency of the calli to be regenerate plants *in vitro*.

In the present study, it has been investigated that the optimal period of desiccation was depended on callus age. In this case, the callus of higher age needed to lower period of desiccation for its maximum efficiency to regenerate plant. On the other hand, higher period of desiccation was needed for the calli of lower age to be desiccated at optimal level.

It was also investigated that partially desiccated calli were more able to be regenerated in salt induced stress condition than un-desiccated calli. Desiccation

improves the efficiency of plant regeneration in date palm (Ibrahim et al. 2012). Such beneficial effect was reported in several plants *viz.* sugarcane (Kaur and Gosal 2009), citrus (Singh 2014) and wheat (Stipesevic and Kladvko 2005, Scotti-Campos et al. 2014).

In the present study, for plant regeneration in NaCl induced stress condition, response of partially desiccated calli was investigated. The results showed that desiccated calli gave higher regeneration than the controls (**Table 21**). At the top level of NaCl stress (200 mM), the variety BRRI dhan47 performed around 2 folds higher regeneration than the control (un-desiccated) when the calli were pretreated for 45 hrs partial air desiccation. The un-desiccated calli of BR10 and BRRI dhan32 were been unable to regenerate plant at 200 mM NaCl level. But, when they were pretreated for 45 hrs desiccation, the calli were been able to be regenerated plant. In this case, 11.11% and 4.74% regeneration was recorded for BR10 and BRRI dhan32 while in both varieties no regenerations were found in control. Makerly et al. (2012) reported that regeneration capability was varied depending on the duration of desiccation. At optimal period of partial air desiccation characterized the cells of the callus to survive and adapt at adverse physiological stress. For application of desiccation pretreatment, the cells of calli lost their water, resulting the calli gained higher osmotic potential (OP). Therefore, the ability was developed for the calli to up take water in NaCl stress condition and hence, they could be able to survive and regenerate plants. However, it could be suggested that application of physical stress factor as well as partial air desiccation pretreatment was more effective for the variety BRRI dhan47 to enhance the capability of regeneration in NaCl stress.

Organogenesis is an important way to regenerate plants using various organs of plant in tissue culture. Some authors have regenerated plant *in vitro* in rice via organogenesis from different explants, such as root (Hoque and Mansfield 2004), leaf (Ramesh et al. 2009, Afrasiab and Jafar 2011), coleoptile (Sahrawat and Chand 2001, Ghobeishavi et al. 2014), mature embryo (Pons et al. 2000) and protoplast (Jelodar et al. 2002). By using the inflorescence (Chen et al. 1985) and root (Abe and Futsuhara 1985) of rice reports on *in vitro* plant regeneration have also been described. Moreover, organogenesis has been done form epicotyl in sweet orange (Filho 2001)

and pummelo cv. Cikoneng (Dewi et al. 2013) and hypocotyl in blackgram (Saini and Jaiwal 2002). To culture *in vitro*, root, hypocotyl, epicotyl shoot tip, shoot apical meristem, nodal and internodal part of stem, leaf, auxillary bud, flower buds, anther, pollen, cotyledons, immature seed and mature seeds were also used by Jha and Ghosh (2009).

In the present study, for organogenesis as well as *in vitro* organ culture, nine types of explants were used to examine the totipotent nature of the cells of the calli. In this case eight types of explants viz. radicle, adventitious root (AR), adventitious root tip (ART), coleoptile, mesocotyl nodal segment (MNOS), mesocotyl internodal segment (MINS), primary leaf (PL) and secondary leaf (SL) were taken from different parts of vegetative organs of *in vitro* germinated rice seedlings and the another was zygotic embryo (ZE). All the explants were cultured in MS + 2.5 mg/l 2, 4-D + 1.0 mg/l kin + 300 mg/l L-proline + 400 mg/l for callus induction. The results showed that 93.13% ZE responded to produce callus and the frequency 19.58% was the lowest recorded for coleoptile (**Table 23, 24**). All the responded explants were been able to produce primary callus (PC); while all the PCs couldn't be proliferated to produce secondary callus (SC). Furthermore, the explants which came from vegetative organs did delay to initiate callusing than ZE. In this case all the responded explants of ZE initiated callus induction within 2-5 days. Hence, it could be express that the cells of ZE influenced quickly by the plant growth regulators than the cells of vegetative organs. However, Deo et al. (2010) reported that several factors i.e. type of explants, plant genotypes, culture conditions including plant growth regulators (PGRs), affect rice somatic embryogenesis and subsequent plant regeneration.

