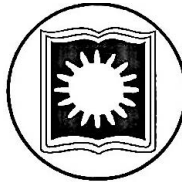


***IN VITRO* REGENERATION AND GUS
GENE TRANSFORMATION IN RICE
(*ORYZA SATIVA* L.)**



**THESIS SUBMITTED FOR THE DEGREE
OF
DOCTOR OF PHILOSOPHY
IN THE
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By
Md. Asaduzzaman

**Plant Biotechnology Laboratory
Institute of Biological Sciences
University of Rajshahi
Rajshahi-6205
Bangladesh**

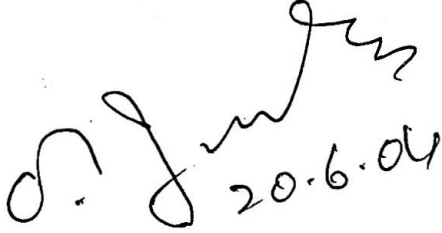
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Certificate

We hereby certify that the thesis entitled “ *In vitro* Regeneration and GUS Gene Transformation in Rice (*Oryza sativa* L.)” has been submitted by **Mr. M. Asaduzzaman** in fulfilment for the requirements of the degree of Doctor of Philosophy in the field of Plant Biotechnology, Institute of Biological Sciences, Rajshahi University, Bangladesh. It is also certified that the research work embodied in this thesis is original and carried out by Mr.M. Asaduzzaman and the work or part of it has not been submitted before as candidature for any other degree.

We would like to clarify that the author has completed his work under our supervision.

1.  20/6/04
Dr. M. A. Bari Miah
Professor
Institute of Biological sciences
Rajshahi University
Rajshahi-6205
Bangladesh

2.  20.6.04
Dr. O. I. Joarder
Professor
Department of Genetics and Breeding
Rajshahi University
Rajshahi-6205
Bangladesh

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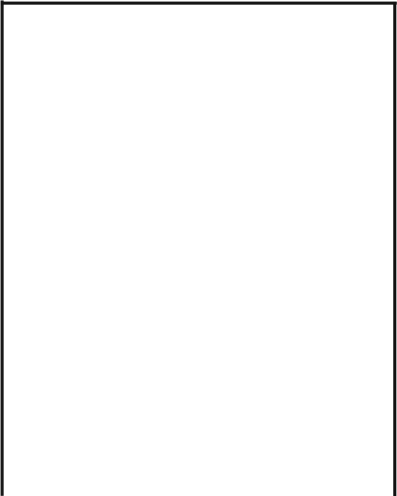
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Summary

CHAPTER-I

1. Summary

Rice is the most important cereal crop in Bangladesh and it was the first attempt in Bangladesh to transform rice plant with target genes. As a new intervention deserves enormous efforts to develop the optimum conditions in order to accomplish effective transformation. Scutellum, leaf base, mesocotyl and root explants of six rice genotypes viz. BR-22, BR-23, BRRI dhan-29, BRRI dhan-30, BRRI dhan-28 and BRRI dhan-31 were cultured in N6, LS and MS basal media containing different concentrations and combinations of 2,4-D to induce callus. 2,4-D plays an important role in callus induction of plant tissue and 2.0 mg/l 2,4-D was the most suitable for callus induction in rice. A wide range of variation in callus induction was marked in this experiment with respect to their explants and treated media. Three varieties BRRI dhan-30, BRRI dhan-28 and BRRI dhan-31 proved more competent ones to induce callus with all of their four explants. On the contrary, the varieties BR-22, BR-23 and BRRI dhan-29 failed to induce callus in all their explants tissue. In respect of explants, the scutellum proved more efficient and gave highest callus formation (77%) followed by root (61%). In respect of nutritional strength, N6 media gave highest percentage of callus formation followed by LS medium. In respect of genotypic performance, BRRI dhan-30 was found as the most efficient variety producing 68% callus induction followed by BRRI dhan-28 (62%).

Calli derived from individual explant were subjected to regeneration media and their comparative performances were evaluated. In the comparative study BRRI dhan-30 proved to be the best variety giving 88% regeneration frequency from somatic embryogenesis. Three explants; scutellum, leaf base and mesocotyl

were used as the target tissue explants and scutellum derived calli gave highest regeneration (88%) followed by mesocotyl (32%). The embryogenic calli were treated to three regeneration media supplemented with different phytohormones and MS medium containing sorbital (3%) was found to be the best medium for regeneration in tested rice varieties. In meristem culture the highest regeneration frequency was observed in excised meristem isolated from four days old seedlings. Variable range of regeneration response was observed in different rice varieties. The varieties BRRI dhan-28 and BRRI dhan-30 gave the highest percentage of regeneration 65% and 56% respectively from meristem culture. Considering the overall performance covering both the purviews of callus induction and regeneration BRRI dhan-30 was selected as the target variety for transformation studies because of its best *in vitro* tissue culture response and economic importance in the country.

Research endeavor has also been intensified to investigate the ability of anthers of seven rice genotypes viz. BR-5, BR-10, BRRI dhan-29, BRRI dhan-31, BRRI dhan-34, BRRI dhan-37 and BRRI dhan-38 for induction of callus and their regeneration. To optimize media composition for rice anther culture, three induction media (Z2, R2 and N6) were used for this research work. After 3-4 weeks of incubation, pollens of the responsive varieties started to produce callus. This study proved Z2 to be more effective over either N6 or R2 for the indica type cultivars in callus induction. It was also observed that only BRRI dhan-38 induced calli from anther culture in all three basal media used for this experiment. The medium Z2 was also found to be the best one produced higher percentage of regenerated green plants and no albino plantlets were formed in this medium. In Z2 medium, use of higher concentration of three vitamins (Thiamine HCl 2.5

mg/l, Pyridoxine HCl 2.5 mg/l and Nicotinic acid 2.5 mg/l) with other components showed better performances in embryo formation.

Efforts were made to improve the anther culture of Bangladeshi rich genotypes by cold pretreatment. The culture condition and duration of cold pretreatment was optimized for five rice genotypes and it was subjected to the rice panicles before culture. For cold pretreatment six durations (1 day, 2 days, 3 days, 4 days, 5 days and 6 days) were used to enhance the embryo formation. 3-4 days for pretreatment was recorded as optimum duration for better embryo yield and their subsequent regeneration

Embryogenic calli derived from anther culture were transferred to two regeneration media (RM) of RMI and RM2. In both the media percentage of regeneration varied from 7% to 34% and highest regeneration was found in BRRI dhan-37 with the addition of Kinetin (0.5 mg/l), BAP (2.0 mg/l) and NAA (1.0 mg/l). RMI medium gave excellent regeneration of plantlets in anther calli. Upon transfer to RMI medium among five genotypes the highest percentage of green plants (33.2%) were produced in BRRI dhan-37 followed by 28.1% green plants recorded in BRRI dhan-38.

Callus induction and regeneration studies revealed that the rice variety BRRI dhan-30 holds the highest potentiality in regeneration property which favors the variety to be selected as the most suitable one for accomplishing genetic transformation. Two plasmid constructs pCL3 and pCL4 harbouring in *Agrobacterium* strain LBA4404 were cloned with the reporter genes GUS and GFP respectively. Conditions for *Agrobacterium*-mediated genetic transformation in rice were optimized for GUS and GFP reporter genes. For standardization of OD in *Agrobacterium* culture, seven OD levels (2.0, 1.8, 1.5, 1.3, 1.0, 0.7, 0.5 and 0)

(control) were tested and the optimum OD was determined by histochemical analysis. The highest numbers of GUS expression calli were observed when the bacterial culture was used at OD 0.5-0.7.

Addition of acteosyringone to the co-cultivation medium is considered to enhance the rate of gene transfer in host plant, particularly for monocot plant like rice. For optimizing the effective concentration of acteosyringone, five different concentrations were tested; 100 ppm, 200 ppm, 400 ppm, 600 ppm and 1000 ppm. Acteosyringone at 1000 ppm was found best efficient in *Agrobacterium*-mediated gene transfer method in rice producing highest number of GUS blue spots on rice calli. Effort was also made to transfer another reporter gene GFP following the same *Agrobacterium* co-cultivation method in rice calli. Under fluorescence microscope the transformed tissue with GFP was clearly demonstrated by distinct green colored images. Under the present investigation of genetic transformation in rice, the blue colored spot and green fluorescence in treated rice calli clearly denominated the genetic integration of GUS gene in calli treated with pCL3 and GFP gene in calli treated with pCL4 which expressed the GUS and GFP activity respectively.

General Introduction

CHAPTER-II

2. General Introduction

Rice is one of the most important cereal and the staple food for more than two billion peoples, predominantly in developing countries (FAO, 1995). World population presently stands at 5.3 billions is likely to exceed 6 billions by the turn of the 21st century and 8 millions by 2020. Due to the faster rate of population, the rice eaters are increasing day by day and the number of rice consumers will probably double by the year of 2020 (Khush and Toenniessen, 1991). It is estimated that about 95 % of the rice is consumed by humans as an unprocessed food and remaining 5 % is used as the processed foods like industrial products and alcoholic beverages. Rice is a widely grown both in upland and in water in tropical and subtropical region.

Rice is a grass belonging to the genus *Oryza* Linn and it is difficult for the Botanists to cite the time when the cultivation was first started in the world. The genus *Oryza* includes twenty five species distributed all over the world including tropical and subtropical regions of Asia, Africa, America and Australia. Among them, two species cultivated widely of which one is *Oryza sativa* and another is *Oryza glaberrima*. *Oryza glaberrima* is cultivated only in small areas in West Africa but *Oryza sativa* is grown worldwide. Rice has well-developed classic genetics having a simple diploid $2n=24$ and has a relatively small genome ($4.2 \cdot 10^8$ bp) in each haploid (Ayres and Park, 1994). Two independent RFLP maps have been established in rice (McCouch *et al.*, 1993 and Saito *et al.*, 1991) that provides important model system for cereal research.

On the basis of geographical distribution and morpho-physiological variations (Kato *et al.*, 1928 and Glaszmann, 1987) the cultivated rice (*Oryza sativa*) is classified by two major sub species, indica and japonica. The indica type accounts for 80 % of the cultivated rice areas in the world covering mostly the tropical areas (Swaminathan, 1982 and Wu *et al.*, 1990) and japonica rice is rather preferred food in temperate regions. Rice grains of indica and japonica differ in starch contents and its starch consists of amylose (Linear polymer of glucose) and amylopectin (branched polymer of glucose), which have the major effect on the physical properties of the grain starch. The amylose content in indica and japonica rice was reported to be 20-30% and 17-22%, respectively (Shimada *et al.*, 1993).

In our country, rice is the most important food grain crop. After Thailand, USA and Pakistan, Bangladesh is the biggest rice producing country in the world. Indica sub species including Basmati varieties are predominantly cultivated in Bangladesh and used as for the preparation for special scented rice dishes. Bangladesh holds a good advantage in producing the high valued aromatic rice from our genetic resources of Kaligira, Badshabhog, Kathari Bhog etc. The aroma of these rice becomes evident when the uncooked grain is grinded, but in the more scented varieties are recognized when the grain is boiled, while the flowers especially the dehiscing anthers and even the vegetative parts, may emit the perfume (Grist, 1986). By traditional breeding a lot of high yielding varieties has been developed in our country (BRRI, 1999). Crossing between BR-6 x Purbachi and BR-5 x Basmati respectively has developed the hybrid BRRI dhan-28 and BRRI dhan-37.

2.1 Tissue Culture of Rice

An efficient and reproducible regeneration of normal and fertile plant has key importance in the application of molecular biology for the improvement of crops (Potrykus, 1990). Tissue culture proved to be difficult for gramineous species because they are recalcitrant to *in vitro* manipulation. The strategies to regenerate rice plants at high efficiency from tissue culture have been evolving steadily during the last decade (Ozawa and Komamine., 1989; Datta *et al.*, 1990a and Kyoizuka *et al.*, 1987; Jain *et al.*, 1995 and Jain *et al.*, 1996). Fujiwara and Ojima (1955) and Amemiya *et al.* (1956) first initiated rice tissue culture by culturing of excised rice immature embryos on nutrients media. Tissue culture limitations in rice were over come by the culturing of immature and undifferentiated tissue and organ explants at different stages of development in nutrient media containing high concentrations of strong synthetic auxins such as 2,4-Dichlorophenoxy acetic acid (2,4-D). Plant regeneration in rice was achieved from organogenesis (Kwata and Ishihara, 1968), somatic embryogenesis (Abe and Futsuhara, 1985) and embryogenesis calli (Wu and Zapata, 1992). Organogenesis generally depends on size of explant and embryogenesis on nutrients media used for the initiation, proliferation of the tissue as well as proper hormonal balance. (Maeda *et al.* 1988).

Rice tissue culture could be used to select resistance regenerates to various abiotic or biotic stresses by adding stressing agent in the culture medium to non-resistant cell level (Dykes and Nabors, 1986). By the addition of sodium salt and polyethylene glycol (PEG) to the culture medium was performed to check the salt and drought tolerant of the cells, respectively (Dykes and Nabors, 1986). The selected regenerates of indica varieties IR8 and IR54 on toxin produced by

Helminthosporum oryza were resistant to brown spot disease (Ling *et al.*, 1988). Resistance was maintained in subsequent generations, which helped in the study of somaclonal variations. The effects of different combinations of growth factors on callus induction were difficult to estimate because due to their dependence on concentration, mutual ratio and genotype of plant (Liang, 1980).

2.2. Somatic Embryogenesis and Regeneration of Rice

Rice was the first major cereal crop to be regenerated into whole plant from tissue culture (Vasil, 1983). Somatic embryogenesis directly from explants could circumvent the tissue culture problems. The culturing conditions, media and selection of explants for somatic embryogenesis were established before regeneration into whole plant (Chaleff and Stolarz., 1981). Re-differentiation of rice explants and organs were studied by Nishi *et al.* (1968). Niiuzeki and Oono (1968) regenerated first rice haploid plant from anther culture and Tamura (1968) from rice embryo originated calli. Effects of different promotive factors were studied on embryogenesis of rice (Heyser *et al.*, 1983; Siriwardana and Nabors, 1983; Gupta *et al.*, 1989; Koetje *et al.* 1989 and Chowdhury *et al.* 1993). The higher genotype differences in the embryo formation from pollen of different rice varieties were reported by Mukherjee (1973) and also found that addition of tryptophan increased the amount of callus formation and embryogenesis.

The embryogenic calli characterized by Vasil and Vasil (1982) as compact but fragile, nodular and white to pale yellow in color. Abe and Futsuhara, (1989) reported that selection and differentiation of embryogenic calli is necessary to get efficient regeneration. Peng and Hodges (1989) genetically analyzed the phenomenon of regeneration in rice and regeneration was achieved by cytoplasmic hybrids of rice by Yang *et al.* (1989). The plant regeneration of rice was achieved

from embryogenic calli initiated from different tissue explants; leaves (Wernicke *et al.*, 1981), roots (Abe and Futsuhara, 1985, Kishore and Reddy, 1986; Zimny and Lorz, 1986), young inflorescences (Chen *et al.*, 1985), rice pollen grains (Chen *et al.*, 1981; 1982; Cho and Zapata, 1988; Kim and Raghavan, 1988; Torrizo and Zapata, 1992), microspore (Datta *et al.*, 1990b), immature embryo (Lai and Liu, 1982), scutellum (Ghosh Biswas *et al.*, 1994 and Rueb *et al.*, 1994) and mature seeds (Siriwadana and Nabors, 1983; Raina *et al.*, 1987; Wang *et al.*, 1987).

Abe and Futsuhara (1986b) tested 66 rice varieties and reported large differences between indica and japonica *in vitro* manipulation. The japonica showed higher response in culture as compared to indica varieties (Abe and Sasahara, 1982; Abe and Futsuhara, 1984). The factors like explant, medium and genotype were characterized and optimized for reproducible and efficient *in vitro* tissue culture system for various rice genotypes (Nishi *et al.*, 1968; Ohira *et al.* 1973; Kamia *et al.*, 1988 and Oinam and Kotharii 1995). The poor response of indica as compare to japonica slowed down the application of cell culture procedures. A comprehensive study of 500 rice varieties showed that both callus formation and plant regeneration were highly genotype dependent (Kamia *et al.*, 1988 and Peng and Hodges 1989). Studies carried on the nutritional requirement of rice cells in culture medium (Schmitz and Lorz 1990) also indicated the involvement of genotypic factors in rice tissue culture. The long term maintenance of embryogenic potentiality of culture is important and the rice plants were successfully regenerated from long term callus cultures by Heyser *et al.* (1983), Nabors *et al.* (1983), Raghava Ram and Nabors (1984) and Kishore and Reddy (1986).

Plant regeneration of indica rice was achieved from embryogenic calli derived from immature embryos by Heyser *et al.* (1983) from mature seeds by Hartke and Lorz (1989) from scutellum by Gupta *et al.* (1989) and from coleoptile by Oinam *et al.* (1995). The meristems of different indica rice were also cultured for obtaining direct regeneration without undergoing the long process of callus formation (Khanum, 1996). Efficient regeneration of indica rice Pusa 169 was achieved by Chowdhury *et al.* (1993). They reported promotion effect of proline and tryptophan on the frequency of callusing and regeneration of rice. It is important to regenerate the indica rice varieties cultivated in most of the tropical regions of the world including Bangladesh. The success for reproducible *in vitro* regeneration of indica rice varieties had been limited (Kyoizuka *et al.*, 1988, Terada and Shimamoto 1990 and Jain *et al.*, 1996). The work on callus induction of Pakistani Basmati varieties was done by Abbas and Qureshi (1985) and regeneration was achieved in Basmati 370 by Zafar *et al.* (1992). There is a need to establish tissue culture of wide range of local rice varieties in order to establish an efficient regeneration system for local varieties of rice either from meristem culture or from somatic embryogenesis using different tissue explants.

2.3. Anther Culture

On the basis of geographical distribution and morphophysiological variations rice (*Oryza sativa* L.) is distinguished into indica and japonica species (Kato *et al.*, 1928 and Glaszmann, 1987). The indica accounts for 80% of the area under cultivation in the world tropical and sub-tropical areas (Swaminathan, 1982). About 200 million tons of world's rice production are spoiled or lost every year due to abiotic factors (Wunn *et al.*, 1996). Rice has been an important food crop in Bangladesh. The population of rice eaters continues to increase at an

alarming rate and the demand for rice is likely to exceed production by the end of this century. To meet this demand rice varieties are needed with a higher yield potential, multiple resistance to diseases, insects and tolerance to abiotic stresses. Recent breakthrough in androgenesis and genetic transformation has provided tools that can increase the efficiency of breeding method and allow unconventional approaches to rice improvement. Applications of this technique will provide us to develop and improve rice varieties for meeting the challenges of increase rice production.

Anther culture has proved to be a very useful tool in plant breeding, providing a rapid method for regeneration of desirable genotypes. This technique opens new vistas of breeding for resistance and quality of crop plants, not only shortening the breeding cycle by immediate fixation of homozygosity, but increasing the selection efficiency, widen genetic variation and allowing the expression of recessive genes. Rice anther culture holds great potential in variety improvement. Anther culture provides a method for the production of homozygous lines over the course of a few months, rather than the several generations required using conventional whole plant techniques. The double haploid plants resulting from anther culture are homozygous and breed true.

Conventional breeding programs typically require several years to develop a new variety. The process begins with a cross-pollination to combine desirable parental traits. The first generations of offspring the F_1 , are heterozygous but genetically uniform. Segregation occurs during reproduction by the F_1 . Segregation is the separation of homologous chromosomes and genes from different parents at meiosis and produces genetic variability within the F_2 population. With successive generations self-pollinating crops such as rice become

progressively more homozygous, because the heterozygosity decreases by half with each generation. By the F_3 generations, plants are nearly 97% homozygous, which is sufficiently homozygous for advanced testing. However, a considerable portion of the time required to develop a new variety entails this production of several generations to achieve a relatively homozygous condition following the initial cross-pollination.

Anther contains pollen and culture involves the culture of these structures *in vitro*. The immature pollen or microspores contained within the anther either gives rise directly to embryos, called androgenesis, or to callus tissue, which in turn is induced to regenerate plants under the influence of growth regulators added to the culture medium. Pollen is haploid, and the cells produced from pollen or microspores during culture are haploid as well. Other methods for the production of haploids include the culture of unfertilized ovules or ovaries, resulting in gynogenesis or by the chromosome elimination method as used in the *Hordium bulbosum* method (Bajaj, 1983). When plants are regenerated from haploid cells, a haploid plant is produced.

Haploid plants also have the gametophytic number of chromosomes that is a single set of chromosomes in the sporophyte. Their significance for plant improvement, and as a tool in various disciplines of plant science has been stressed (Kasha, 1974). Since haploids are of great importance, especially in studies on the induction of mutations, genetic transformation and also for the production of homozygous plants, they needed in large numbers. However, the conventional methods (Kimber and Riley, 1963; Magoon and Khanna, 1963), employed by plant breeders for their productions are cumbersome, laborious and not very efficient. The introduction of techniques for the induction of androgenesis

by the culture of excised anthers (Guha and Maheshwari, 1964), isolated pollen (Nitsch, 1974). It has become increasingly evident that tissue culture methods could considerably speedup the production of haploids for breeding programs.

2.3.1 *In vitro* Androgenesis

The development of haploid plants derived from immature pollen or microspore (anther or microspore culture) is termed as androgenesis. Since the discovery by Guha and Maheshwari (1964, 1966) the immature pollen could be induced to bypass normal development within the anther and the production of haploid plants, first realized in *Datura innoxia* Mill. Considerable efforts have been made to extend the technique to other species. In some species it is possible to produce haploids through the culture of isolated microspores (Keller *et al.* 1987). Today androgenetic haploids have been produced in more than 50 genera, but the greatest effort has been given to economically important crops such as the vegetable crops and cereals (Cao *et al.* 1995; Veilleux 1994). Via androgenesis new varieties have been developed in a number of agricultural crops such as *Brassica sp.*, tobacco, potato, asparagus, wheat, rice, maize, barley etc. (Bajaj 1990). The frequency of the induction of androgenesis in cultured anther in general is rather low. In order to utilize anther culture in crop breeding programs, it is essential that the haploids be produced in large numbers.

Production of haploid plants of rice (*Oryza sativa* L.) through anther culture was first reported by Niizueki and Oono in 1968. Since then, extensive research has been carried out in many laboratories around the world. Now the techniques for anther culture of rice have advanced to the level that large numbers of haploids and spontaneously doubled haploids can be obtained from many cultivars and hybrids. The theoretical basis for the utilization of anther derived plants for

breeding has also been established (Lee and C.C Chen 1982, C.M. Chen *et al* 1982, C.C. Chen *et al.*, 1983). Thus rice has become one of the few crops in which anther culture can be readily applied to breeding programs.

The first event in androgenesis is a shift of the microspores from gametophytic to sporophytic development. After this induction phase, the microspores of *Datura innoxia* (Guha and Maheshwari, 1966) and tobacco (J.P. Nitsch and C. Nitsch, 1969) develop into plants directly via embryogenesis. Unlike in *Datura* and tobacco, androgenesis in rice usually has an intermediate step of callus formation (Niizueki and Oono, 1968); organogenesis and / or embryogenesis of the callus give rise to plants. The process of plant formation from rice microspores, *in vitro* androgenesis is the most convenient and common technique used to produce DH plants in rice.

Some factors are closely related to the anther culture of rice and predominantly determine the success of culture. i) genotype dependency (Schaeffer *et al.*, 1979; Lazar *et al.*, 1984; Barnabas *et al.*, 1989); ii) donor plants growth conditions (Bajaj 1983; Schmid and Keller, 1986); iii) stage of microspore and anther development (Wenzel and Foroughi-Wher, 1982; Dunwell, 1986; He and Ouyang, 1984); iv) pretreatment of anthers (Schmid, 1990; Picard and De Buyes, 1975; Pan *et al.*, 1975; Hu, 1986) and v) culture media (Chuang *et al.*, 1978; Chu, 1978; Wang and Hu, 1984; Fadel and Wenzel, 1990). However, under present investigation effort has been intensified on the development of methods for production of haploids by anther culture in rice.

2.3.2 Cold Treatment

There are differences of opinion in the literature regarding the effects of cold treatment in anther culture. Most researchers obtained enhanced callus induction by the application of cold shock, but there are differences of opinion in the literature regarding the optimum duration and temperature to use. Chen *et al.*, (1976) observed as slightly higher frequency of callus induction in rice anthers treated at 5°C for 24 hours and at 10°C for 48 hours than untreated anthers. Wang *et al.*, (1974) reported the pre-treatment of rice panicles at 10°C for 48 hours greatly increase the frequency of callus induction. Chaleff *et al.*, (1980) reported that rice anthers when treated at 6°C for 5 days produced twice as many calli than untreated anthers. Cold shock before or after plating rice anthers significantly increased the frequency of callus induction. Low temperature treatment after inoculation altered the normal path of microspore development in rice by inducing equational division with positively influenced the callus induction. Chen (1985) showed that pretreated anther produced not only higher number of calli but also produced more haploid plants.

In some cases, especially after low temperature pretreatment symmetrically dividing microspores were found to be main sources of androgenic haploids (Zheng and Ouyang 1980). Lazar *et al.* (1985) showed that variation in intensity and duration in cold pretreatment lead to significant difference in callus formation frequency. Ouyang *et al* (1987) concluded that the most suitable culture temperature for anther culture of field-grown material was about 2°C higher than that of the greenhouse-grown material irrespective of genotype. They also noticed that the anthers of the green house grown material did not develop as well as those of the field grown material. Cold treatment (3°C) of the tillers soaked in water

significantly improves the androgenic success mainly during the less favorable winter period (Picard and De Bbyser, 1975; Pan *et al.*, 1975). Marsotais and Kasha (1983) also reported that cold pretreatment is not always necessary to obtain the higher rates of androgenetic plant.

2.3.3 Factors Affecting Androgenesis

The responses of anthers in cultures greatly depend on genotype of the plant species. Striking variations in haploid plant production have been obtained from anthers of different genotypes. Several factors affect the success of anther culture, especially the genotype of the plant from which anthers are obtained (Bajaj, 1983; Croughan, *et al.*, 1989) and the composition of nutrient media. Guha-Mukherijee (1973) selected 20 different rice varieties from a wide genotypic background. Only four of the 20 varieties produced embryoids while 12 varieties failed even to produce callus. The main advantage of anther culture technique for haploid production is the rapid and complete homozygosity on the offspring, because phenotype selection for quantitative and particularly for quantitative inherited characters is much easier and therefore, breeding is more efficient.

Niiuzeki and co-workers (1968) in the National Institute of Agriculture Science, Japan were the first research works to produce haploid successfully by anther culture from rice. Their results were reported at a semi-annual meeting of the Japanese Society of Breeding (April 1968). Since haploid plant formation through anther and microspore culture have been successfully applied in different cereal crops; rice (Genovesi and Maggill, 1979) wheat (Picard and De Buyser, 1973; Ouyang, 1986). In cereals, production of haploid through anther culture has major obstacles with low generation rate and albino plant production. However,

applications of different method and treatments may improve induction and regeneration efficiency.

Anther culture technique has long been recognized as a valuable tool in plant breeding and has been profitable employed in various crop improvements like wheat, rice, ray etc. But its application is still limited by many factors which influence the culture efficiency such as the genotypes, the growing conditions by the donor plants, the development stage of microspores, pre-treatments of the panicles, the culture method, the media and the *in vitro* conditions. One of the major constraints in the application of anther culture in rice improvement program is the identification of responsive anthers for callus formation. Limited work has been reported on the response of morphological markers such as panicle length, spikelet position in the panicle, anther position in the spikelet and anther color. Low plant regeneration frequency is one of the most serious limitations in anther culture techniques.

2.4. Transformation of Rice

Plant biotechnology provides biologically safer crops carrying agronomically useful genes for different biotic and abiotic stresses. Transformation is a technique by which functional foreign genes are inserted into genome of the crop plants from diverse biological system. Plant transformation could be defined as delivery, integration and expression of foreign genes into plant cells, which ultimately regenerated into whole plant. 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 (Swaminathan,

1982; Khush, 1984). Molecular improvement of cereals has been thoroughly reviewed by Vasil (1994).

The techniques such as pollen tube method (Luo and Wu, 1988), microinjection (Potrykus *et al.*, 1985), dry seeds imbibed in DNA solutions (Aryon *et al.*, 1991) and DNA delivery into intact tissue by electroporation (Dekeyser *et al.*, 1990) had resulted in transient gene expression. Fertile transgenic indica rice variety IR36 plants were obtained after electroporation of the seed embryo cells (Xu and Li 1994). These approaches with the exception of the pollen tube pathway require a reproducible cell culture and plant regeneration system in order to produce fertile transgenic plants.

In dicots, a strong wound response to a tumour formation and which facilitates subsequent *Agrobacterium* mediated transformation. However cereals lack wound response and leads towards the cell death but some researchers had reported *Agrobacterium* mediated transformation in rice (Raineri 1990; Li *et al.*, 1992 and Chan *et al.*, 1992; Hiei *et al.*, 1994). Among them only Chan *et al.* (1993) was able to regenerate transgenic indica rice plants. Chan *et al.* (1992) reported that *Agrobacterium* mediated transformation was highly genotype dependent. The *Agrobacterium* mediated transformation of different japonica cultivars were also achieved by Hiei *et al.* (1994) and Vijaya Chandra *et al.* (1995) by co-cultivation of scutellum derived callus of rice with *Agrobacterium*.

Genome mapping and biotechnology research in recent years offer powerful tools in crop improvement including genetic transformation and molecular marker-assisted selection. These techniques have opened enormous opportunities to meet the challenges of food production. More than 20 genes for

resistance to various plant diseases have been isolated from a wild range of plant species including monocotyledonous and dicotyledonous species including tomato, rice, tobacco and maize. Genes for resistance to various insects have been identified in many crop species and their wild relatives, including gall midge and brown planthopper resistance in rice and pink borer resistance in cotton. Many insect resistance transgenic cotton, corn and rice plants have been developed through gene transformation procedure. Metabolic engineering of carotenoid biosynthesis has been achieved recently on rice and engineering high levels of β -carotene in the endosperm of golden rice is a major breakthrough. Golden rice is an excellent example of how genetic engineering of plants can be of direct benefit to the consumer, especially the poor and the disadvantaged in developing countries. All these beneficial genes including insect resistance, drought resistance and gene for metabolic pathway have routinely been transferred to different crop plants along with some reporter and marker genes. Agronomically importance genes (gene of interest) are fused with reporter genes in order to screen out the putative transformants towards obtaining the stable transformed target plants. The protocol of transfer of reporter gene is one of important tool for success in DNA transgenic technology to establish. Selection of transformed calli or plants is an important factor in plant transgenic technology. A simple but efficient protocol is essential in selecting transformants from which transgenic plants can be regenerated. A number of reporter genes Beta-glucuronidase (GUS), Luciferase (LUC), chloromphenical acetyl transferase (CAT) and anthocynins are being used in cereal transformation to analyze gene expression. In rice, the most commonly used reporter gene is GUS (Peng *et al.*, 1992). In this technique, the GUS gene usually isolated from *E. Coli* (Jefferson *et al.*, 1987) and then the intrinsic GUS like activity is observed in rice tissue as alkaline pH 8.0 or by including 20 %

methanol in the assay (Kosugi *et al.*, 1990). Selectable markers such as antibiotic resistance are increasingly used for the transformation screening. The neomycin phosphotransferase (NPTII) confers resistance to kanamycin (Uchimiya *et al.*, 1986) and hygromycin phospho transferase (hpt also called hph) to hygromycin antibiotic (Blochlinger *et al.*, 1984).

Early selection and screening of putative transformants for stable transformation is essential and GUS expression could be used as a rapid early screening method (Hu *et al.*, 1990). The success of producing transgenic plants depends upon transmission of functional foreign genes from primary transformants to their progenies (Hayashimoto *et al.*, 1990). Mendelian segregation of foreign genes is being possible to observe in first progeny of primary transformants (T1) by Christou *et al.* (1991). The inheritance and Mendalian ratio 3:1 was reported in progeny of GUS (Li *et al.*, 1992; Peng *et al.*, 1992). The size of the introduced DNA also affected the stable transformation and integration of DNA into rice genome (Uchimiya *et al.*, 1986). The transformation with large fragments of DNA is introduced for identifying clones that contained genes of interest because many agronomic traits controlled by cluster of genes.

2.5.Objectives

Application of plant biotechnology is gaining momentum in developed as well as in developing countries to improve the agricultural crops. In our country there have tremendous scopes for developing its agricultural crops by the application of plant DNA transgenic technology, in particular, to develop the high yielding, disease resistant and aromatic rice production would possible.

Most of the rice transformation work reported was based on japonica varieties and indica rice despite of its importance was still under studied. However very little work had been done on improving Bangladeshi local rice varieties using modern biotechnological methods. There is an urgent need to undertake such studies to produce local transgenic rice varieties in order to exploit the prospective quality of aroma and ability to combating biotic and abiotic stresses. Bangladesh, as poor nation, holds the promise to develop metabolically engineered rice for important micronutrients like golden rice for supporting vitamin A. But for genetic transformation to be accomplished in our local cultivars of rice it is urgently needed to develop the regeneration protocol and the efficient methods of gene transfer technology in our local varieties of rice.

Application of transgenic technology in agriculture demands an efficient *in vitro* regeneration system of plant and standardization of transformation tools for the specific crop plant like rice. The main objectives of the present study were (1) to screen the local Bangladeshi rice varieties for their *in vitro* culture potentialities either from somatic tissue and from anther culture and (2) to establish an efficient regeneration system for target rice variety to be transformed and (3) to standarize the conditions for stable transformation of rice by *Agrobacterium* co-cultivation.

In vitro
Tissue Culture of Rice

CHAPTER-III

3. *In vitro* Tissue Culture of Rice

3.1 Introduction

In vitro tissue culture is an experimental approach for basic and applied research. It is an essential component of plant breeding, biotechnology and genetic improvement of plants. Every plant cell is considered to be totipotent. The potential to readily regenerate plants from cultured tissue has been considered as a powerful tool for crop improvement and the simplest from of genetic engineering (Larkin and Scowcroft, 1981). Tissue culture techniques are well established for most monocotyledonous plants but difficult to culture gramineous species because of their extremely recalcitrant behavior *in vitro* manipulation. The prerequisite of *in vitro* culture is the identification of tissues of cells, which are competent for regenerating the whole plant.

Regeneration of plant is possible via somatic embryogenesis or organogenesis. Plant somatic embryos develop from embryogenic calli derived from somatic cells. Embryogenic term applied to those cells that do not require auxin to produce the somatic embryos. The cells in transition between somatic cells and embryogenic cells are called competent cells. The competent cells still require exogenous auxin can be forced to become somatic embryos not only by exposure to phytohormones but also by pH, heat shocks, desiccation and treatments with various chemicals. An efficient and reproducible regeneration of normal and fertile plant has key importance in the application of molecular biology for the improvement of crops (Potrykus, 1990).

Rice is one of the world's major cereal, its importance in agriculture and plant science make it a favourite target for establishing tissue culture methods. *In vitro* tissue culture of rice was started by Fujiwara and Ojima, (1955) with the culture of excised roots and immature rice embryos (Amemiya *et al.*, 1956). Tissue culture constraints were reduced by culturing of immature and undifferentiated mass of cells (Callus) and meristem at defined stages of development in nutrient media supplemented with varying concentrations of phytohormones (Vasil and Vasil, 1982 and Vasil, 1988). Rice was the first cereal to regenerate into whole plant (Vasil, 1983). Morphogenic and regeneration studies of 66 rice varieties was done by Abe and Futsuhara (1986) and they observed large differences for the tissue culture ability of japonica and indica. They reported that Japonica varieties showed high callus yield and regeneration ability while indica gave very poor response *in vitro* tissue culture. Their study of *in vitro* tissue culture of rice was intended to screen different indica rice varieties for their ability to produce embryogenic calli and regenerate into whole plant (Abe and Sasahara., 1982 and Brar *et al.*, 1985; Datta *et al.*, 1990b).

Indica rice is widely cultivated in Bangladesh and despite of their importance only little work has been done on artificial culture of Bangladeshi indica varieties. In present study broad range of indica varieties were screened for their ability to regenerate into whole plant either from embryogenesis or meristem culture. Different explants were used for the induction of regenerable rice culture including meristem, mesocotyl, leaf base, root and mature seed scutellum. The present study addressed genotypic variation in callus formation and regeneration by organ culture or somatic embryogenesis.

3.2 Materials and Methods

3.2.1 Materials

Six rice (*Oryza sativa* L.) varieties BR-22, BR-23, BRRI dhan-28, BRRI dhan-29, BRRI dhan-30 and BRRI dhan-31 were used in the present work. Seeds of these varieties were collected from Regional Rice Research Institute, Rajshahi, Bangladesh.

3.2.2 Methods

3.2.2.1 Dehusking and Sterilization of Mature Seeds

Dehusked mature seeds of *Oryza sativa* varieties were surface sterilised with 70% (v/v) ethanol for one minute and commercial bleach containing sodium hypochloride (NaOCl) for five minutes, seeds were again sterilised with 0.1% (v/v) mercuric chloride (HgCl₂) for 5 minutes. After mercuric chloride the seeds were thoroughly washed with autoclaved distilled water for several times. Following surface sterilisation the seeds were sown on different growth media for germination and callus induction.

3.2.2.2 Preparation and Sterilization of Tissue Culture Media

Culture media for the growth of different explants consist of various inorganic and organic nutrients. Inorganic nutrients consisted of a number of major elements such as N, P, K, Ca, Mg, S, and minor elements such as Fe, Mn, Zn, B, and I etc. These inorganic nutrients were supplied as water-soluble compounds. Organic nutrients included carbon source, which were usually supplied with sucrose as glucose and organic growth substances mainly of B group vitamins. The medium mixed with fundamental inorganic and organic nutrients is called the basic medium. In order to regulate the growth and differentiation of the cultured cells, it is always

necessary to supplement the basic medium with various physiologically active substances, phytohormones such as auxins and cytokinins. The basic medium not only supplies nutrients but also provides suitable pH and osmotic pressure conditions for the growth of culture.

The media used in tissue culture studies were prepared according to the composition described in **Table 3.1**. The various combinations of growth regulators in addition to the basic media were used. Tissue culture and regeneration media were primarily based on the formulations developed by Murashige and Skoog (MS; 1962), Linsmair and Skoog (LS; 1965), Chu *et al.*, (N6; 1975), These media were solidified with 0.8% purified agar (Sigma). The pH was adjusted to 5.8 with 1N HCl or 1N KOH prior to sterilisation and the pH was always adjusted before the addition of agar.

Various media sterilisation procedures were employed. N6, LS, MS, and RM media were sterilised at 121°C at 15 lbs for 20 minutes in an autoclave. Glassware and other general equipment's were steam sterilised (121°, 15 lbs of steam pressure, 25 minutes). All tissue culture work was carried out in sterilised environment on a laminar airflow cabinet.

3.2.2.3 Callus Induction from Different Explants

Surface sterilised seeds were germinated on MS medium in dark at $25 \pm 2^\circ\text{C}$ for harvesting explants. For callus induction, LS medium supplemented with 2.5-mg/l 2,4-D, N6 medium supplemented with 2.0-mg/l 2,4-D and MS medium supplemented with 2.0-mg/l 2,4-D was used. The media were solidified with 0.8% agar and pH of the medium was adjusted to 5.8 before addition of agar.

Table 3.1. Composition of plant tissue culture media

Constituents	Concentration in medium (mg/L)		
	MS	LS	N6
Macronutrients			
(NaH ₄) ₂ SO ₄	-	-	-
KNO ₃	1900	1900	2830
NH ₄ NO ₃	1650	1650	-
MgSO ₄ .7H ₂ O	370	370	185
KH ₂ PO ₄	170	170	400
CaCl ₂ .2H ₂ O	440	440	166
(NH ₄) ₂ .SO ₄	-	-	463
KCl	-	-	-
NaH ₂ PO ₄ .H ₂ O	-	-	-
Micronutrients			
H ₃ BO ₃	6.2	6.2	1.6
MnSO ₄ 4H ₂ O	22.3	22.3	4.4
ZnSO ₄ .7H ₂ O	8.6	8.6	1.5
NaMoO ₄ . 2H ₂ O	0.25	0.25	-
CuSO ₄ .5H ₂ O	0.025	0.025	-
CoCl ₂ .6H ₂ O	0.025	0.025	-
KI	0.83	0.83	0.8
FeSO ₄ .7H ₂ O	27.8	27.8	27.8
Na ₂ EDTA	37.3	37.3	37.3
Vitamins and Sugars			
Thiamine HCl	0.1	1.0	05
Pyridoxine HCl	0.5	-	0.5
Nicotinic acid	0.5	-	1.0
Myo-inositol	100	100	100
Sucrose	30000	30000	30000
Amino acids			
Glycin	2.0	-	2.0
L-glutamine	-	-	-
L-aspartic acid	-	-	-
L-arginine	-	-	-
Hormones			
2-4, D	-	2.5	2.0
Kinetin	-	-	-
GA3	-	-	-
NAA	-	-	-
Zeatin	-	-	-
Agar	10000	10000	10000
pH	5.8	5.7	-

Explants (scutellum, root, leaf base and mesocotyl) from seven days old seedlings and mature embryos were cultured on LS, N6 and MS media and incubated at $25\pm 2^{\circ}\text{C}$ in dark for callus induction. Calli were sub cultured on the same medium after fifteen days interval. The embryogenic callus inducing frequency was determined after three weeks of incubation of different explants on different callus inducing media in the dark. The compact but fragile, nodular, creamy yellowish and light yellow in colour calli were considered as embryogenic.