The frequency of primary callus induction (PCI) was measured and maximum for ZE (79.06%) and the lowest value was recorded for coleoptile (15.42%). Therefore, the rage of variation was remarkable and it was statistically significant at $p \leq 0.001$. The secondary callus induction (SCI) frequencies were ranged as 12.50 - 70.63%; and ANOVA expressed the highly significant variations among the explants tested. Among the explants ZE showed highest CI (70.63%); and out of the rest eight different explants obtained from the vegetative organs of seedlings, MNOS (61.11%) gave the maximum value (**Table 24**). The result indicated that the source of explants

affected CI, and a clear indication was observed that ZE is the best explants considering its callusing efficiency and earlier initiation of CI. Radhesh et al. (2013) reported that 39.6, 53.6, 47.3 and 99.0% CI for root, coleoptile, leaf base and mature seed in indica rice cultivar IR36, respectively. In their reports mature seed (zygotic embryos) were performed the best than the others. Therefore, the present investigations agreed well with their reports and claimed that the cells of ZE were more totipotent to be dedifferentiated as well as efficient to induce calli. Sahrawat and Chand (2001) recorded 72.8% embryogenic callus from the explant coleoptile of indica rice seedlings. No callus induction was found using the explant leaf blade rice cultivar IR36 (Ramesh et al. 2013). The present study, 12.50% callus induction was recorded by coleoptile which was lowest among the explants examined. On the other hand 22.86% and 27.14% callusing was recorded from primary leaf and secondary leaf respectively. The findings argued with the previous reports. This argument would be happened due to difference in genotype, age of explants and/or composition of medium along with the combinations of PGRs. Sahrawat and Chand (2001) also reported that the explants age of 7 days produced callus with lower frequency than the explants age of 3 and 4 days.

The present study investigated the efficiency of the calli of four age groups (3, 4, 5 and 6 w) derived from different explants and the results showed that the calli induced by MNOS age of 6 w regenerated plants with the highest frequency (90.63%). Whereas, 55.00% regeneration was recorded for 4 w old calli of ZE and the lowest was for the calli age of 3 w derived from AR (32.50%) (**Table 25**). The results showed significant difference to regeneration among the explants along with the ages of the calli at $p \leq 0.01$. By observing the data, it was clearly invented that although ZE was more efficient for callus induction, its calli was not so efficient to regeneration than the calli derived from MNOS; yet MNOS was poor to induce calli. Hence, it could be claimed that the cells of different explants of rice specially the variety BRRI dhan29 didn't carried equal ability in both de-differentiation (callus induction) and re-differentiation (regeneration). Verdeil et al. (2007) described the mechanisms of de-differentiation of somatic cell into a totipotent embryogenic cell which can either be regenerated or develop an embryo and subsequent entire plant.

The importance of totipotency has been investigated earlier *in vitro* plant regeneration by Haberlandt (1901). In 1838, Schwann and Scheiden reported that on totipotency theory as cells are autonomic, and in principle, are capable of regenerating to give a complete plant. Moreover, Hossain (2010) reported that although each cell of a plant body carries the potentiality to regenerate plant, yet the meristematic cells are the best one to prove the presence of totipotency. However, it was observed that ZE, the calli of lower age were more efficient than higher age. On the other hand opposite phenomenon was found for other eight types of explants which came from vegetative organs. In this case, calli of higher age showed maximum efficiency than lower age calli. Hence, the age of calli is an important factor to influence *in vitro* culture. Importance of callus or embryo was described by several authors (He et al. 1988, Islam 2010b, Siddique et al. 2014). Hoque and Mansfield (2004) reported that on the effect of age of explants in regeneration root derived callus of indica rice. Hossain (2010) expressed that explants are consisted of various types of cells which are stimulated when treated with suitable growth regulators, then the mRNA of cells are stimulated produce protein-enzymes related to cell division. An explant which can perform better to induce callus, might not be able enough to regenerate plants. In contrast, the calli derived from the explant which lower efficient to callusing, could be able to be re-differentiated at high rate and produce plant. Among the explants tested, MNOS, MINS, coleoptile, PL and SL are the explants carrying such features.

Genetic transformation using helicase gene *PDH47* (pea DNA helicase 47) was conducted for the genotype BRRI dhan29. For this purpose embryogenic calli age three to four weeks were used and agro-infection was conducted by *Agrobacterium tumefaciens* (LBA4404) which carried the targeted gene construct followed by the standard protocol (Sahoo et al. 2011). Putatively transgenic plants were regenerated from infected calli known as T₀. In this case, two types of calli were used which derived from different explants i.e. zygotic embryos (ZE) and mesocotyl nodal segment (MNOS). Frequencies of putative transgenic regeneration were recorded as 23.33% and 18.33 ZE and MNOS respectively.