3.2.2.4 Callus Regeneration from Different Tissue Explants

The embryogenic calli were induced from different tissue explants for three weeks in N6, LS, and MS media and dissected swollen scutellum from growing seeds were used for regeneration studies. The calli induced from different tissue explants were sub cultured on the same medium for three days under 1000 lux light at $25\pm 2^{\circ}\text{C}$ for better proliferation. The proliferated calli were finally transferred to regeneration media MS and N6 supplemented with different concentrations of phytohormones. The calli were kept in light 14h day /10h night cycle at $25\pm 2^{\circ}\text{C}$. Regenerated plants were transferred to MS medium for production of elaborated roots.

3.3 Meristem Regeneration

In order to assess meristem regeneration, about 2-3 mm leaf base pieces from 4,5 and 7-day-old etiolated seedlings were isolated and kept on MS medium. The plates were wrapped with parafilm and incubated in light 14h day/10h night cycle at $25\pm 2^{\circ}\text{C}$. The effect of different phytohormones was also studied on meristem regeneration. The leaf bases isolated from 4-day-old etiolated seedling were cultured on MS medium supplemented with different concentrations of NAA and BAP ranges between 0.5 –2.5 mg/l. Regenerated plants originated from these leaf

bases were transferred to MS medium supplemented with NAA 0.5mg/l and BAP 2.5mg/l in magenta vessels for multiple shoot formation.

3.4 Establishment of Regenerated Plants in Hydroponic

The regenerated plants were transferred to hydroponic medium (**Appendix**) before transferring into soil. The pH of hydroponic medium is important and which was checked after every two days. Before transfer into hydroponic medium, plants were washed in tap water in order to remove the agar from the roots. The plants were kept in water for two days and shifted into hydroponic medium covered with polythene sheets under light 1000lux and humidity 68%. The hydroponic medium was changed weekly. After two weeks the plants were taken out of polythene sheets and transferred into soil.

3.4.1 Establishment of Regenerated Plants in Soil

The regenerated plants derived from callus or meristem after maintaining in hydroponic medium were transferred to soil containing peat moss in plastic baskets and incubated at a temperature of 30°C under 14h day/10h night cycle. After four weeks plants were transferred into pots of regenerated plants.

3.5 Result and Discussion

3.5.1 Embryogenesis

Embryogenesis was studied in six rice varieties from different tissue explants of scutellum, mesocotyl, leaf base and root on different callus inducing media. All explants produced callus at different frequencies on different callus inducing media (Fig. 3.1.).

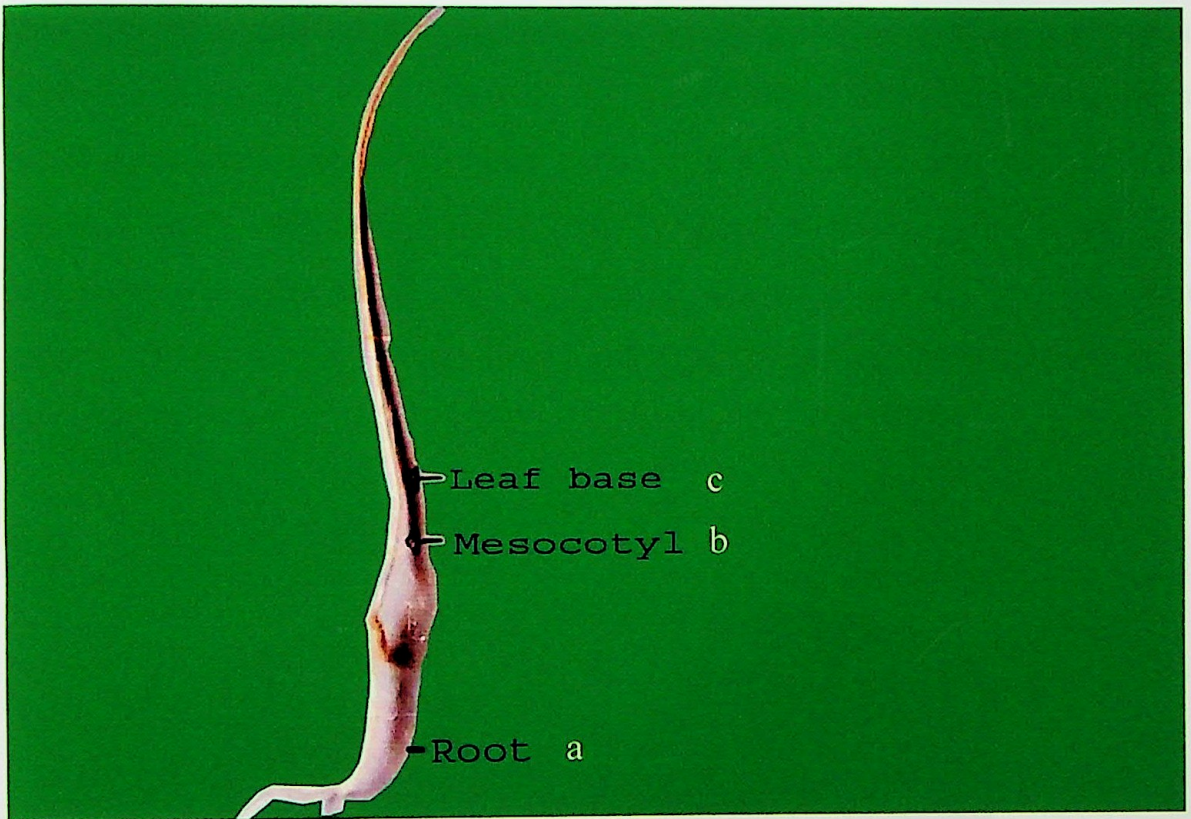


Fig. 3.1. Seven days old seedlings of rice showing different explant portions

a. Root

b. Mesocotyl

c. Leaf base

3.5.2 Callus Induction Response in Different Media from Different Tissue Explants

The plant growth regulator 2,4-D plays an important role in callus induction of plant tissue and 2.0 mg/l 2,4-D was reported as the most suitable for callus induction in rice (Farzana Khanam, 1996). Six rice varieties were tested on three callus induction media of LS, MS and N6 supplemented with 2.0 mg/l of 2, 4-D. Response in callus induction of six varieties each with four explants were studied and their results are presented in **Table 3.2**. A wide range of variations in callus induction was marked in this experiment with respect to their explants and treated media. Three varieties, BRRI dhan-28, BRRI dhan-30 and BRRI dhan-31 proved to be more competent ones to induce callus with all of their four explants. The varieties BR-22, BR-23 and BRRI dhan-29 failed to induce callus in all their explant tissues. The mean values of callus induction for six varieties were individually calculated over the media and explants and the results are shown in **Fig.3.2**.

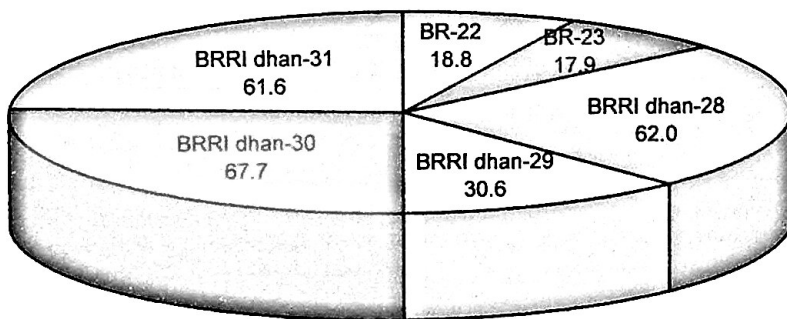


Fig.3.2. Mean values of callus induction of six varieties over media and explants.

BRRI dhan-30 was found as the most efficient variety. Producing 67.70% callus induction followed by BRRI dhan-28 (62.00%) and BRRI dhan-31 (61.60%). Lowest value was noted for BR-22 (18.80%) and BR-23 (17.90%). It is important to note that the explants scutellum and root produced callus irrespective of varieties and media.

Table 3.2. Callus induction performance of different explants of six rice varieties growing on three callus induction media.

Variety	Explants	% $\pm$ S.E. of callus formation			Mean $\pm$ S.E.
		N6	LS	MS	
BR-22	Scutellum	30.8 $\pm$ 1.7	27.1 $\pm$ 2.1	18.9 $\pm$ 0.8	25.6 $\pm$ 3.5
	Leaf base	-	-	-	-
	Mesocotyl	-	-	-	-
	Root	16.6 $\pm$ 1.6	12.9 $\pm$ 1.0	6.7 $\pm$ 0.9	12.1 $\pm$ 2.8
Mean $\pm$ S.E.		23.7 $\pm$ 7.1	20.0 $\pm$ 7.1	12.8 $\pm$ 6.1	18.8 $\pm$ 3.2
BR-23	Scutellum	42.1 $\pm$ 2.0	32.6 $\pm$ 1.9	20.9 $\pm$ 1.0	31.8 $\pm$ 6.1
	Leaf base	-	-	-	-
	Mesocotyl	6.1 $\pm$ 0.7	3.5 $\pm$ 0.5	-	4.8 $\pm$ 1.3
	Root	17.4 $\pm$ 1.2	13.2 $\pm$ 1.8	9.7 $\pm$ 1.1	13.4 $\pm$ 2.2
Mean $\pm$ S.E.		21.9 $\pm$ 10.6	16.4 $\pm$ 8.5	15.3 $\pm$ 5.6	17.9 $\pm$ 2.0
BRRI dhan-28	Scutellum	94.1 $\pm$ 1.6	88.3 $\pm$ 2.3	72.3 $\pm$ 2.0	84.9 $\pm$ 6.5
	Leaf base	59.5 $\pm$ 4.1	63.0 $\pm$ 6.4	30.6 $\pm$ 3.6	51.0 $\pm$ 10.2
	Mesocotyl	56.0 $\pm$ 3.0	60.2 $\pm$ 4.3	20.7 $\pm$ 1.2	45.6 $\pm$ 12.5
	Root	74.8 $\pm$ 2.7	84.4 $\pm$ 3.1	40.8 $\pm$ 3.9	66.5 $\pm$ 13.1
Mean $\pm$ S.E.		71.1 $\pm$ 8.6	73.9 $\pm$ 7.2	41.1 $\pm$ 11.1	62.0 $\pm$ 10.4
BRRI dhan-29	Scutellum	45.7 $\pm$ 2.2	40.6 $\pm$ 2.7	37.9 $\pm$ 1.9	41.4 $\pm$ 2.2
	Leaf base	-	-	-	-
	Mesocotyl	-	-	-	-
	Root	21.3 $\pm$ 1.5	23.2 $\pm$ 1.2	15.1 $\pm$ 1.4	19.9 $\pm$ 2.4
Mean $\pm$ S.E.		33.5 $\pm$ 12.2	31.9 $\pm$ 8.7	26.5 $\pm$ 11.3	30.6 $\pm$ 2.1
BRRI dhan-30	Scutellum	97.3 $\pm$ 0.5	92.3 $\pm$ 2.0	79.9 $\pm$ 3.0	89.8 $\pm$ 5.1
	Leaf base	61.0 $\pm$ 5.5	71.9 $\pm$ 3.9	35.0 $\pm$ 2.8	55.9 $\pm$ 10.9
	Mesocotyl	60.5 $\pm$ 4.2	65.8 $\pm$ 4.6	25.9 $\pm$ 1.7	50.7 $\pm$ 12.5
	Root	85.8 $\pm$ 2.4	86.2 $\pm$ 3.4	51.0 $\pm$ 3.0	74.3 $\pm$ 11.6
Mean $\pm$ S.E.		76.2 $\pm$ 9.1	79.1 $\pm$ 6.1	47.9 $\pm$ 11.8	67.7 $\pm$ 9.9
BRRI dhan-31	Scutellum	90.4 $\pm$ 1.5	84.7 $\pm$ 3.4	72.7 $\pm$ 3.9	82.6 $\pm$ 5.2
	Leaf base	59.5 $\pm$ 4.4	69.9 $\pm$ 7.6	30.6 $\pm$ 1.8	53.3 $\pm$ 11.7
	Mesocotyl	52.7 $\pm$ 3.2	51.9 $\pm$ 2.5	21.8 $\pm$ 1.4	42.1 $\pm$ 10.1
	Root	81.0 $\pm$ 2.0	78.5 $\pm$ 3.1	45.6 $\pm$ 2.4	68.4 $\pm$ 11.4
Mean $\pm$ S.E.		70.9 $\pm$ 8.8	71.3 $\pm$ 7.1	42.7 $\pm$ 11.1	61.6 $\pm$ 9.4

The experimental results revealed that somatic tissue in different explants of rice hold tremendous physiological variations in responding callus induction under different media compositions. Three poor responded varieties were discarded and only the three varieties with better response were screened for further study.

An intensive study has been made to find the relative performance of callus induction of different explants of three varieties using different concentrations of 2, 4-D in three media. Results of callus induction performance of scutellum taken from three varieties tested in three media supplemented with different concentrations of 2, 4-D was presented in **Table 3.3**. The table indicated that variations of hormonal concentrations played a major role in callus induction. N6 supplemented with 2.0 mg/l 2, 4-D was found most effective in callus induction in all varieties and it gave 94.1%, 97.3% and 90.4 % induction in BRRI dhan-28, BRRI dhan-30 and BRRI dhan-31 respectively. Lowest range (34-40%) was observed in lower concentrations (0.5 mg/l) of the hormone. In case of LS medium the hormonal concentration of 2.5 mg/l was found to be optimum in callus induction and lowest values observed in 0.5 mg/l of 2,4-D. While in MS medium 2 mg/l of 2,4-D gave the highest result of callus induction but lowest concentration (0.5 mg/l) fail to induce any callus. Irrespective of hormonal concentrations, the N6 medium supplemented with 2, 4-D was found more effective in callus induction followed by LS. On the other hand, MS supplemented with 2, 4-D showed lowest response in callus induction. The callus induction variation among three genotypes of rice was not varied severely. However, BRRI dhan-30 showed the highest response. When scutellum was used as explants callus formation started within 12-15 days of induction (**Fig. 3.3**).

Table 3.3. Induction of callus from scutellum explants of three rice varieties on different callus inducing media (each value is an average of 12-16 explants and the experiment was repeated twice).

Treatment (mg/l)	% of the callus formation (mean $\pm$ S.E.)			Mean $\pm$ S.E.	Days of callus initiation	Texture of the callus	Colour of the callus
	BRRJ dhan-28	BRRJ dhan-30	BRRJ dhan-31				
N6 + 2, 4-D 0.5	34.0 $\pm$ 1.0	40.7 $\pm$ 2.8	35.4 $\pm$ 1.4	36.7 $\pm$ 2.0	12-16	Compact	Pale Yellow
N6 + 2, 4-D 1.0	68.6 $\pm$ 2.1	76.4 $\pm$ 3.4	60.4 $\pm$ 2.3	68.5 $\pm$ 4.6	12-15	Compact	Yellowish
N6 + 2, 4-D 2.0	94.1 $\pm$ 1.6	97.3 $\pm$ 0.5	90.4 $\pm$ 1.5	93.9 $\pm$ 1.9	13-13	Compact	Yellowish
N6 + 2, 4-D 3.0	89.5 $\pm$ 1.1	91.1 $\pm$ 1.7	88.3 $\pm$ 2.8	89.6 $\pm$ 0.8	12-14	Compact	Yellowish
Mean $\pm$ S.E	71.5 $\pm$ 13.6	76.4 $\pm$ 12.7	68.6 $\pm$ 13.0	72.1 $\pm$ 2.3			
LS+2, 4-D 0.5	27.7 $\pm$ 2.7	32.2 $\pm$ 3.5	26.8 $\pm$ 1.9	28.9 $\pm$ 1.7	10-15	Compact	Light Yellow
LS+2, 4-D 1.0	51.5 $\pm$ 2.8	59.9 $\pm$ 3.4	55.0 $\pm$ 4.3	55.5 $\pm$ 2.4	10-13	Compact	Yellowish
LS+2, 4-D 2.5	88.3 $\pm$ 2.3	92.3 $\pm$ 2.0	84.7 $\pm$ 3.4	88.4 $\pm$ 2.1	10-12	Compact	Yellowish
LS+2, 4-D 3.0	79.3 $\pm$ 4.2	86.5 $\pm$ 2.9	79.7 $\pm$ 2.5	81.8 $\pm$ 2.3	10-12	Compact	Yellowish
Mean $\pm$ S.E	61.7 $\pm$ 13.7	67.7 $\pm$ 13.8	61.5 $\pm$ 13.2	63.6 $\pm$ 13.5			
MS+2, 4-D 0.5	—	—	—	—	10-13	Compact	Creamy
MS+2, 4-D 1.0	14.1 $\pm$ 1.8	25.7 $\pm$ 3.5	20.7 $\pm$ 2.7	20.1 $\pm$ 3.3	10-12	Compact	Creamy
MS+2, 4-D 2.0	72.3 $\pm$ 2.0	79.9 $\pm$ 3.0	72.7 $\pm$ 3.9	74.9 $\pm$ 2.4	10-11	Compact	Creamy
MS+2, 4-D 3.0	46.5 $\pm$ 4.4	70.7 $\pm$ 5.3	55.3 $\pm$ 4.3	57.5 $\pm$ 7.1	10-11	Compact	Creamy
Mean $\pm$ S.E	44.3 $\pm$ 16.8	58.7 $\pm$ 16.7	49.5 $\pm$ 15.2	50.8 $\pm$ 16.1			

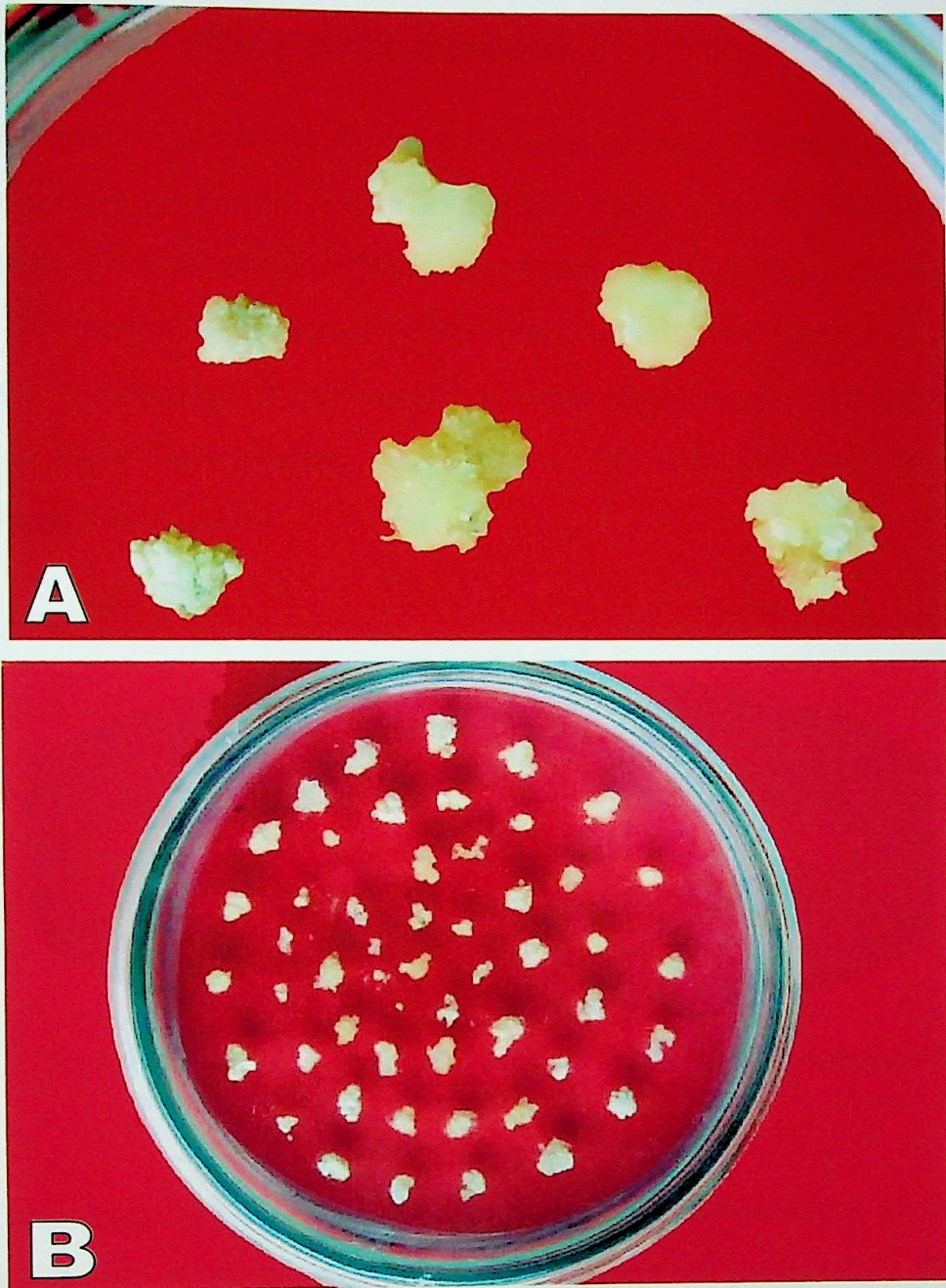


Fig. 3.3. Different types of scutellum derived calli.

A. Embryogenic calli.

B. Transfer of embryogenic calli.

The texture and colour of callus did not show any remarkable variation in three varieties under different media preparations.