PDH47 gene was cloned to pGEM-T vector of DH5-alpha and confirmed by colony PCR using by gene specific primers. From the colony PCR, all ten (10) cases out of

ten positive results were shown (**Fig. 27, 28**). Thereafter, restriction digestion of the targeted gene was done with *Bam*HI / *Xba*I (insert) and *Hind*III internal cutter of gene and vector. In this case, two positive results were found out of two (**Fig. 29**), and hence the presence of targeted gene was confirmed in vector pBI121 which was used for the infection made by *Agrobacterium tumefaciens* (LBA4404). Hiei et al. (1994) gained maximum gene expression in rice by scutellum callus using *A. tumefaciens* EHA101 (pIG121Hm). Aananthi et al. (2010) reported that efficient transformation and subsequent regeneration dependent on several factors such as explants hormonal compositions, nutritional supplements, temperature and duration of co-cultivation, virulence of the *Agrobacterium* strain and concentration of antibiotic selection marker in rice. The similar reports have been published by several authors (Katiyar et al. 1999, Saharan et al. 2004, Tyagi et al. 2007). In tobacco Bakhsh et al. (2014) examined the effectiveness of *Agrobacterium* strains, and reported that LBA4404 performed the maximum number of transgenic shoots followed by GV2260, EHA105, C58C and AGL1.

In this investigation putative transgenics were analyzed by PCR, and out of eleven (11) plants tested, four (4) plants showed positive results. On the other hand, no bands were shown by seven lanes as they were considered the non-transgenic plants. Based on the numbers of inoculated or co-cultivated calli, 6.67% and 3.33% transgenic plants were obtained from the calli of ZE and MNOS respectively. Thereafter, the transgenics were further analyzed by southern hybridization to be confirmed the targeted gene integration into the PCR positive plants. In this case, all positive PCR showed positive results to the southern blot analysis (**Fig 33 b**). Hence, the plants were considered as the transgenic holding the targeted gene *PDH47* of BRRI dhan29. Previously, Lin et al. (2009) reported about 6.0% transformation efficiency for indica rice varieties. In the present investigation approximately similar frequency on transformation was recorded for BRRI dhan29 (6.67%) by southern hybridization through described techniques. Gould et al. (1991) described the transfer of genes for NPT and GUS into shoot apices of corn, subsequent regeneration of plants, and detection of the transferred genes in the F₁ progeny by southern hybridization. Raineri et al. (1990) obtained transformed rice cells expressing neomycin-phosphotransferase (NPT) and β -glucuronidase (GUS) activities and they suggested that T-DNA had been transferred to, integrated in, and expressed in rice cells.

However, transgenic plants were tested for leaf disk senescence (LDS) using 0 mM (control), 100 mM and 200 mM salt (NaCl) levels to check the tolerance ability. By the visual observation it was found that 28.16% of the PCR and southern positive plants (transgenic) were able to hold their chlorophyll contents or other plant pigments at 200 mM stress for one week. Hence, they would be considered as the transgenic of *PDH47* having tolerance ability to salt. On the other hand a large amount of transgenic couldn't perform better as shown in non-transgenic plants of BRRI dhan29 (control). The transgenic plants would be considered for further molecular analysis related to gene expression. It is claimed that the expression of integrated gene, tolerance to drought stress was not to be experimented due to time limitation while it could be conducted in further research work. However, the standardized protocol of *Agrobacterium*-mediated plant transformation for BRRI dhan29 may be helpful to advance biotechnological research to develop highly efficient transgenics characterized on expected agronomical traits and further works would be taken based on present investigations.

Chapter VIII

Summary



8. Summary

The present study was conducted to improve rice (*Oryza sativa* L.) variety considering the features related to abiotic stress tolerance through biotechnological approaches. For this purpose seventeen Bangladeshi indica rice varieties viz. BR3 (Biplob), BR4 (Brrishail), BR8 (Asha), BR10 (Progoti), BR11 (Mukta), BR23 (Dishari), BR24 (Rahamot), BRRI dhan27, BRRI dhan28, BRRI dhan29, BRRI dhan32, BRRI dhan34, BRRI dhan37, BRRI dhan42, BRRI dhan43, BRRI dhan47 and BRRI dhan57 were selected which (mature seeds) obtained from Bangladesh Rice Research Institute (BRRI), Regional Center, Rajshahi, Bangladesh.