In respect of scutellum the respective performance of three varieties were studied and their results are shown in **Fig.3.4**. BRRI dhan-30 exhibited highest performance (67.60%) of callus induction, which was 59.87% higher than BRRI dhan-31 followed by BRRI dhan-28 (59.17%). Similarly, the results also indicated the relative performance of three media in callus induction for scutellum and N6 was found highest performance, which was 11.78% increased over the LS and 30.65% increased over MS.

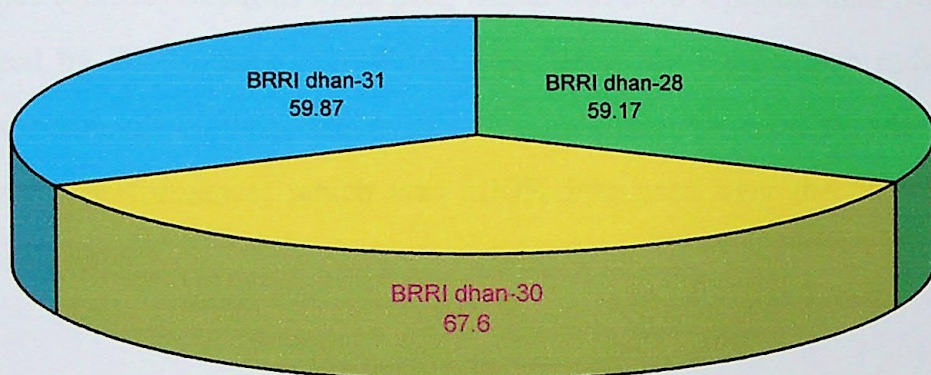


Fig.3.4: The relative performance of three rice varieties in callus induction for scutellum explant.

Callus induction response obtained from leaf base explant of three rice varieties culturing on different callus induction media are presented in **Table 3.4**. Like scutellum leaf base explant failed to show high level of induction efficiency. N6 supplemented with 2.0mg/l 2, 4-D showed the better result in callus induction in all three varieties. But LS supplemented with 2.5 mg/l 2, 4-D produced highest percentage of callus induction in all varieties and it gave 63.0%, 71.9% and 69.9 %

induction in BRRI dhan-28, BRRI dhan-30 and BRRI dhan-31 respectively. Lowest range (18.5-20.0%) was observed in lower concentrations (0.5 mg/l) of the hormone. All the hormonal concentrations used in MS medium was found to give low level of callus induction in three varieties. While in MS medium 2 mg/l of 2,4-D gave the highest result of callus induction but lowest concentration (0.5 mg/l) fail to induce any callus. When leaf base was used as explants callus formation started within 22-27 days of induction (**Fig. 3.6**). The texture and color of callus did not show any remarkable variation in three varieties under different media preparations.

In respect of leaf base the respective performance of three varieties were studied and their results are shown in **Fig. 3.5**. BRRI dhan-30 exhibited highest performance (38.5%) of callus induction, which was 34.6% higher than BRRI dhan-28 followed by BRRI dhan-31 (34.5%). Similarly, the results also indicated the relative performance of three media in callus induction for scutellum and LS was found highest performance, which was 5.10% increased over the N6 and 44.8% increased over MS.

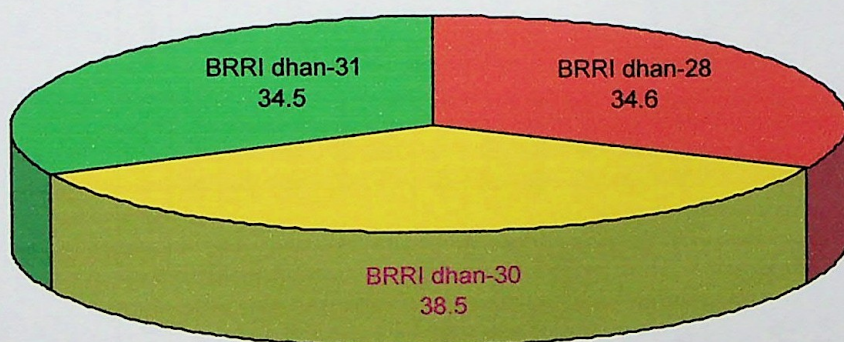


Fig. 3.5. The relative performance of three rice varieties in callus induction for leaf base explant.

Table 3.4. Induction of callus from leaf base explants of three rice varieties on different callus inducing media (each value is an average of 12-16 explants and the experiment was repeated twice).

Treatment (mg/l)	% of the callus formation (mean $\pm$ S.E.)			Mean $\pm$ S.E.	Days of callus initiation	Texture of the callus	Colour of the callus
	BRRJ dhan-28	BRRJ dhan-30	BRRJ dhan-31				
N6 + 2, 4-D 0.5	22.1 $\pm$ 2.5	24.2 $\pm$ 3.8	21.2 $\pm$ 2.3	22.5 $\pm$ 0.9	23-27	Friable	Light Yellow
N6 + 2, 4-D 1.0	34.4 $\pm$ 3.5	40.1 $\pm$ 3.7	39.5 $\pm$ 5.1	38.0 $\pm$ 1.8	23-25	Friable	Yellowish
N6 + 2, 4-D 2.0	59.5 $\pm$ 4.1	61.0 $\pm$ 5.5	59.5 $\pm$ 4.7	60.0 $\pm$ 0.4	23-24	Friable	Yellowish
N6 + 2, 4-D 3.0	51.8 $\pm$ 4.2	49.8 $\pm$ 3.5	45.1 $\pm$ 4.6	48.9 $\pm$ 1.9	23-25	Friable	Yellowish
Mean $\pm$ S.E	41.9$\pm$8.4	43.7$\pm$7.7	41.3$\pm$7.9	42.3$\pm$7.9			
LS+2, 4-D 0.5	20.9 $\pm$ 2.9	25.6 $\pm$ 3.5	18.5 $\pm$ 2.5	21.6 $\pm$ 2.1	22-26	Friable	Light Yellow
LS+2, 4-D 1.0	37.4 $\pm$ 4.6	40.3 $\pm$ 5.6	31.7 $\pm$ 3.5	36.4 $\pm$ 2.5	23-25	Friable	Yellowish
LS+2, 4-D 2.5	63.0 $\pm$ 6.4	71.9 $\pm$ 3.9	69.9 $\pm$ 7.6	68.3 $\pm$ 2.6	22-23	Friable	Yellowish
LS+2, 4-D 3.0	51.3 $\pm$ 7.2	55.2 $\pm$ 5.7	50.7 $\pm$ 2.3	52.3 $\pm$ 1.4	22-24	Friable	Yellowish
Mean $\pm$ S.E	43.1$\pm$9.1	48.2$\pm$9.9	42.7$\pm$11.2	44.6$\pm$2.1			
MS+2, 4-D 0.5	--	--	--	--	23-26	Friable	Creamy
MS+2, 4-D 1.0	5.4 $\pm$ 0.9	10.7 $\pm$ 0.7	6.4 $\pm$ 1.2	7.4 $\pm$ 1.5	23-25	Friable	Creamy
MS+2, 4-D 2.0	30.6 $\pm$ 3.6	35.0 $\pm$ 2.8	30.6 $\pm$ 1.8	32.1 $\pm$ 1.4	23-24	Friable	Creamy
MS+2, 4-D 3.0	20.5 $\pm$ 2.0	25.4 $\pm$ 1.5	22.0 $\pm$ 1.9	22.6 $\pm$ 1.4	23-24	Friable	Creamy
Mean $\pm$ S.E	18.8$\pm$7.3	23.6$\pm$7.1	19.6$\pm$7.0	20.6$\pm$1.5			

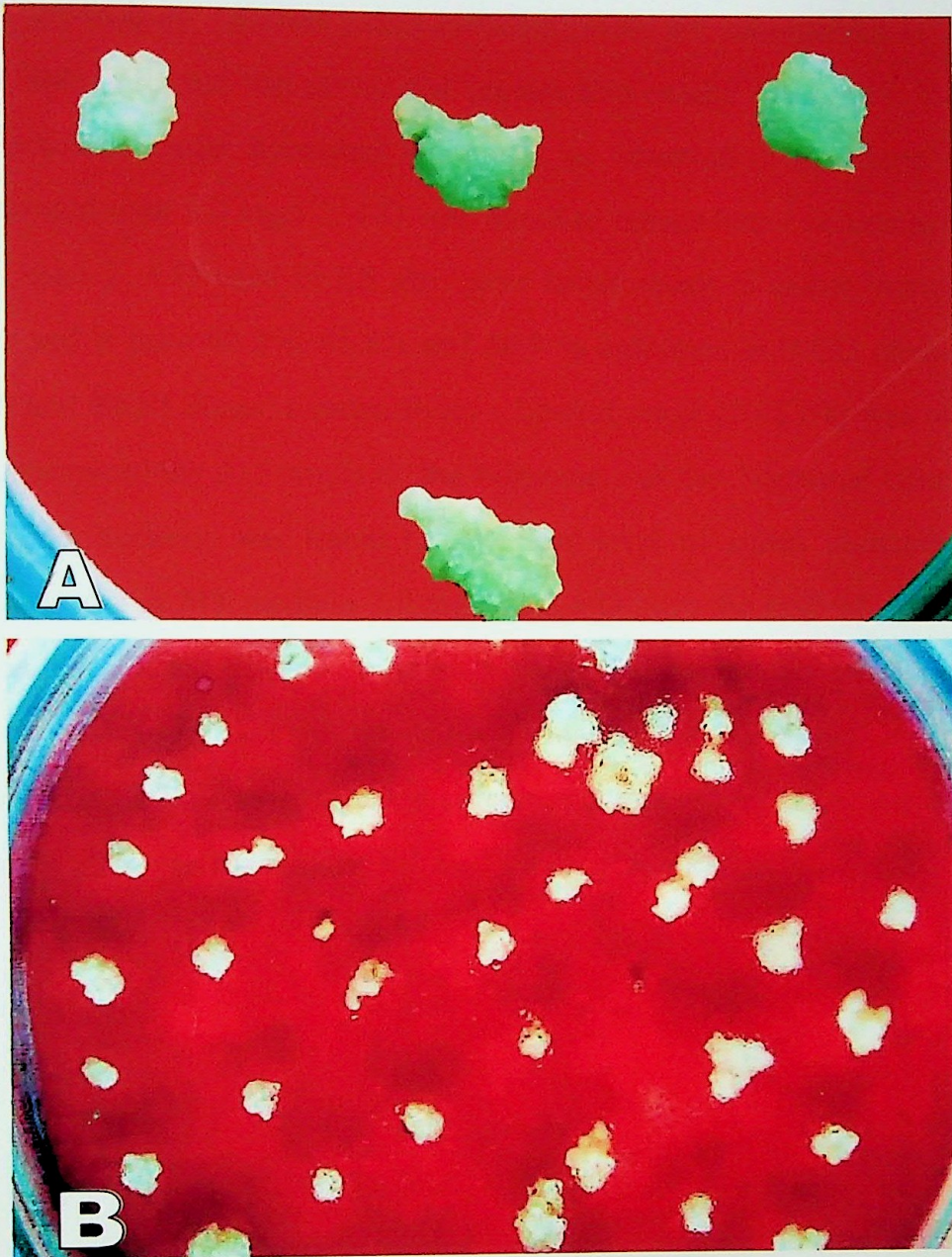


Fig. 3.6. Different types of leaf base derived calli.

A. Embryogenic calli.

B. Transfer of embryogenic calli.

Callus induction performance of mesocotyl explant taken from three rice varieties growing on different callus inducing media are summarized in **Table 3.5**. and the table indicated that mesocotyl explant also exhibit the similar low level of callus induction under hormonal treatments as observed in leaf base. Here, N6 supplemented with 2.0 mg/l 2, 4-D was found to induce better percentage of callus and 2.5 mg/l 2, 4-D used in LS also gave better result in callus induction in all varieties and it gave 60.2%, 65.8% and 50.9 % induction in BRRI dhan-28, BRRI dhan-30 and BRRI dhan-31 respectively. Lowest range (20-23%) was observed in lower concentrations (0.5 mg/l) of the hormone. All the hormonal concentrations used in MS medium was found to give low level of callus induction in three varieties. While in MS medium 2.0 mg/l of 2,4-D gave the highest result of callus induction but lowest concentration (0.5 mg/l) fail to induce any callus. When mesocotyl was used as explants callus formation started within 21-28 days of induction (**Fig. 3.7**). The texture and colour of callus did not show any remarkable variation in three varieties under different media preparations.

Table 3.5. Induction of callus from mesocotyl explants of three rice varieties on different callus inducing media (each value is an average of 12-16 explants and the experiment was repeated twice).

Treatment (mg/l)	% of the callus formation (mean $\pm$ S.E.)			Mean $\pm$ S.E.	Days of callus initiation	Texture of the callus	Colour of the callus
	BRRi dhan-28	BRRi dhan-30	BRRi dhan-31				
N6 + 2, 4-D 0.5	25.9 $\pm$ 1.5	36.2 $\pm$ 2.2	24.4 $\pm$ 3.3	28.8 $\pm$ 3.7	21-25	Friable	Yellow
N6 + 2, 4-D 1.0	39.6 $\pm$ 3.3	40.1 $\pm$ 2.1	31.5 $\pm$ 3.6	31.1 $\pm$ 2.7	21-24	Friable	Yellowish
N6 + 2, 4-D 2.0	56.0 $\pm$ 3.0	60.5 $\pm$ 4.2	52.7 $\pm$ 3.2	56.4 $\pm$ 2.2	21-23	Friable	Yellowish
N6 + 2, 4-D 3.0	42.9 $\pm$ 3.3	45.6 $\pm$ 5.6	35.8 $\pm$ 2.1	41.4 $\pm$ 2.9	21-27	Friable	Yellowish
Mean $\pm$ S.E	41.1 $\pm$ 6.1	45.6 $\pm$ 5.3	36.1 $\pm$ 6.0	40.9 $\pm$ 2.7			
LS+2, 4-D 0.5	23.4 $\pm$ 2.9	20.1 $\pm$ 2.2	21.1 $\pm$ 1.5	21.5 $\pm$ 0.9	21-26	Friable	light Yellow
LS+2, 4-D 1.0	47.4 $\pm$ 1.7	48.3 $\pm$ 4.1	24.8 $\pm$ 1.4	40.1 $\pm$ 7.6	21-25	Friable	Yellowish
LS+2, 4-D 2.5	60.2 $\pm$ 4.3	65.8 $\pm$ 4.6	50.9 $\pm$ 2.5	59.3 $\pm$ 4.0	21-22	Friable	Yellowish
LS+2, 4-D 3.0	48.2 $\pm$ 2.0	50.3 $\pm$ 2.8	49.2 $\pm$ 1.8	49.5 $\pm$ 0.9	21-28	Friable	Yellowish
Mean $\pm$ S.E	44.8 $\pm$ 7.7	46.3 $\pm$ 8.5	36.7 $\pm$ 8.0	42.6 $\pm$ 2.9			
MS+2, 4-D 0.5	—	—	—	—	23-28	Friable	Creamy
MS 2, 4-D 1.0	4.1 $\pm$ 0.3	5.1 $\pm$ 0.7	3.2 $\pm$ 0.2	4.1 $\pm$ 0.5	23-26	Friable	Creamy
MS 2, 4-D 2.0	20.7 $\pm$ 1.2	25.9 $\pm$ 1.7	21.8 $\pm$ 1.4	22.8 $\pm$ 1.5	22-24	Friable	Creamy
MS 2, 4-D 3.0	16.8 $\pm$ 1.5	20.7 $\pm$ 1.6	11.7 $\pm$ 1.1	16.4 $\pm$ 2.6	24-28	Friable	Creamy
Mean $\pm$ S.E	13.8 $\pm$ 5.0	17.2 $\pm$ 6.2	12.2 $\pm$ 5.3	14.4 $\pm$ 1.4			

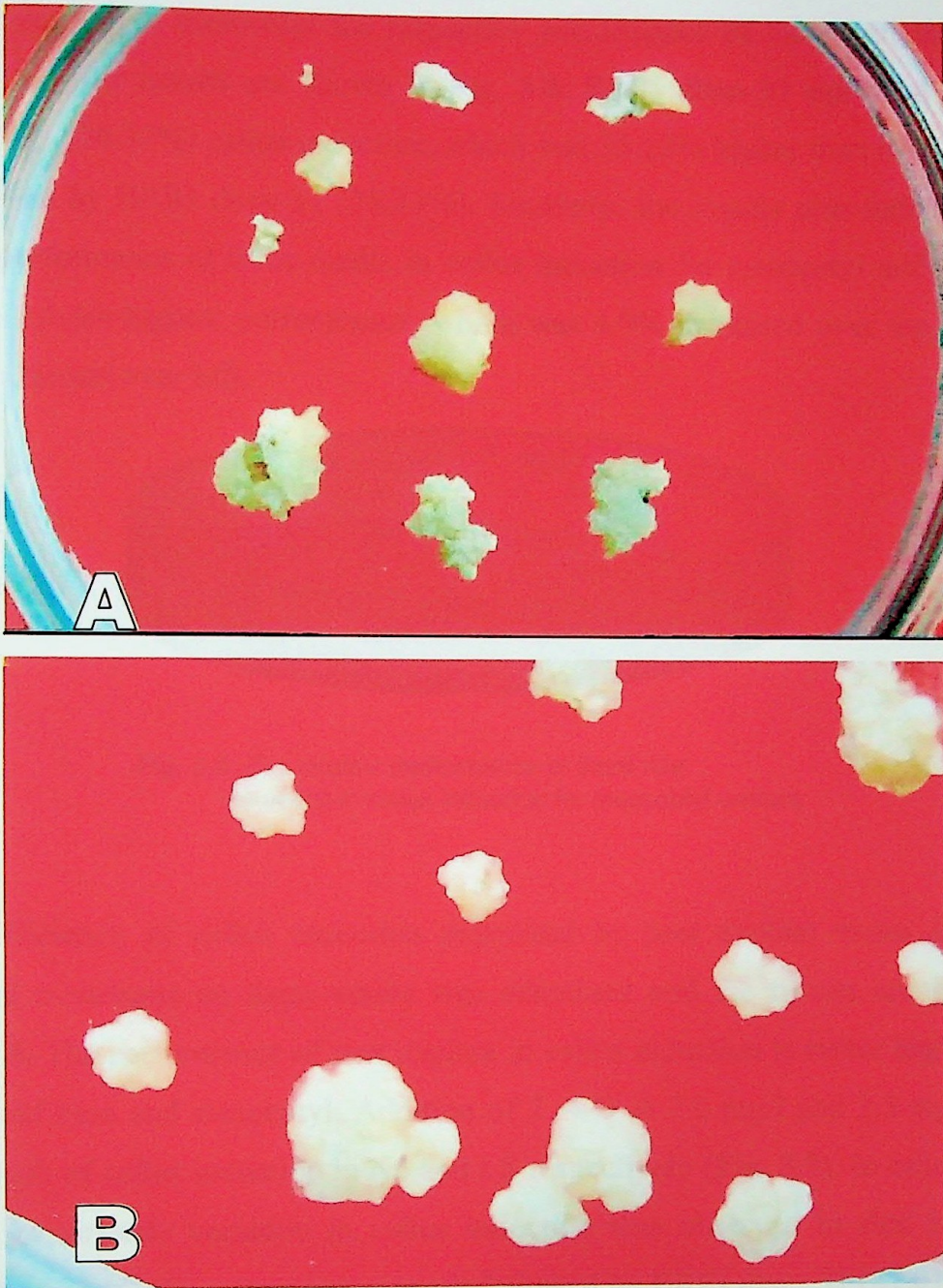


Fig. 3.7. A-B: Mesocotyl derived calli.

In respect of mesocotyl the respective performance of three varieties were studied and their results are shown in **Fig. 3.8**. BRRI dhan-30 exhibited highest performance (36.37%) callus induction, which was 33.23% higher than BRRI dhan-28 followed by BRRI dhan-31 (28.33%). Similarly, the results also indicated the relative performance of three media in callus induction for mesocotyl and LS was found to exhibit highest performance, which was 4.0% increased over the N6 and 66.0% increased over MS.

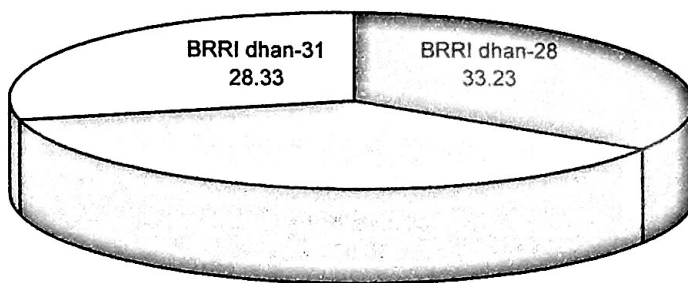


Fig. 3.8: The relative performance of three rice varieties in callus induction for mesocotyl explant.

Efficiency of callus induction expressed by root explant under different hormonal treatments on three media was examined and the results are given in **Table 3.6**. The performance of root explant in callus induction is rather higher than that of leaf base and mesocotyl. Addition of 2, 4-D in 2.0 mg/l and 2.5 mg/l gave better result of callus induction in N6 and LS respectively (**Fig. 3.9**). In case of root explant, remarkable variation in callus induction was observed in rice varieties. BRRI dhan-30 (86.2%) show better result followed by BRRI dhan-31 (81.0%). Low percentage of callus formation was observed in MS medium. Callus derived from different explants in rice were well developed and their colour varies from yellow, yellowish to light yellow. A remarkable difference was observed in their structure. The structure of callus was found hard when it was derived from scutellum but the calli gave friable structure when they were derived from other explant.

Table 3.6. Induction of callus from root explants of three rice varieties on different callus inducing media (each value is an average of 12-16 explants and the experiment was repeated twice).

Treatment (mg/l)	% of the callus formation (mean $\pm$ S.E.)			Mean $\pm$ S.E.	Days of callus initiation	Texture of the callus	Colour of the callus
	BRR1 dhan-28	BRR1 dhan-30	BRR1 dhan-31				
N6 +2, 4-D 0.5	24.8 $\pm$ 2.1	31.1 $\pm$ 1.8	30.3 $\pm$ 1.2	28.7 $\pm$ 1.9	20-25	Friable	light Yellow
N6 +2, 4-D 1.0	45.5 $\pm$ 3.6	50.8 $\pm$ 3.4	48.5 $\pm$ 2.5	48.2 $\pm$ 1.5	20-23	Friable	light Yellow
N6 +2, 4-D 2.0	74.8 $\pm$ 2.7	85.8 $\pm$ 2.4	81.0 $\pm$ 2.0	80.5 $\pm$ 3.1	20-22	Friable	light Yellow
N6 +2, 4-D 3.0	65.6 $\pm$ 2.8	75.2 $\pm$ 1.5	70.5 $\pm$ 2.9	70.4 $\pm$ 2.7	20-23	Friable	light Yellow
Mean $\pm$ S.E	52.6 $\pm$ 11.1	60.7 $\pm$ 12.2	57.5 $\pm$ 11.3	56.9 $\pm$ 2.3			
LS+2, 4-D 0.5	30.6 $\pm$ 1.7	32.2 $\pm$ 1.4	28.6 $\pm$ 1.0	30.4 $\pm$ 1.0	20-24	Friable	light Yellow
LS+2, 4-D 1.0	50.7 $\pm$ 1.8	52.6 $\pm$ 2.5	48.9 $\pm$ 2.7	50.7 $\pm$ 1.1	20-23	Friable	light Yellow
LS+2, 4-D 2.5	84.4 $\pm$ 3.1	86.2 $\pm$ 3.4	78.5 $\pm$ 3.1	83.0 $\pm$ 2.3	20-21	Friable	light Yellow
LS+2, 4-D 3.0	70.4 $\pm$ 2.6	74.8 $\pm$ 2.7	68.8 $\pm$ 5.6	71.3 $\pm$ 1.7	20-22	Friable	light Yellow
Mean $\pm$ S.E	59.0 $\pm$ 11.7	61.4 $\pm$ 11.9	56.0 $\pm$ 11.0	58.8 $\pm$ 1.6			
MS+2, 4-D 0.5	—	—	—	—	20-24	Friable	Yellowish
MS+2, 4-D 1.0	15.3 $\pm$ 1.8	20.0 $\pm$ 1.8	17.8 $\pm$ 2.4	17.7 $\pm$ 1.3	20-24	Friable	Yellowish
MS+2, 4-D 2.0	40.8 $\pm$ 3.9	51.0 $\pm$ 3.0	45.6 $\pm$ 2.4	45.8 $\pm$ 2.9	20-23	Friable	Yellowish
MS+2, 4-D 3.0	24.9 $\pm$ 2.3	30.9 $\pm$ 3.2	27.4 $\pm$ 2.2	27.7 $\pm$ 1.7	20-23	Friable	Yellowish
Mean $\pm$ S.E	26.9 $\pm$ 7.4	33.9 $\pm$ 9.0	30.2 $\pm$ 8.1	30.3 $\pm$ 2.0			

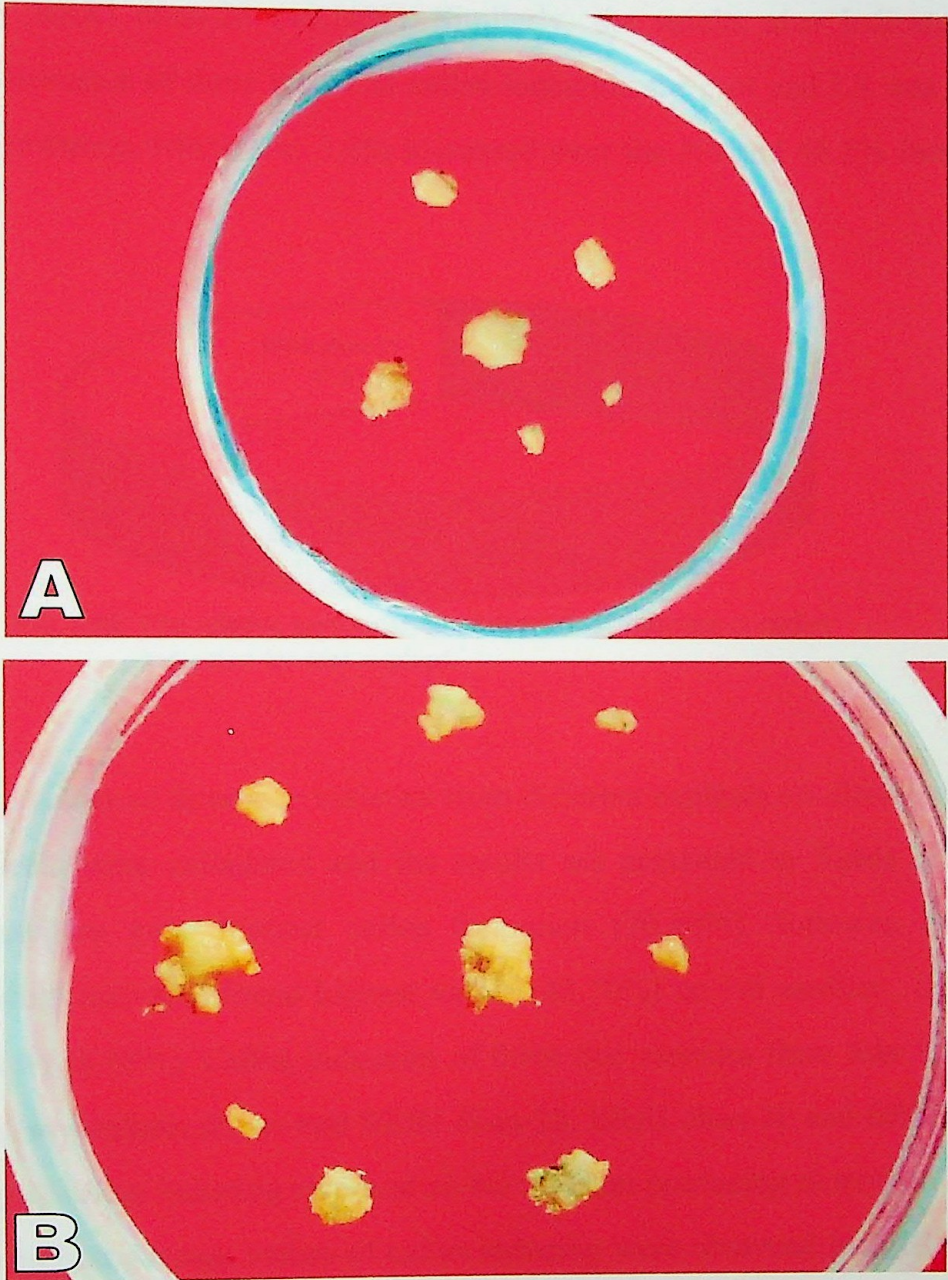


Fig. 3.9. A-B: Root explant derived calli.

In respect of root the respective performance of three varieties were studied and their results are shown in Fig. 3.10. BRRI dhan-30 exhibited highest performance (52.0%) callus induction, which was 47.9% higher than BRRI dhan-31 followed by BRRI dhan-28 (46.16%). Similarly, the results also indicated the relative performance of three media in callus induction for root and LS was found highest performance, which was 3.2% increased over the N6 and 48.5% increased over MS.

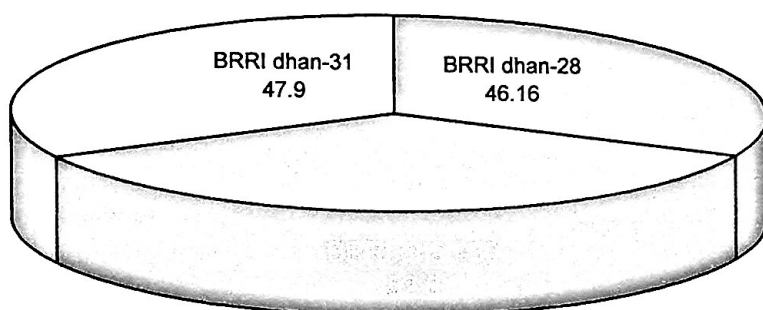


Fig.3.10: The relative performance of three rice varieties in callus induction for root explant.

Mean values of three rice varieties from different explants on different callus inducing media were calculated and the results are presented in Table 3.7. The explant scutellum gave highest callus formation (76.40%), followed by root (60.70%) while leaf-base found highest value (43.70%) on N6 medium, followed by mesocotyl (36.10%). Callus induction of three rice varieties from four different explants on N6 medium shows that BRRI dhan-28, BRRI dhan-30 and BRRI dhan-31 gave higher callus inducing response on N6 medium producing more than 53.0% callus. The highest average values of callus inducing were observed in BRRI dhan-30 (56.60%) followed by BRRI dhan-28 (51.50%) and BRRI dhan-31 (50.80%).

Table 3.7. Mean values of callus induction of different explants taken from three rice varieties on different callus inducing media.

Explants ↓ Media→	Varieties												Mean±S.E
	BRRJ dhan-28			BRRJ dhan-30			BRRJ dhan-31						
	N6	LS	MS	N6	LS	MS	N6	LS	MS	N6	LS	MS	
Scutellum	71.5 ±13.6	61.7±13.7	44.3±16.8	76.4±12.7	67.7±13.8	58.7±16.7	68.6±13.0	61.5±13.2	49.5±15.2	62.2±3.4			
Leaf base	41.9±8.4	43.1±9.1	18.8±7.3s	43.7±7.7	48.2±9.9	23.6±7.1	41.3±7.9	41.1±10.9	19.6±7.0	35.8±3.8			
Mesocotyl	40.1±6.1	44.8±7.7	13.8±5.0	45.6±5.3	46.3±8.5	17.2±6.2	36.1±6.0	36.7±8.0	12.2±5.3	32.5±4.7			
Root	52.6±11.1	59.0±11.7	26.9±7.4	60.7±12.2	61.4±11.9	33.9±9.0	57.5±11.3	56.0±11.0	30.2±8.1	48.6±4.7			
Grand mean±S.E	51.5±7.2	52.1±4.7	25.9±6.6	56.6±7.6	55.9±5.1	33.3±9.1	50.8±7.4	49.2±5.7	27.8±8.0				

In LS medium, mean values indicated that the explant scutellum gave highest callus formation (67.75%), followed by root (61.40%) and lowest value was recorded in mesocotyl (36.70%), followed by leaf base (48.20%). The highest average values of callus induction was observed in BRRI dhan-30 (55.9%) followed by BRRI dhan-28 (52.10%) and BRRI dhan-31 (49.20%) and their mean value was calculated by 52.4%. It is important to note that overall performance of callus induction obtained from leaf base, mesocotyl and root explants on N6 found lesser than that of LS medium.

The callus inducing response of scutellum on MS medium was found poor and it varies from 44.3 to 58.7%. The explants of leaf base, mesocotyl and root produced less than 21% callus in all varieties in this medium. In the mean values of explant in all varieties scutellum gave highest percentage of callus (50.8%), followed by root (30.33%). Better response was found in leaf base (20.66%) than that of mesocotyle (14.40%). The mean values of callus formation of three varieties irrespective of any explant on MS medium shows that only BRRI dhan-30 variety gave 33.9% of callus and the remaining two varieties (BRRI dhan-28, BRRI dhan-31) produced less than 26.0% of callus.

From foregoing discussion it may be concluded that the media N6 and LS exhibited more or less similar level of callus induction in all rice varieties. But in case of scutellum explant N6 induced better callus induction over LS although N6 and LS media exhibited similar level of callus induction. While the scutellum derived calli grown on N6 medium more suitably than LS. On the other hand, LS medium proved to be more favorable for leaf base, mesocotyl and root explants derived calli than that of N6 medium. Results obtained from different experiments

confirmed that MS medium supplemented with 2, 4-D was less efficient in callus induction in rice varieties in comparison to the performances of N6 and LS.

3.5.3 Varietal Response in Callus Formation

The mean values of callus induction of three varieties over the values of explants and media were computed and the results are presented in **Fig. 3.11**. The figure shows that BRRI dhan-30 variety proved to be more efficient in callus induction and the result was found to be 48.60% on artificial media. In respect of callus induction efficiency the variety BRRI dhan-28 stood 2nd position (43.20%) and BRRI dhan-31 variety was found less efficient in callus induction.

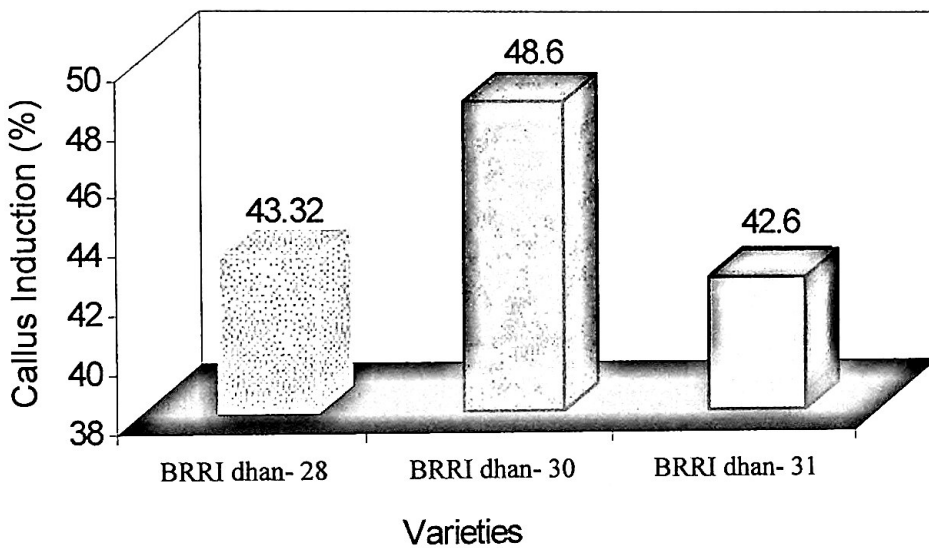


Fig. 3.11: Callus induction performance of three rice varieties.

3.5.4 Explant Response in Callus Formation

The callus inducing capability of different explants was also studied over all varieties in different media and the results are presented in **Table 3.8**. The table shows that scutellum induced highest percentage of embryogenic calli.

Table 3.8. Mean values of explants (over varieties) on different callus inducing media.

Media	Explants				Mean % $\pm$ S.E.
	Scutellum % $\pm$ S.E.	Leaf base % $\pm$ S.E.	Mesocotyl % $\pm$ S.E.	Root % $\pm$ S.E.	
N6	72.1 $\pm$ 2.2	42.3 $\pm$ 0.7	40.6 $\pm$ 2.7	56.9 $\pm$ 2.3	53.0 $\pm$ 7.3
LS	63.6 $\pm$ 2.0	44.6 $\pm$ 1.7	42.6 $\pm$ 2.9	58.8 $\pm$ 1.5	52.4 $\pm$ 5.1
MS	50.8 $\pm$ 4.2	20.6 $\pm$ 1.4	14.4 $\pm$ 1.4	30.3 $\pm$ 2.6	29.0 $\pm$ 7.9
Mean $\pm$ S.E.	62.1 $\pm$ 6.1	35.8 $\pm$ 7.6	32.5 $\pm$ 9.0	48.6 $\pm$ 9.1	

The scutellum produced 72.1% callus in N6 medium 63.60% in LS and 50.80% in MS medium. Scutellum produced 29.58% and 20.13% increased callus in N6 and LS media over the value of MS medium respectively. The callus obtained on N6 and LS media from scutellum explant in all tested varieties showed compact texture, globular structure, yellowish in colour and embryogenic in nature. The varieties of BRRI dhan-28, BRRI dhan-30 and BRRI dhan-31 produced more compact, yellowish calli derived from scutellum explant but all varieties on MS medium produced creamy coloured callus in case of scutellum.

The root explant produced more than 57.85% callus on N6 and LS media but 30.30% on MS medium. The calli obtained from root explants on N6 and LS media from all varieties had almost same texture. The calli were light yellow in colour friable in nature and globular in behaviour. The root-derived calli on MS medium were brownish in colour and less friable which ultimately died after 3-4 weeks. The leaf base and mesocotyl explants produced calli at a very low frequency producing less than 42.50% callus on N6 and LS media and less than 17.50% on MS medium. The calli obtained from leaf base explants on N6 and LS media were globular, friable but not compact. In BRRI dhan-28, BRRI dhan-30 and BRRI dhan-31 varieties the leaf base derived calli were yellowish and friable.

The leaf base derived calli obtained on MS medium were yellowish and friable in all varieties.

Mean values of callus producing potentiality of different explants are shown in **Fig. 3.12**. The figure shows that scutellum explant produced highest (62.10%) callus, followed by root explant (48.60%). Leaf base explant also shows better response (35.80%) over mesocotyl explant (32.5%). The results indicated that the scutellum explant appeared as the most suitable callus inducing explant in rice producing two-fold callus formation over the values of mesocotyl.

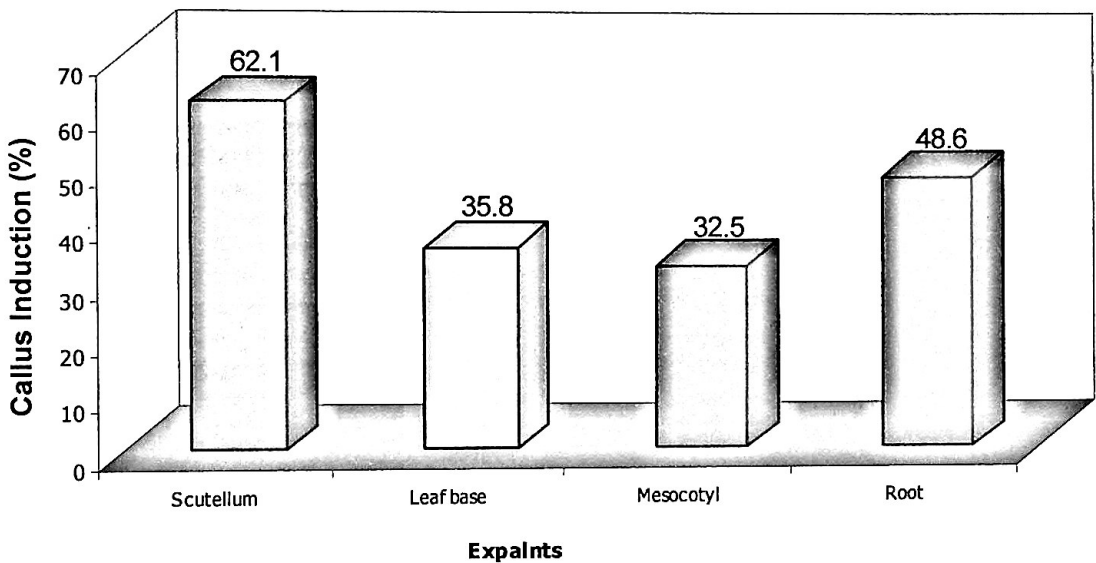


Fig. 3.12: Mean values of explants (over varieties) on three callus inducing media.

3.3.5 Response of Different Varieties and Explants to Different Media

The mean values of callus induction of three different media are presented in **Fig. 3.13**. The figure shows that among the three tested callus inducing media, the N6 and LS media showed higher callus inducing response and produced 53.0% and 52.40% callus respectively over the values of varieties and explants. There was two fold increased in callus induction on N6 (53.0%) and LS (52.4%) media over the values of MS medium (29.0%). Experimental results proved that N6 and LS media were more suitable callus inducing media for the explants of rice varieties.

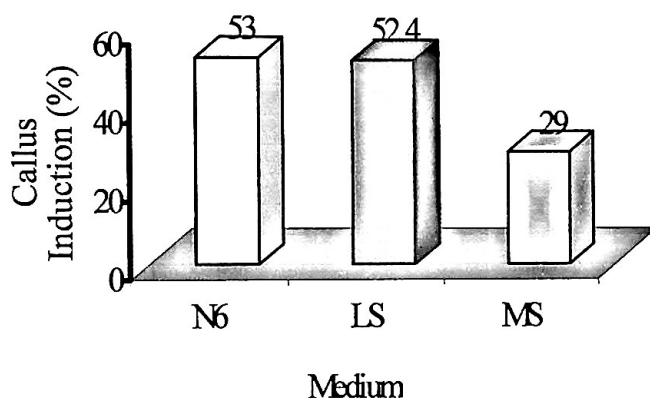


Fig. 3.13: Mean values of callus induction (over varieties and explants) on three different callus inducing media.