In this study, the experiments were done on somatic embryogenesis (SE), organogenesis (organ culture), relative growth rate (RGR), tolerance index (TI), relative water content (RWC), test of viability of calli in salt stress, effect of partial air desiccation to enhance regeneration, enhancement of regeneration in salt stress applying desiccation pretreatment and *Agrobacterium*-mediated genetic transformation. Experiments on somatic embryogenesis (Chapter IV) were conducted for screening a suitable variety to culture *in vitro* along with the advance biotechnological researches. For this purpose the experiments on the effect of plant growth regulators (PGRs) to callus induction (CI) (Section 4.1.3.1), effect of media to CI (Section 4.1.3.2), effect of PGRs to plant regeneration (PR) (Section 4.1.3.3) and effect of media to regeneration (Section 4.1.3.4) were worked out. In case of CI, fifteen types of hormonal concentration and combination (T_1 - T_{15}) with MS medium was used. The results showed that out of seventeen varieties, BRRI dhan29 performed the highest number of CI (78.67%) in T_5 (2.5 mg/l 2,4-D) followed by BRRI dhan32 (77.33%) in T_5 , BRRI dhan29 (74.67%) in T_6 (3.0 mg/l 2,4-D), BRRI dhan29 (73.33%) in T_4 (2.0 mg/l 2,4-D), BRRI dhan32 (72.00%) in T_6 , BRRI dhan32 72.00% in T_4 , BRRI dhan29 (70.67%) in T_{12} (2.5 mg/l 2,4-D + 0.5 mg/l Kin.), BR10 (69.33%) in T_6 and BRRI dhan28 (69.33%) in T_5 (Section 4.1.3.1). On the other hand BR11 and BRRI dhan27 both varieties gave the lowest number of CI 33.33% in T_1 (0.05 mg/l 2, 4-D).

To observe the effect of media on CI, four types of basal media such as MS, N6, LS and SK1 supplemented with 2.5 mg/l 2,4-D were tested; and the highest frequency of CI was recorded for BRRI dhan29 (77.33%) in MS; followed by 74.67% ,72.00% and 68.00% for BRRI dhan32, BR10 and BRRI dhan28 in MS respectively (Section 4.1.3.2). On an average, the highest value of callusing was recorded from MS (59.76%) out of four media; and the lowest was in SK1 (46.98%).

Another experiment was conducted to find out the effect of PGRs on plant regeneration by adding fifteen types of hormonal combinations (H_1 - H_{15}) with MS (Section 4.1.3.3). The maximum performance was found for BRRI dhan29 (70.67%) when 3 weeks old calli were transferred to H_{11} (2.0 mg/l BAP + 1.0 mg/l Kin + 1.0 NAA). On the other hand, the variety BRRI dhan32 (22.67%) showed the lowest regeneration in H_{15} (4.0 mg/l BAP + 0.5 mg/l Kin + 0.5 mg/l NAA).

Experiment on the effect of media to regenerate plant was done in addition of H_{11} (2.0 mg/l BAP + 1.0 mg/l Kin + 1.0 NAA) with four types of basal media viz. MS, N6, LS and SK1. The results expressed that the calli of BRRI dhan29 were more efficient to be regenerated at the maximum frequency of 69.33% when the calli age of 3 weeks were transferred to MS medium supplemented with H_{11} (Section 4.1.3.4). In case of other media, maximum 62.67, 61.33 and 56.00% regenerations were recorded from N6, SK1 and LS respectively. The medium LS gave the lowest regeneration for BR10 (24.00%). Moreover, BRRI dhan29 gave the maximum values of plant regeneration for all media. On an average the highest regeneration was recorded (51.14%) from MS and the lowest was in LS (40.23%).

In Section 4.2.3.2.1, another experiment was conducted related to *in vitro* abiotic stresses factors to improve callus ability to be existed in stress condition. In this case, calli were cultured up to four weeks on callus induction medium with addition of four concentrations of NaCl (50, 100, 150 and 200 mM). The varieties BRRI dhan47, BR10 and BRRI dhan32 gave 53.33, 14.67 and 2.67% viable calli after one week cultured in the highest concentration of NaCl (200 mM) respectively. When the calli of BRRI dhan32 were cultured for 4 weeks there was no calli viable; while in case of BRRI dhan47, 45.33% calli were alive at 200 mM salt level.