3.6 Regeneration of Rice

For regeneration study three rice varieties of BRRI dhan-28, BRRI dhan-30 and BRRI dhan-31 was selected on the basis of their greater percentage of callus induction. Investigation on the comparative regeneration efficiency of a variety needs enormous collection of induced calli. Under the present investigation, regeneration study has been undertaken on the selected rice varieties through somatic embryogenesis.

3.6.1 Callus Regeneration Response

The embryogenic calli obtained from different explants of BRRI dhan-28, BRRI dhan-30 and BRRI dhan-31 was subjected to different regeneration media. The calli obtained from different explants had three morphological phenotypes. Calli with a dry, compact and nodular appearance formed numerous globular structures and were embryogenic nature. Second type of calli was friable, translucent and somewhat slimy and never formed embryos called non-embryogenic. The third type had a pronounced rhizogenic capacity and some of them were entirely composed of root primordia.

3.6.2 Regeneration from Scutellum Calli

Plant regeneration from scutellum derived calli depends on a number of factors e.g., age of scutellum, type of derived calli and varieties etc. The effect of age of scutellum derived calli for three rice varieties were tested and the results are given in **Table 3.9**.

Table 3.9. Age effect of calli derived from scutellum on regeneration performance.

Age of scutellum callus	Regeneration percentage in different rice varieties (mean % $\pm$ S.E.)		
	BRRI dhan -28	BRRI dhan -30	BRRI dhan -31
5-Days old	4.4 $\pm$ 0.7	5.2 $\pm$ 1.0	3.1 $\pm$ 0.5
8- Days old	20.7 $\pm$ 1.6	21.1 $\pm$ 2.8	16.9 $\pm$ 1.4
21-Days old	85.3 $\pm$ 3.0	88.2 $\pm$ 2.8	86.6 $\pm$ 3.8

The 21 days old calli of BRRI dhan-30 gave the highest percentage of regeneration (88.2%) while BRRI dhan-31 gave 86.6% and BRRI dhan-28 gave 85.3% regeneration. Regeneration efficiency of 5 days old calli was very low, only

3.1%. While 8-days old calli showed 16 to 21% of regeneration in all varieties of rice. In 21-days old calli regeneration percentage was found highest (86.40%) (Fig. 3.14.).

The regeneration response of scutellum derived calli were studied and the results are presented in **Table 3.10**. The table shows that the variety BRRI dhan-30 gave highest regeneration response on medium RM (88.2%) while regeneration response on medium RM3 was the lowest (33.0%). The regeneration response on medium RM 1 was 72.3% and 42.2% regeneration was recorded on RM 2 for BRRI dhan-30. The variety of BRRI dhan-31 gave highest regeneration response (86.3%) on medium RM while 69.4% on medium RM1. The regeneration response on media RM3 and RM2 varied from 29.1 to 39.1%. The variety of BRRI dhan-28 gave the highest regeneration response on medium RM (85.8%) and it was 65.2% on RM1. The lowest regeneration response recorded on media RM 3 and RM 2 varied from 30.5 - 35.4%.

Individual regeneration percentage of three varieties for each medium was computed and presented in **Fig. 3.15**. The figure shows that BRRI dhan-30 gave 88.2% regeneration in medium RM, which is two times more regeneration response than the average value of media RM1, RM2 and RM3 (49.2%). In case of BRRI dhan-28 the regeneration percentage was highest on medium RM (85.8%), which was two times more than the average values of media RM1, RM2 and RM3 (43.7%). In case of BRRI dhan-31 the regeneration percentage was highest on medium RM (86.3%) and the average value of media RM1, RM2 and RM3 was 45.9%. The figure shows that in the comparative study of regeneration media, the medium RM gave best regeneration response (86.8%), two times more than the average value of media RM1, RM2 and RM3 (46.2%).



Fig. 3.14: Scutellum derived plants via regenerable callus.
A. Plantlets developed from the embryoids.
B. Scutellum derived plants.

Table 3.10. Regeneration response of three rice varieties from scutellum derived calli on BAP with NAA in MS and N6 basal media.

Regeneration Media (RM)	Media formulation Mg/l	Varieties					
		BRRI dhan-28		BRRI dhan-30		BRRI dhan-31	
		%± S.E. of shoot formation	No. of shoot/callus	% ± S.E. of shoot formation	No. of shoot/ of shoot/ callus	%± S.E. of shoot formation	No. of shoot/ callus
RM	MS+2 mg/l BAP+ 0.05 mg/l NAA+3% sorbitol	85.8±2.3	5.00	88.2±2.1	5.25	86.3±1.5	4.65
RM1	MS+0.5mg/l BAP+ 0.05 mg/l NAA	65.2±2.6	4.00	72.3±2.7	4.25	69.4±3.9	4.00
RM2	MS+0.5 mg/l BAP	35.3±2.2	3.00	42.2±1.3	2.50	39.1±3.6	3.29
RM3	N6+0.5 mg/l BAP+ 0.05 mg/l NAA	30.5±1.6	4.25	33.0±1.8	3.75	29.1±1.5	3.15
Mean±S.E		54.2±13.0	4.1	58.9±12.8	3.9	55.9±13.2	3.8

RM = MS+2 mg/l BAP+ 0.05 mg/l NAA+3% sorbitol

RM1 = MS+0.5mg/l BAP+ 0.05 mg/l NAA

RM2 = MS+0.5 mg/l BAP

RM3 = N6+0.5 mg/l BAP+ 0.05 mg/l NAA

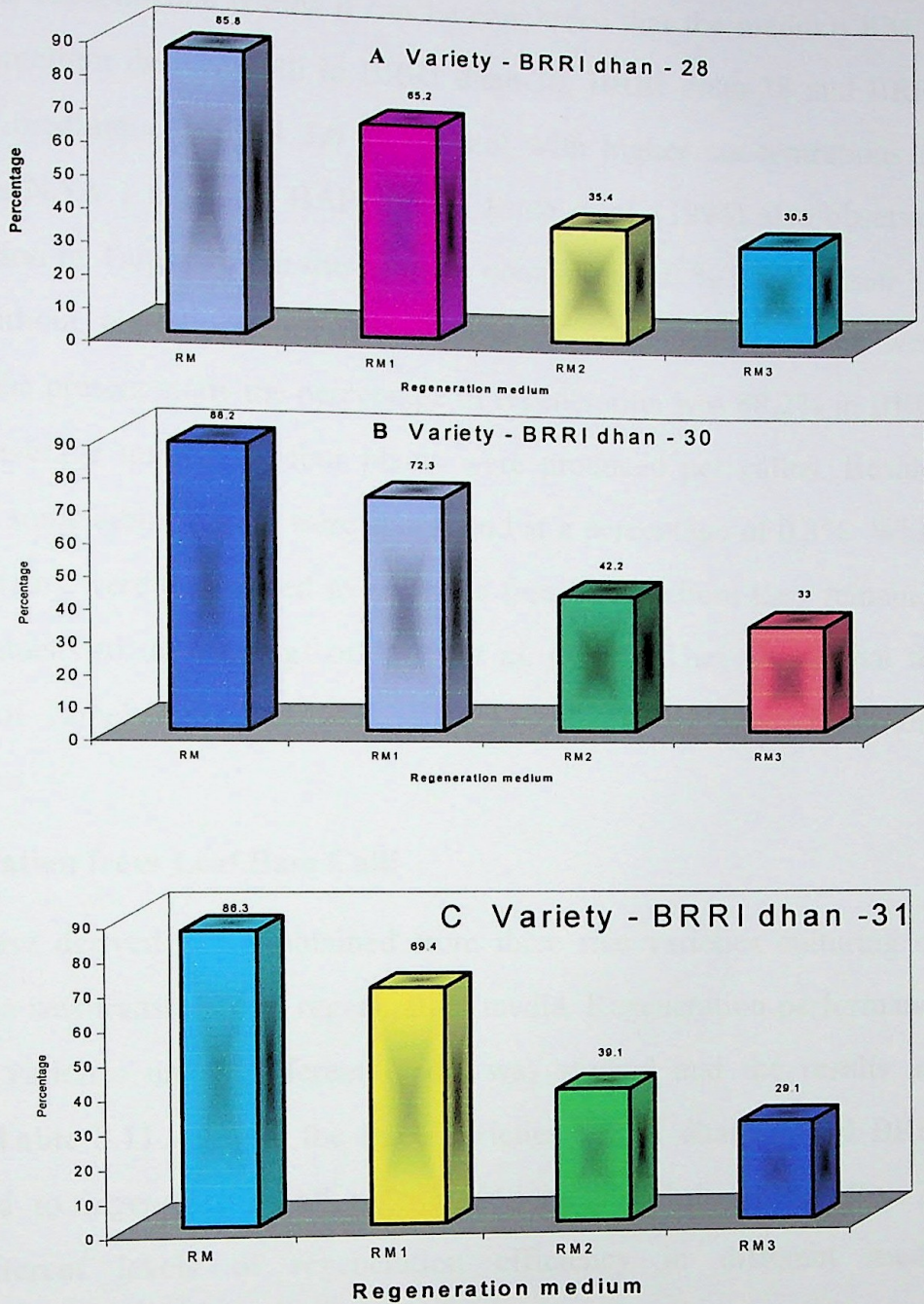


Fig. 3.15: Comparative regeneration response of scutellum derived calli on four different regeneration media (RM)

A. Comparative regeneration response of BRR I dhan - 28

B. Comparative regeneration response of BRR I dhan - 30

C. Comparative regeneration response of BRR I dhan - 31

From the experimental results it can be concluded that the medium RM is the best for scutellum derived calli in BRRI dhan-30, BRRI dhan-28 and BRRI dhan-31. This medium contained 3.0 % sorbitol with higher concentrations of phytohormones NAA 1 mg/l and BAP 2 mg/l. Rueb *et al.* (1994) also observed 63% regeneration in Taipei 309 in the medium containing 2.0 % sucrose and 3.0 % sorbitol without phytohormones and average of 2 plants per callus were produced. In the present study the percentage of regeneration was 88.2% in BRRI dhan-30. An average number of four plants were produced per callus. Besides normal plants, some albino plants were also found at a percentage of 0.3%. When these albino plants were transferred to hormone free MS medium they remained albinos in contrasts to the findings of Rueb *et al.* (1994). They found that the albino plants of Taipei 309 gradually became green after shifting into hormone free LS medium.

3.6.3 Regeneration from Leaf Base Calli

Leaf base derived callus obtained from three rice varieties culturing on different media was transferred to regeneration media. Regeneration performance of three rice varieties under different media was studied and the results are presented in **Table 3.11**. Among the three varieties, BRRI dhan-28 and BRRI dhan-30 failed to regenerate in all regeneration media. Only BRRI dhan-31 exhibited different levels of regeneration efficiency in different media preparations. On transference to MS and N6 basal media without any growth regulator, yellowish nodular calli became dark green or blackish and no further morphogenic changes were noticed. In some cases, external portion of calli started to become blackish and consequently the calli become necrotic.

Table 3.11. Regeneration response of BRRI dhan-31 rice variety from leaf base derived calli on BAP with NAA in MS and N6 basal media.

Regeneration Media(RM)	Media formulation (mg/l)	Variety BRRI dhan-31		
		%± S.E. of shoot formation	No. of shoot/callus	Shoot length (cm)
RM	MS+2.0mg/l BAP+ 0.5 mg/. NAA	12.8 ± 1.6	3.10	3.59
RM1	MS+0.5 mg/l BAP + 0.05 mg/l NAA	24.3 ± 2.2	3.25	4.00
RM2	MS+0.5 mg/l BAP	8.7 ± 1.2	3.00	4.00
RM3	N6+0.5 mg/l BAP +0.05 mg/l NAA	8.2 ± 0.6	3.25	4.00
Mean± S.E		13.5 ± 3.7	3.15	3.89

RM = MS+2.0 mg/l BAP+ 0.5 mg/l NAA

RM1 = MS+0.5mg/l BAP+ 0.05 mg/l NAA

RM2 = MS+0.5 mg/l. BAP

RM3 = N6+0.5 mg/l BAP+ 0.05 mg/l NAA

When the calli of BRRI dhan-31 were transferred to medium RM1 (BAP 0.5 mg/l and NAA 0.05 mg/l) shoot proliferation was started within two weeks of subculture. Highest percentage (24.3%) of callus produced plantlets/ shoots and maximum number (3.25) of shoots per culture were obtained in medium RM1 (**Fig. 3.16**). The percentage of shoot formation was 12.8% on RM medium. The regeneration was found less in media RM2 and RM3 (8.5%). The result shows that the leaf base explant gave poor response in callus formation as well as poor response in regeneration. It may be concluded that the explant, which gave poor callus response, would give poor regeneration response.

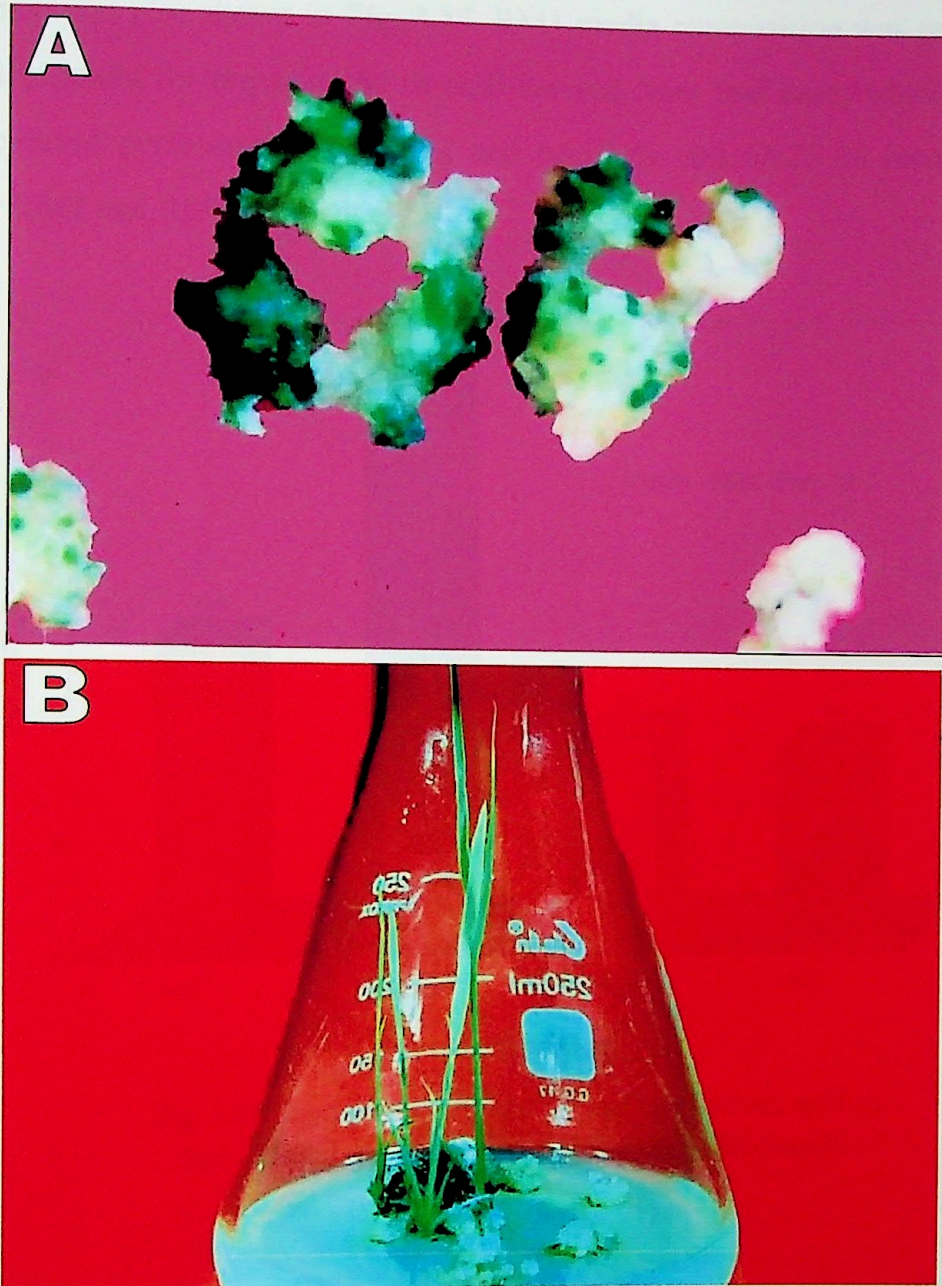


Fig. 3.16: Leaf base derived plants via regenerable callus.

A. Plantlets developed from the embryoids.

B. Leaf base derived plants.

The regeneration efficiency was highly affected by different media composition and the mean value of the efficient performance of four media used in the experiment was also shown in **Fig. 3.17**. RM1 media composition was proved as the most efficient for regeneration of calli derived from leaf base, which was 47.3 % increased performance over RM and 64.19 % and 66.25 % increased values over RM2 and RM3 respectively.

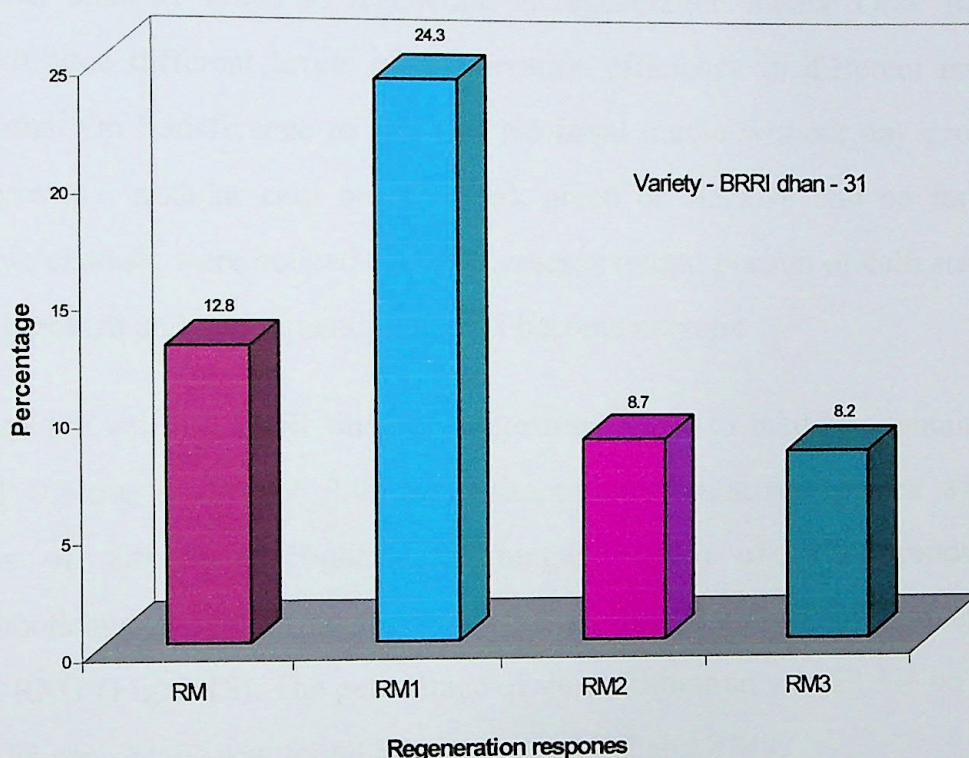


Fig. 3.17: Regeneration response of leaf base derived calli on four different regeneration media (RM) in BRRI dhan-31 .

The explant root produced calli, which looked like embryogenic in nature but failed to regenerate into whole plant in tested regeneration media.

3.6.4 Regeneration from Mesocotyl Calli

Mesocotyl derived callus obtained from three rice varieties culturing on different media was transferred to four regeneration media. Regeneration performances of three rice varieties under different media were studied and the results are presented in **Table 3.12**. Among the three varieties studied BRRI dhan-28 and BRRI dhan-31 failed to regenerate in regeneration media. Only BRRI dhan-30 exhibited different levels of regeneration efficiency in different media concentrations. On transference to MS and N6 basal media without any growth regulator, creamy, nodular calli become dark green or blackish and no further morphogenic changes were noticed. In some cases, external portion of calli started to become blackish and consequently the calli become necrotic.

When the calli of BRRI dhan-30 were transferred to medium containing RM1 (BAP 0.5 mg/l and NAA 0.05 mg/l) shoot proliferation was started within two weeks of subculture. Highest percentage (32.4%) of callus produced plantlets/shoots and maximum number (4.55) of shoots per culture were obtained in medium RM1 (**Fig. 3.18**). The percentage of shoot formation was 22.3% on RM medium. The percentage was found less in media RM2 and RM3.

In the present investigation it was observed that different concentrations of 2,4-D alone produced callus in good frequency from mesocotyl explants of rice. It is also noted that, in the present study, there were no albino plants obtained from mesocotyl derived callus. Albino plants are unwanted and their production greatly limits the objects of tissue culture technique (Torriso and Zapata, 1986).

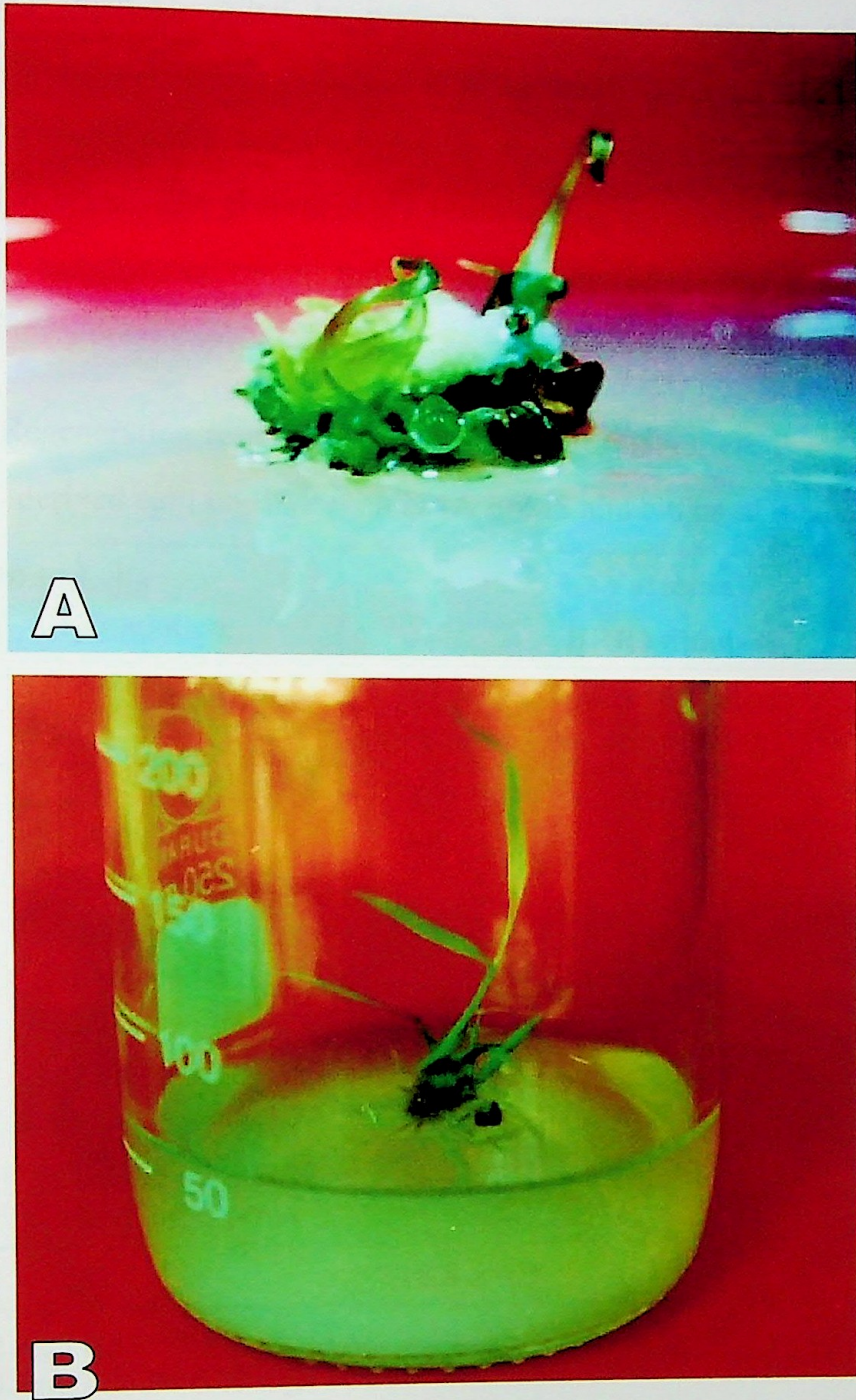


Fig. 3.18: Mesocotyl derived plants via regenerable callus.

A. Plantlets developed from the embryoids.

B. Mesocotyl derived plants.

Among the three varieties of rice studied in this investigation BRRI dhan-28 and BRRI dhan-31 failed to perform a complete process of artificial regeneration under mesocotyl culture. However BRRI dhan-30 experienced an efficient performance under both the previews of callus induction and regeneration in mesocotyl culture. The present study revealed that the variety BRRI dhan-30 proved to be a suitable rice variety holding potentiality of genetic manipulation to be applied in mesocotyl explant culture.

Table 3.12. Regeneration response of BRRI dhan-30 rice variety from mesocotyl derived calli on BAP with NAA in MS and N6 basal media.

Regeneration Media(RM)	Media formulation (mg/l)	Varieties BRRI dhan-30		
		%± S.E of shoot formation	No. of shoot/callus	Shoot length (cm)
RM	MS+2mg/l BAP+0.5 mg/l. NAN	22.3 ± 1.7	2.00	
RM1	MS+0.5 mg/l BAP + 0.05 mg/l NAA	32.4 ± 3.0	4.55	4.00
RM2	MS+0.5 mg/. BAP	8.3 ± 0.6	3.25	4.00
RM3	N6+0.5 mg/l BAP +0.05 mg/l NAA	12.4 ± 1.1	3.69	3.50
Mean± S.E		18.8 ± 5.3	3.37	4.03

RM = MS+2 mg/l BAP+ 0.5 mg/l NAA
 RM1 = MS+0.5mg/l BAP+ 0.05 mg/l NAA
 RM2 = MS+0.5 mg/l. BAP
 RM3=N6+0.5mg/lBAP+0.05mg/lNAA

3.7 Meristem Regeneration Response

The meristem (leaf base) of six rice varieties was isolated from different aged seedlings and cultured on MS medium for meristem regeneration. The results of regeneration of six varieties are presented in **Table 3.13**. The result shows that all tested varieties gave regeneration from meristem in the range of 31.3 - 64.7% when meristem was isolated from 4 days old seedlings. The variety BRRI dhan-28 gave higher regeneration (64.7%), followed by BRRI dhan-30 (56.1%). The variety BR-22 gave the lowest value of meristem regeneration (31.3%), followed by BR-23 (38.6%). The varieties of BRRI dhan-29, BRRI dhan-30 and BRRI dhan-31 gave moderate response in meristem regeneration about 49.4%. The mean values of meristem regeneration from different aged seedlings shows that BRRI dhan-28 gave highest meristem regeneration response (49.0%), followed by BRRI dhan-30 (43.9%), BRRI dhan-31 (39.7%) and BRRI dhan-29 (31.3%). This difference of meristem regeneration response might be due to difference of genotype. The variety BR-22 gave lowest response among tested varieties (25.0%), followed by BR-23 (31.1%).

Table 3.13. Effect of different age of seedlings on meristem regeneration.

Age of seedlings	Varieties					
	BR-22 %± S.E.	BR-23 %±S.E.	BRRI dhan-28 %± S.E.	BRRI dhan-29 %± S.E.	BRRI dhan-30 %± S.E.	BRRI dhan-31 %± S.E.
4 – day	31.3±2.3	38.6±0.6	64.7±2.9	40.1±1.6	56.1±2.4	51.2±2.4
5 – day	29.6±3.8	34.3±2.4	55.0±2.1	32.6±2.1	50.3±3.4	45.2±2.9
7 – day	14.3±2.6	20.5±2.9	27.3±2.4	21.2±1.7	25.3±3.2	22.7±2.8
Mean ± S.E.	25.0±5.4	31.1±5.4	49.0±11.2	31.3±5.4	43.9±9.4	39.7±8.6

The effect of different concentrations of phytohormones of NAA and BAP was observed on meristem regeneration. Meristems of six varieties were isolated from 4 day old seedlings and subjected to MS medium supplemented with different concentrations of phytohormones and the results of the experiment are shown in **Table 3.14**. The table is presenting the meristem regeneration response of six varieties on MS medium with 10 different concentrations of BAP and NAA. The table shows that the response of meristem regeneration ranging from 31.3 – 64.7 % on MS medium without any hormone. With the addition of NAA and BAP the meristem regeneration was decreased. The meristem regeneration ranging from 10.3 – 30.2% in all tested varieties at 0.5 mg/ l NAA and 0.5 mg/l BAP. With increasing concentration of BAP while NAA kept constant the meristem regeneration was slowly decreased. But with the increasing concentrations of NAA while BAP kept constant the meristem regeneration was decreased considerably. The percentage of meristem regeneration became very low at hormone level of 0.5 mg/1 NAA and 2.5 mg/1 BAP (8.7%) than that of hormone concentration of 2.5 mg/1 NAA and 0.5 mg/1 BAP. The meristem of varieties BRRI dhan-28 and BRRI dhan-30 gave half the value of regeneration when cultured on MS medium supplemented with 0.5 mg/1 NAA and BAP. The varieties BR-22 and BR-23 gave 3 times less meristem regeneration on MS medium with 0.5 mg/1 NAA and BAP. The variety BRRI dhan-29 and BRRI dhan-31 gave two times less regeneration in this medium when compared with MS without hormones. The varieties BR-22, BR-23, BRRI dhan-28, BRRI dhan-29, BRRI dhan-30 and BRRI dhan-31 gave meristem regeneration within the range of 1.0 – 31.0% on all cultured media.

Table 3.14. Regeneration efficiency of meristem on MS medium supplemented with different concentrations of NAA and BAP (mg/l).

Media formulation	Varieties (mean % $\pm$ S.E.)					
	BR-22	BR-23	BRRIdhan-28	BRRIdhan-29	BRRIdhan-30	BRRIdhan-31
MS without hormones	31.3 $\pm$ 2.3	38.6 $\pm$ 0.6	64.7 $\pm$ 2.9	40.1 $\pm$ 1.6	56.1 $\pm$ 2.4	51.2 $\pm$ 2.4
MS+0.5mg/l NAA+0.5mg/l BAP	10.3 $\pm$ 1.8	14.1 $\pm$ 1.8	28.5 $\pm$ 3.1	15.5 $\pm$ 2.0	30.2 $\pm$ 3.0	25.0 $\pm$ 2.0
MS+0.5mg/l NAA+1.0mg/l BAP	11.8 $\pm$ 1.6	17.1 $\pm$ 1.6	25.0 $\pm$ 3.0	16.3 $\pm$ 2.6	27.1 $\pm$ 3.2	27.4 $\pm$ 3.3
MS+0.5mg/l NAA+1.5mg/l BAP	8.5 $\pm$ 1.6	13.2 $\pm$ 1.5	22.3 $\pm$ 3.3	12.2 $\pm$ 1.3	23.6 $\pm$ 3.1	22.5 $\pm$ 0.9
MS+0.5mg/l NAA+2.0mg/l BAP	7.7 $\pm$ 0.5	9.2 $\pm$ 0.8	18.3 $\pm$ 1.2	10.5 $\pm$ 0.8	17.3 $\pm$ 0.7	14.5 $\pm$ 0.6
MS+0.5mg/l NAA+2.5mg/l BAP	8.3 $\pm$ 0.3	7.6 $\pm$ 0.8	10.0 $\pm$ 0.7	8.2 $\pm$ 0.6	11.2 $\pm$ 0.6	7.1 $\pm$ 0.9
MS+1.0mg/l NAA+0.5mg/l BAP	7.6 $\pm$ 1.0	7.2 $\pm$ 0.9	11.4 $\pm$ 1.9	9.2 $\pm$ 0.7	14.9 $\pm$ 2.0	5.3 $\pm$ 0.8
MS+1.5mg/l NAA+0.5mg/l BAP	7.3 $\pm$ 0.8	7.0 $\pm$ 0.7	10.1 $\pm$ 1.0	6.3 $\pm$ 0.6	9.1 $\pm$ 0.5	6.2 $\pm$ 0.8
MS+2.0mg/l NAA+0.5mg/l BAP	5.5 $\pm$ 0.3	8.1 $\pm$ 0.4	10.1 $\pm$ 0.2	3.2 $\pm$ 0.2	5.3 $\pm$ 0.6	4.8 $\pm$ 0.4
MS+2.5mg/l NAA+0.5mg/l BAP	2.5 $\pm$ 0.3	4.2 $\pm$ 0.7	2.2 $\pm$ 0.2	1.5 $\pm$ 0.1	2.4 $\pm$ 0.5	1.9 $\pm$ 0.6

The effect of different hormones on meristem regeneration was studied and the mean values of meristem regeneration with different concentrations of hormones and without hormones are shown in Fig. 3.19. The figure revealed that the hormones NAA and BAP when used at different concentrations decreased the meristem regeneration. In case of BRRIdhan-28 and BRRIdhan-30 four times

decrease in meristem regeneration was observed. In varieties BR-22, BR-23, BRRi dhan-29 and BRRi dhan-31 meristem regeneration were decreased in four folds. Different stages of meristem regeneration are shown in Fig. 3.20. The figure shows that when meristem was cultured on MS medium, the root formation started at basal portion and shoot formation at upper portion (Fig. 3.20). The meristem directly regenerated into whole plant without forming any callus.

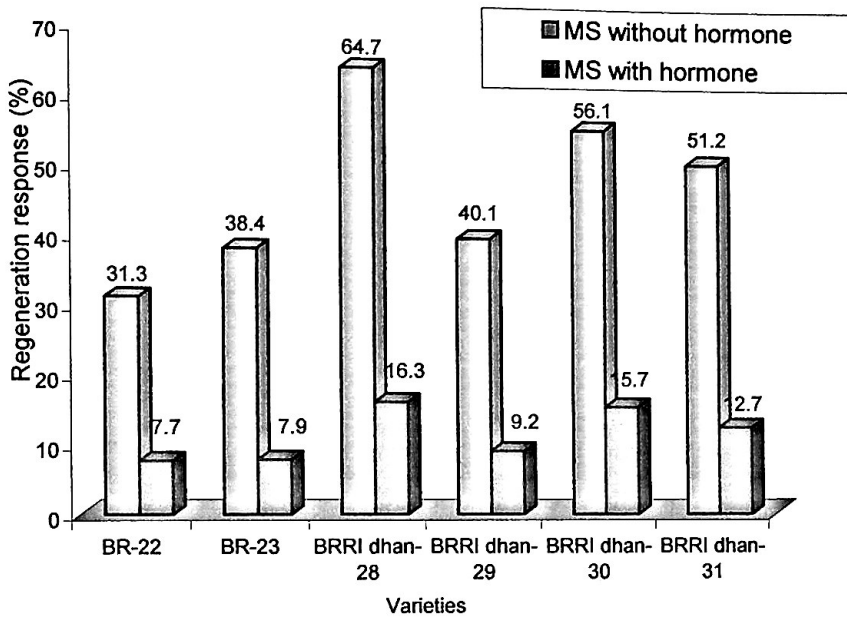


Fig. 3.19. Comparison of meristem regenerated plants with and without hormones.

Bobak *et al.* (1995) also observed such type of response in *Dorser rotundifolia* L. (a carnivorous plant) during shoot regeneration from isolated leaves. They observed best direct shoot regeneration on MS growth regulators free medium or at very low concentration of NAA. The similar findings were also found by Simola, (1987) and Anthony, (1992), they reported that hormone free medium induced regeneration in leaf culture of *Droser rotundifolia* L.

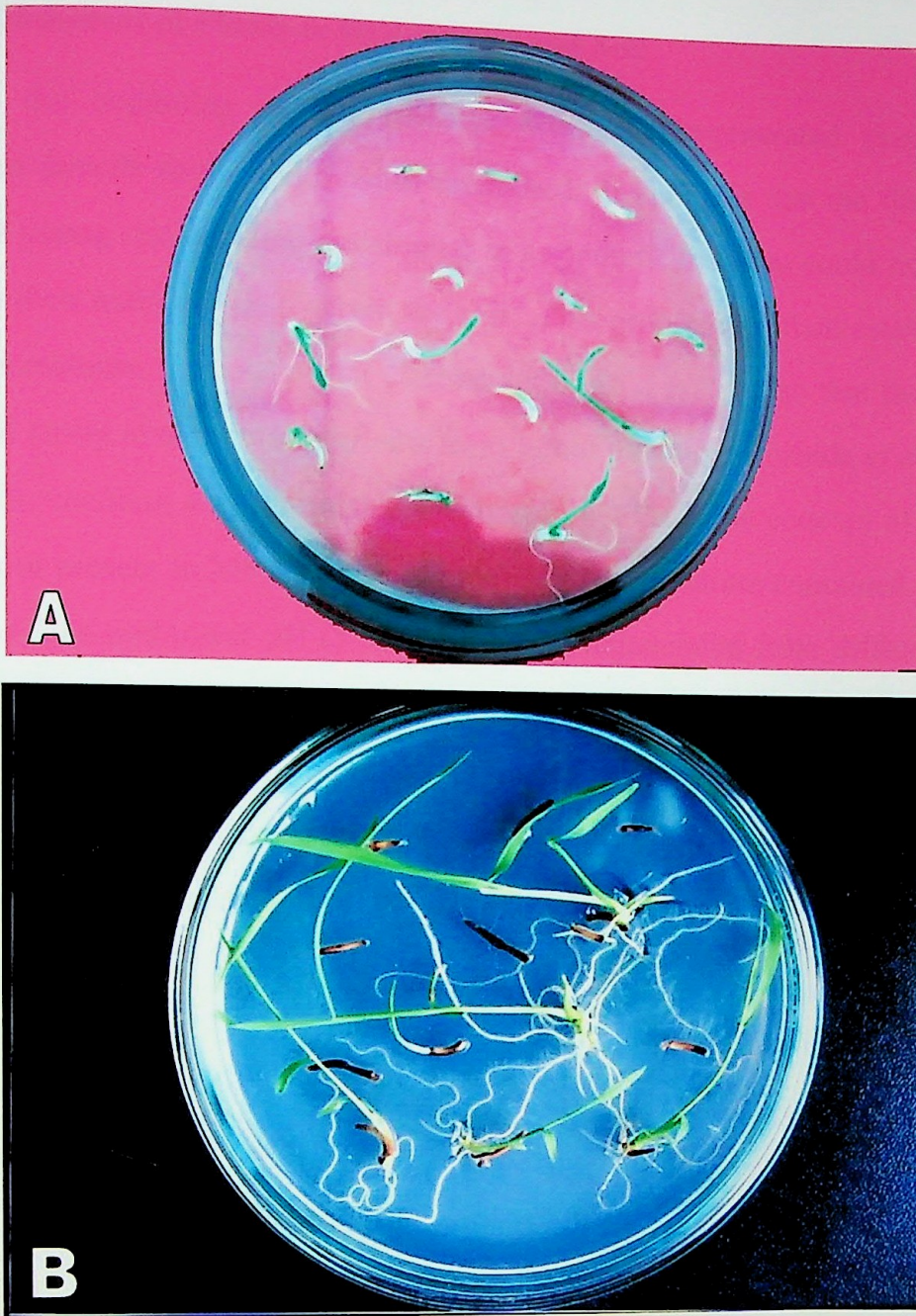


Fig. 3.20: Different stages of meristem regeneration of BRRI dhan-30

- A. Four days old cultured leaf bases on MS medium producing root and shoot.
- B. Seven days meristem regenerated plants.

3.8 Establishment of Plants in Hydroponic and Soil

In the beginning of this research work the regenerated plants when transferred directly to the soil most of them died. It indicates that the fibrous root system of young rice seedling developing in a synthetic medium could not be able to absorb their nutrients easily from the soil. This problem of plant establishment in soil necessitates formulating an intermediary liquid system, so that the delicate root could easily absorb their nutrients from liquid medium (Yoshida *et al.*, 1976). Hydroponic medium was formulated by considering the basic concept of nutrients requirement present in MS medium. The hydroponic medium contained of macro and micro salts and lower pH (5.5). The plants regenerated from different tissue explants were transferred to hydroponics medium for their better establishment. Within two weeks regenerated plants produced elaborated roots in hydroponic medium (Fig. 3.21.). After proper hardening when the plants developed root system they were transferred from hydroponic into soil (Fig. 3.22).



Fig. 3.21: Establishment of BRRI dhan-30 plant in hydroponic medium.