Three weeks old calli were cultured in 50, 100, 150 and 200 mM NaCl concentrations for 3 weeks also; and RGR, TI and RWC were determined. The recorded RGR values were 1.03, 0.23 and 0.11 at 200 mM salt for BRRI dhan47, BR10 and BRRI dhan32 respectively. In control (without salt) the varieties BRRI dhan47, BRRI dhan32 and BR10 showed RGR values as 5.12, 4.61 and 4.02 respectively (Section 4.2.3.2.2). BRRI dhan47 showed the highest ability to grow in salt stress showing TI of 0.20. Comparatively lower TI numbers were recorded in other two genotypes of BR10 (0.02) and BRRI dhan32 (0.06). The WRC values were also lower for BR10 (7.22%) and BRRI dhan32 (7.03%) than BRRI dhan47 (10.23%). The growth pattern for BRRI dhan47 was determined where RGR was increased up to two weeks for all NaCl levels tested. After two weeks, RGR was restricted at 100, 150 and 200 mM stress levels. In case of 50 mM level, RGR values were gradually increased up to four weeks.

Calli of different age groups (3, 4, 5 and 6 w) were partially desiccated (15, 30, 45 and 60 hrs) and cultured on regeneration medium. Among the studied genotypes 4 weeks old calli of BRRI dhan32 performed around 2 folds higher regeneration (76.19%) than control (38.10%) when it was pretreated by 45 hrs air desiccation. The variety BR10 and BRRI dnan47 also gave more than 2 folds higher regeneration than the undedicated calli (Section 4.2.3.2.3). Moreover, it was investigated that the calli of lower age needed to higher desiccation pretreatment within a range than comparatively callus of higher age to perform maximum regeneration.

The calli desiccated at optimal level be able to regenerate plant in NaCl stress. In this case four weeks old calli were pretreated at 45 hrs air desiccation and transferred to regeneration medium that supplemented with NaCl levels (50, 100, 150, 200 mM). After desiccation pretreatment the calli of BRRI dhan47 gave 1.98 folds higher regeneration (26.98%) compared to control (14.29%) in 200 mM NaCl (Section 4.2.3.2.4). In the same stress level (200 mM), other two varieties BR10 and BRRI dhan32 could not be regenerated from undedicated calli; whereas, after desiccation pretreatment they were been capable to regenerate plants with frequency of 11.11% and 4.76%, respectively.

The experiments on organogenesis (Chapter V) were conducted to investigate the efficiency of various types of explants on regeneration. In this case, nine different types of explants of which eight obtained from vegetative organs of *in vitro* germinated seedlings age 10 days viz. radicle, adventitious root (AT), adventitious root tips (ART), mesocotyl nodal segments (MNOS), mesocotyl internodal segments (MINS), coleoptile, primary leaf (PL) and secondary leaf (SL); and another one from mature seeds (zygotic embryo, ZE). All the explants were cultured for callus induction in MS supplemented with 2.5 mg/l 2, 4-D + 1.0 mg/l kin + 300 mg/l L-proline + 400 mg/l. The results indicated that ZE was the best one than others showing both features of callus initiation earlier (within 2 - 5 days) and producing the maximum number (79.06%) of primary callus induction (Section 5.3.1 and 5.3.2). The rest explants initiated calli within 10 - 17 d, while after inoculation till 13 day, no initiation of callus was found for coleoptile, it initiated at 14 - 17 d. The frequencies of primary callus induction were 63.33, 53.50, 30.36 and 26.43% for the explants MNOS, MINS, SL and PL which came from vegetative organs, respectively. The lowest value of primary callus was recorded for coleoptiles (15.42%). Secondary callus was developed by proliferation the primary callus of different explants; and the frequencies of secondary callus induction were 70.63, 61.11, 51.00, 27.14 and 22.86% for ZE, MNOS, MINS, SL and PL respectively, while the explants coleoptile produced the lowest number of secondary callus (12.50%). In this case, it was observed that a large amount of primary callus couldn't be proliferated for producing secondary callus of ZE. On the other hand almost all the primary callus produced secondary one for MNOS and MINS.

It was observed that using three weeks old calli derived from nine different explants were cultured to determine the relative growth rates. The results showed that the calli derived from ZE grew with the highest RGR (3.86); while 2.29, 2.20, 1.69, 1.63, 1.55, 1.52 and 1.15 RGRs were recorded for the explants of MNOS, MINS, ART, AR, SL, PL and coleoptile respectively and lowest for radicle (1.12) (Section 5.3.3). Furthermore, the relative water content (RWC) of calli for all the explants were determined as 0.18 (AR), 0.16 (ART, radicle, ZE), 0.12 (PL), 0.11 (MINS, coleoptile, SL) and 0.10 (MNOS).

The calli of four age groups (3, 4, 5 and 6 weeks) induced from different explants were separately transferred to regeneration medium (MS + 2.0 mg/l BAP + 0.5 mg/l NAA + 1.0 mg/l Kin). Among nine explants, significant difference to plant regeneration was found; and the calli age of 6 w, induced by MNOS regenerated at the highest frequency (90.63%). The calli age of 5 w, induced by PL, SL, coleoptile, AR and radicle gave 75.00, 72.92, 71.88, 47.50 and 45.00% regeneration respectively (Section 5.3.4). In the case of ZE, maximum 55.00% plant regeneration was recorded from the calli age of 4 w. Without consideration of callus age, on an average the calli came from MINS performed with the highest frequency of plant regeneration (83.60%) and 38.13% in AR.

Survival ability in field culture of *in vitro* plants, produced from the calli of different explants were determined separately and the results were differed significantly at $p \leq 0.001$. The highest survival rate (90.63%) was recorded for the plantlets obtained from 6 w old calli of MINS. The plantlets regenerated from radicle, coleoptile, PL and SL showed approximately similar survival ability. On the other hand the plants developed from the calli age of 6 w by ZE survived at the lowest rate (47.50%) among the explants tested under this study.

Agrobacterium-mediated transformation was conducted using BRRI dhan29 using targeted gene of *PDH47*. The co-cultured calli derived from ZE and MNOS produced 23.33 and 18.33% putatively transgenic plants respectively after infection of *Agrobacterium tumefaciens* strain of LBA4404 (Section 6.4.3). In case eleven putatively transgenics plants were obtained when the calli derived from ZE were used, and out of eleven four plants showed PCR positive, analyzed through genomic DNA isolation. Further studied have been done and all four showed positive results that confirmed gene integration by southern hybridization analysis. It was observed that the potentiality of explants to regenerate transgenic plants were varied 6.67% for ZE and 3.33% for MNOS. In the experiment of leaf disk senescence (LDS), the transgenics were able to hold chlorophyll contents in their mesophyll tissues and remaining healthy with green in color.

Chapter IX

References



9. References

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Curriculum Vitae

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B. Educational background

Examination	Year	Board / University	Division/Class
SSC	1988	Rajshahi Board	First
HSC	1991	Rajshahi Board	Second
B. Sc (Hons) in Botany	1994	National University	First Class (Second position)
M. Sc (Thesis on Cytogenetics)	1995	National University	First Class (First position)

C. List of Publications

i) Published articles

1. **Abu Baker Siddique**, Israt Ara, S M Shahinul Islam and Narendra Tuteja (2014) Effect of air desiccation and salt stress factors on in vitro regeneration of rice (*Oryza sativa* L.). Plant Signaling Behavior 9:12 (e977209- 1-10).
2. **Abu Baker Siddique** and S. M. Shahinul Islam (2015) Effect of light and dark on callus induction and regeneration in tobacco (*Nicotiana tabacum* L.). Bang. J. Bot. 44(4): 643-651.
3. Mozidul Haque, **Abu Baker Siddique** and S. M. Shahinul Islam (2015) Effect of silver nitrate and amino acids on high frequency plants regeneration in barley (*Hordeum vulgare* L.). Plant Tissue Cult. & Biotech. 25(1): 37-50.
4. Selim Morshed, **Abu Baker Siddique**, S. M. Shahinul Islam (2014) Efficient Plant Regeneration using Mature and Immature Embryos of Maize (*Zea mays* L.). Int. Journal of Agriculture Innovations and Research 3(3): 2319-1473.
5. Supria Saha, **Abu Baker Siddique**, Bakul Bhattacharjee, Md. Shahadat Hossain, S. M. Shahinul Islam (2015) Enhanced callus induction and regeneration by PGRs in Bangladeshi wheat (*Triticum aestivum* L.) cultivars. SKUAST Journal of Research 17(1): 29-36.

ii) Abstracts (Conference/Workshop) 04

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4. **Abu Baker Siddique**, Israt Ara, S M Shahinul Islam (2015) Efficient plant regeneration using callus derived from mature seeds and different vegetative organs in rice (*Oryza sativa* L.). Ann. Plant Tiss. Cult. & Biotech Conf. 2014, Org. by BAPTC&B, March 28, Abst. 29, p 42.