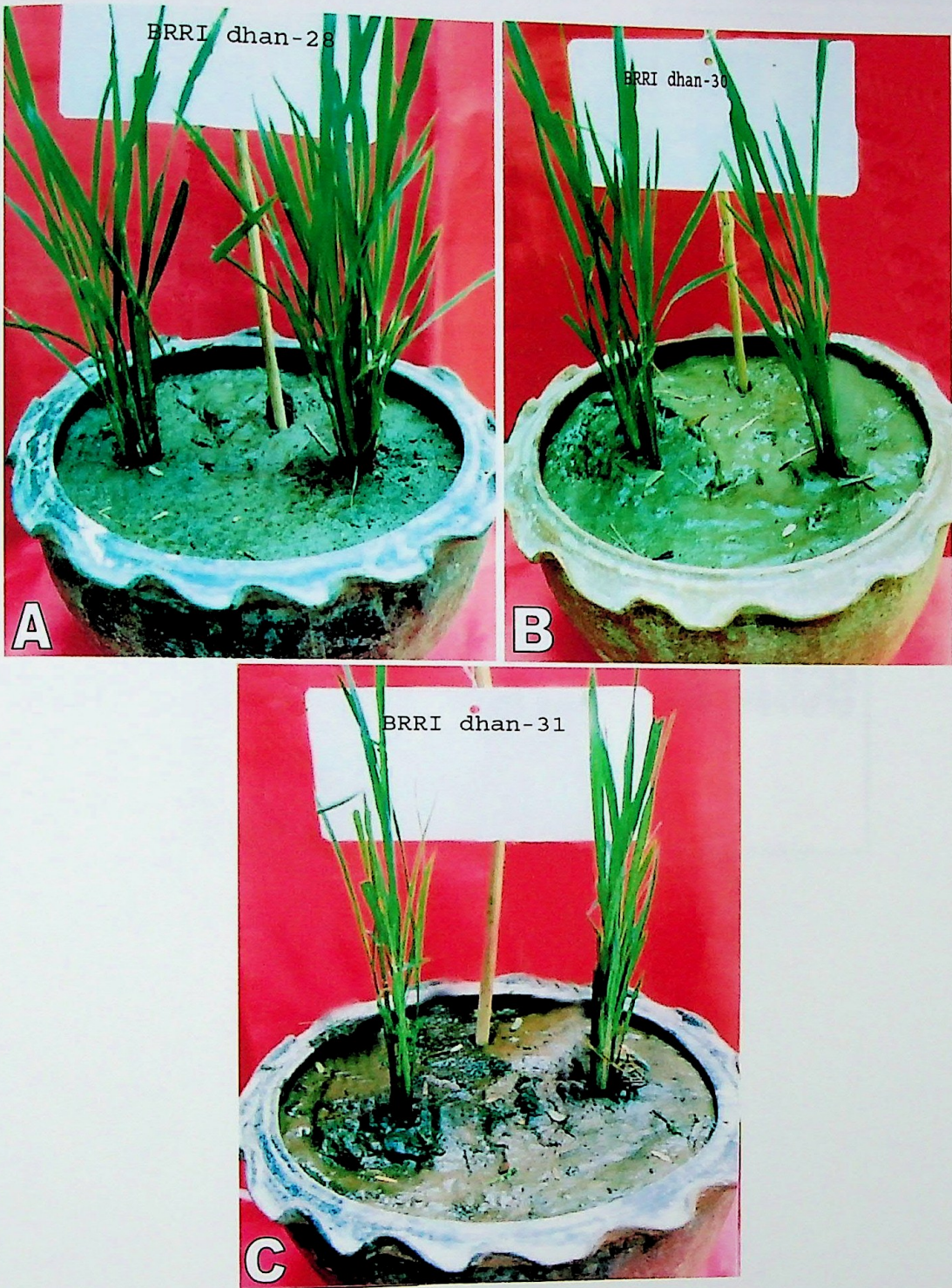
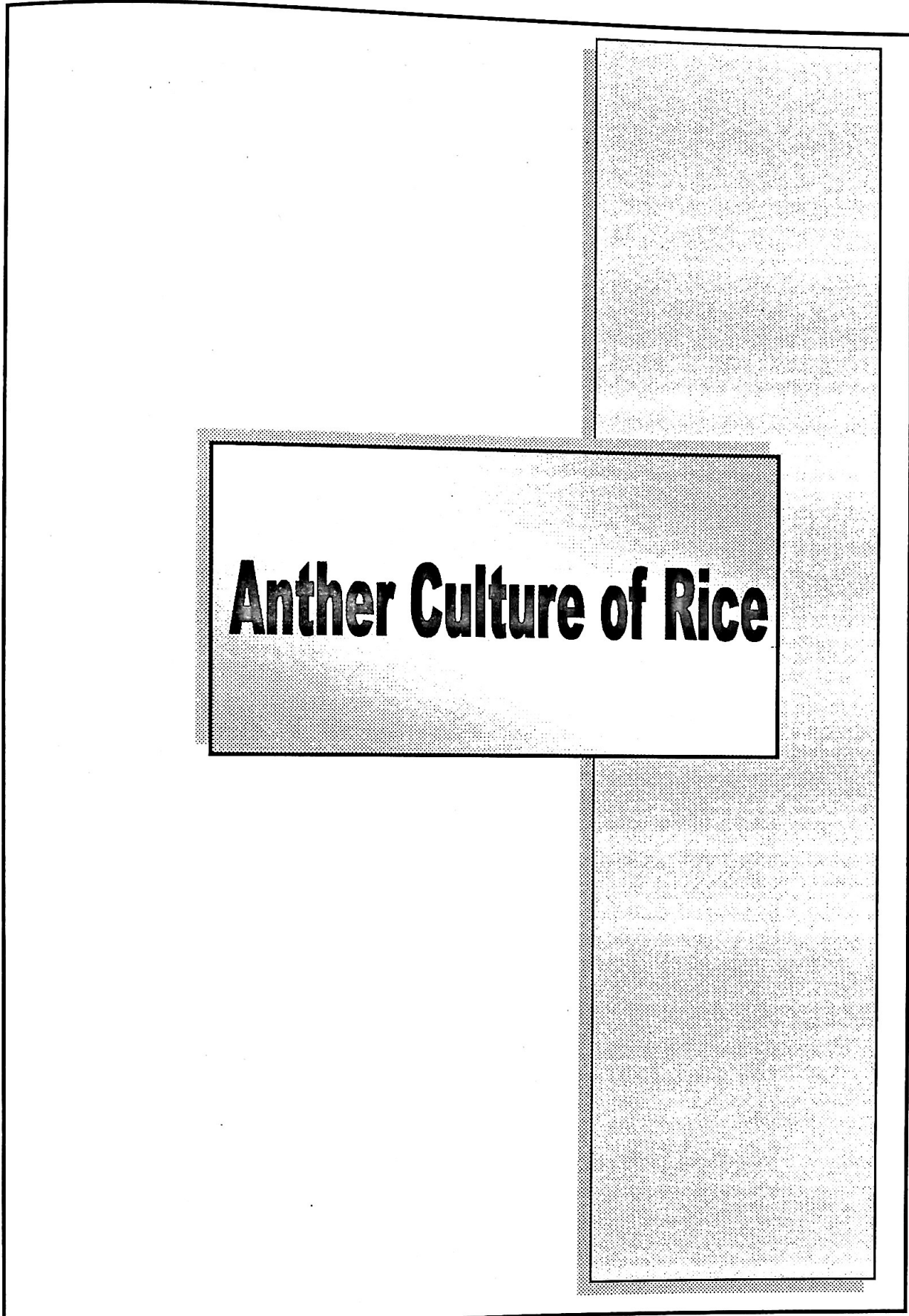


Fig. 3.22: Establishment of regenerated plants in soil.

- A. Regenerated plant of BRRI dhan-28.
- B. Regenerated plant of BRRI dhan-30
- C. Regenerated plant of BRRI dhan-31



Anther Culture of Rice

CHAPTER-IV

4. Anther Culture of Rice

4.1 Introduction

Haploids obtained from anther culture are very potential breeding material in crop improvement (Collings and Genovesi, 1981). The haploid plant formation through anther and microspore culture has been reported in different cereal crops wheat (Picard and De Buyser, 1973; Ouyang, 1986), rice (Wang *et al.*, 1979; Genovesi and Maggill, 1979), maize (Petolino and Jones, 1986), barley (Foroughi-Wher *et al.*, 1982) and rye (Wenzel *et al.*, 1977). After a decade of intensive tissue culture research on rice and other plants, the uses and limits of tissue culture and related research have been clearly defined. Researchers working with rice have successfully produced callus and plants from plated ovaries, anthers and from isolated pollen (Niiuzeki and Oono, 1968; Chu *et al.*, 1975; Oono, 1977; Hu, 1978; Wang *et al.*, 1979; Chaleff, 1980; Chen *et al.*, 1980; Kurcherenko, 1980). Production of haploid plants of rice (*Oryza sativa* L.) through anther culture was first reported by Niiuzeki and Oono in 1968. Since then, extensive research has been carried out in many laboratories around the world. Now the techniques for anther culture of rice have advanced to the level that large numbers of haploids and spontaneously doubled haploids can be obtained from many cultivators and hybrids. The theoretical basis for the utilization of anther derived plants for breeding has also been established (Lee and Chen 1982; Chen *et al.*, 1982; Chen *et al.*, 1983). Thus rice has become one of the few crops in which anther culture can be readily applied to breeding programs.

Certain cold pretreatment has a positive effect on androgenesis. The low temperature pretreatment effect on callus formation and several investigators have studied regeneration in rice anther culture (Genovesi and Maggill, 1979 and Cornejo-Martin and Primo-Millo, 1981). The 4°C or lower temperatures have been considered too severe for obtaining an optimum response in rice (Genovesi and Maggill, 1979).

By using anther and pollen culture techniques it is possible to produce haploid and homozygous plants from single gametes. An important advantage of anther culture is that the phenotype of the haploid plants is the expression of single copy genetic information and there is no masking of trait through gene dominance. Hence these plants are useful in plant breeding programs for the selection of desirable characteristics. In view of the stated above the present research was performed in anther culture of seven rice varieties for knowing their ability for induction of calli, regeneration of pollen and effect of cold treatment as well.

4.2 Materials and Methods

The genotype of donor plant has a great influence on the response of anther culture. Anther donor plants of seven rice varieties commonly cultivated in the country viz. BR-5, BR- 10, BRRI dhan-29, BRRI dhan-31, BRRI dhan-34, BRRI dhan-37 and BRRI dhan-38 were taken from the field of Regional Rice Research Institute, Rajshahi, Bangladesh during the main crop season (June and November, 1999).

4.2.1 Panicle Collection and Sterilization of Boot

Panicles were collected from the donor plants when the microspore condition was at the uninucleate stage. In all cereals investigated the early or mid-uninucleate stage of microspore development was found to give the best results in androgenesis (Bajaj, 1983; Wenzel and Foroughi-Wher, 1982; Dunwell, 1986 and He and Ouyang, 1984). Prior to culture, precise stage of microspore of mid to late-uninucleate stage was determined by squashing anther from a floret close to the middle of the panicles. Microspore development was observed at the different days of culture initiation by acetocarmin staining. Certain pretreatment of the inflorescence can have a positive effect on the development of microspores. For cereals, cold pretreatment was found most effective. Therefore, the selected tillers containing panicle with microspore at mid to late-uninucleate stage were wrapped in foil and kept at 6°C chamber for 3-7 days. The upper and lower anthers of the panicles were discarded and only those at the appropriate in the middle were used in culture. Leaf sheath enclosed the panicles were sterilized by dipping intact in 70% ethylalcohol for one minute just before inoculation of anther. They were then carefully removed, anthers were separated with a forceps and then put in 6 cm petridishes containing callus induction medium. Petridishes were sealed with parafilm and were incubated at 26±1°C in dark for callus induction. Anther derived calli were sub-cultured in regeneration medium and were exposed to 12 hours photoperiod maintained by fluorescent tube light. The pH of the media was adjusted to 5.8 and all the media were solidified with agar. The development of anthers in each experiment was counted and the frequency of callus induction was calculated by the following formula:

$$\% \text{ of callus induction frequency} = \frac{\text{Number of anther produced callus}}{\text{Number of anther cultured}} \times 100$$

4.2.2 Optimize the Time for Cold Pretreatment

Commonly cultivated seven varieties of BR-5, BR-10, BRRI dhan-29, BRRI dhan-31, BRRI dhan-34, BRRI dhan-37 and BRRI dhan-38 rice was taken as donor from the field of Regional Rice Research Institute, Rajshahi, during the main crop season (June and November, 1999). Cold pretreatment was performed according to the modified method of Chen *et al.*, 1982. Briefly, panicles were harvested shortly after the flag leaf just emerged and when the microspores were at the early to mid uninucleate stage as observed by 1:3 aceto-carmin staining. For this experiment six treatment (T) were performed along with control. For the treatments, harvested panicles were stored for 1-day (T1), 2-day (T2), 3- day (T3), 4- day (T4), 5- day (T5) and 6- day (T6) at 6°C in dark chamber. For control, panicles were harvested on the day of culture initiation.

Sterilization of panicles was carried out by dipping intact boots in 70% ethanol for 2 min just before the inoculation of anthers. The outer covering of the panicle was removed with a sharp scalpel and panicles were cut at the base to excise the anthers. On an average 50 anthers were inoculated in each 6 cm petridishes containing 15 ml of semi solid callus induction medium and sealed with parafilm. Three callus induction media of N6 (Chu *et al.*, 1975), Z2 which, modified from H5 (Huang *et al.*, 1978) and R2 (Chaleff, 1980) supplemented with NAA, Kin and 2,4-D. The cultures were incubated at $26 \pm 1^{\circ}\text{C}$ for 4-5 weeks in the dark for callus induction. Anther derived calli subcultured on regeneration semi solid medium and subsequently transferred to the culture room at 16h/8h, light/dark regimes for good shoot and root formation. Data of anther response and green plantlet regeneration were recorded in each experiment by counting the number of calli/anther, number of green plants/callus and albino plants respectively.

4.2.3 Preparation and Sterilization of Media for Rice Anther Culture

Culture media for the growth of different explants consist of various inorganic salts. Inorganic nutrients consisted of number of major elements such as N, P, K, Ca, Mg, S and minor elements such as Fe, Mn, Zn, B and I etc. These inorganic nutrients were supplied as water soluble compounds and organic nutrients included as carbon sources, which were usually supplied as sucrose and organic growth substances mainly vitamins of B group.

The basic media used in tissue culture studies were prepared as described in the **Table 4.1**. The basic medium supplies nutrients and provides suitable pH and osmotic pressure for the growth of culture. In order to regulate the growth and differentiation of the cells always necessary to supplement the basic medium with various physiologically active substances, phytohormones such as auxins and cytokinins. The Z2, N6 (Chu *et al.*, 1975) and R2 (Chaleff, 1980) and regeneration medium RM derived from MS media were prepared and the pH of all these media was adjusted to 5.8 with 1N NaOH prior to sterilization. Then it was solidified with agar and sterilized at 121°C at 15 lbs for 20 minutes.

Table 4.1. Ingredients of different improved callus induction media used in the study.

Constituents	Concentration in medium (mg/L)		
	Z2	R2	N6
Macronutrients			
KNO ₃	3181.5	2530	2830
NH ₄ NO ₃	-	400	-
MgSO ₄ .7H ₂ O	185	370	185
KH ₂ PO ₄	540	170	400
CaCl ₂ .2H ₂ O	166.0	440	166
(NH ₄) ₂ SO ₄	231.0	-	463
KCl	-	-	-
NaH ₂ PO ₄ .H ₂ O	-	-	-
Micronutrients			
H ₃ BO ₃	6.0	6.2	1.6
MnSO ₄ 4H ₂ O	22.33	16.8	4.4
ZnSO ₄ .7H ₂ O	10.6	6.62	1.5
NaMoO ₄ .2H ₂ O	0.25	0.25	-
CuSO ₄ .5H ₂ O	0.025	0.25	-
CoCl ₂ .6H ₂ O	0.025	0.025	-
KI	0.80	0.83	0.8
FeSO ₄ .7H ₂ O	55.7	27.8	27.8
Na ₂ EDTA	74.5	37.2	37.3
Vitamins and Sugars			
Thiamine HCl	2.5	1.0	0.5
Pyridoxine HCl	2.5	0.5	0.5
Nicotinic acid	2.5	0.5	1.0
Myo-inositol	100	100	100
Cascien hydrolysate	500	-	-
Sucrose	30000	30000	30000
Amino acids			
Glycin	2.0	-	2.0
L-glutamine	-	-	-
L-aspartic acid	-	-	-
L-arginine	-	-	-
Hormones			
2-4, D	0.5	-	2.0
Kinetin	0.5	2.0	-
GA3	-	-	-
NAA	2.5	0.3	-
Zeatin	-	-	-
Agar	10000	10000	10000
pH	5.8	5.7	5.8

4.2.4 Callus Induction in Different Media

The culture medium is one of the most important factors for the induction of androgenesis and the subsequent development of new structure and plants. In the present experiment three types of media viz. Z2, N6 and R2 were used for induction of rice anther culture. For induction of callus from anthers and its subsequent regeneration different concentrations of auxins and cytokinin were used. These hormonal concentrations were tested in three media viz. Z2 from H5 (Huang *et al*, 1978), N6 (Chu *et al.*, 1975) and R2 (Chaleffs, 1980). These three media was supplemented with 2.5 mg/l NAA, 0.5 mg/l kinetin and 0.5 mg/l 2,4-D. Again the media were solidified with agar and pH was adjusted to 5.8 prior to add the agar. All the experiments for induction of callus from anthers of rice cultures were incubated in dark condition. After 3-4 weeks of incubation, pollens of the responsive varieties started to produce callus. Data of both the anther inoculation and callus formation were recorded.

4.3 Regeneration of Rice

For regeneration, anther derived calli were transferred to two regeneration media. RM1 and RM2 and these regeneration media are the simply modified MS medium having difference in hormone concentrations. RM1and RM2 media supplemented with MS+ kinetin 0.5 mg/l+ BAP 2.0 mg/l+ NAA 1.0 mg/l and MS+kinetin 1.0 mg/l +1.0mg/l NAA and was exposed to 12 hours photoperiod maintained by fluorescent light. The percentage of regeneration of plantlets was calculated and recorded.

4.4 Results and Discussion

4.4.1 Callus Induction

Seven varieties of BR-5, BR-10, BRRI dhan-29, BRRI dhan-31, BRRI dhan-34, BRRI dhan-37 and BRRI dhan-38 rice were tested for callus induction in three basal media as shown in **Table 4.2** and **Fig. 4.1**. Callus induction was recorded in five varieties among seven in Z2 medium; however, the induction frequency was varied. BR-5 and BRRI dhan-38 were induced in N6 medium while in R2 medium induced only BRRI dhan-38. On the other hand BRRI dhan-38 produced lower percentage of callus in all of three media. The highest percentage of callus (53.4%) and highest embryogenic structures (93.2%) was observed in BRRI dhan-38 in Z2 medium. Out of the three media tested Z2 was found to be most effective than N6 and R2 and it showed that callus induction efficiency in Z2 was higher than that of either H5 or Z2 respectively. This result supported by Shahjahan *et al.*, 1985 and they suggested that Z2 media appeared better for efficient callus induction of indica varieties of rice. Z2 medium prepared by changing the amounts of pyridoxine HCl, nicotinic acid, yeast extract and sucrose in the original H5 medium was found more efficient than H5 in callus induction of cultivars belonging to the indica genotype. This study proved Z2 to be more effective than either N6 or R2 for the indica type cultivars. It was also observed that only BRRI dhan-38 induced calli in all three basal media used for this experiment. The result indicated that different varieties hold different degrees of potentiality of anther induction in different media. The genotype of the pollen plant has the greatest influence on the frequency of pollen callus formation (Chu, 1982).

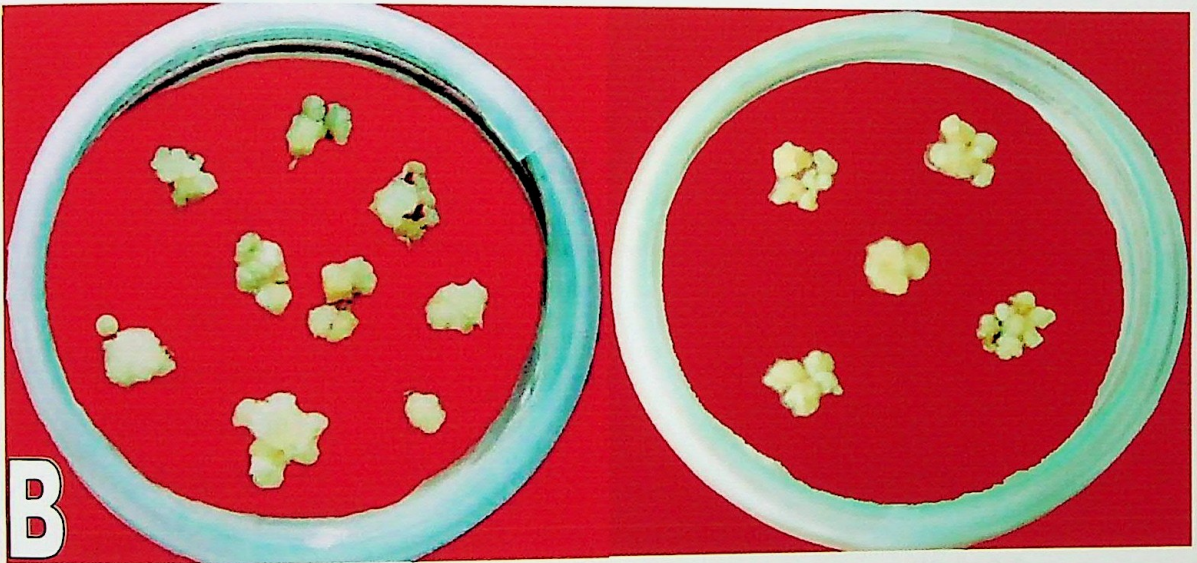
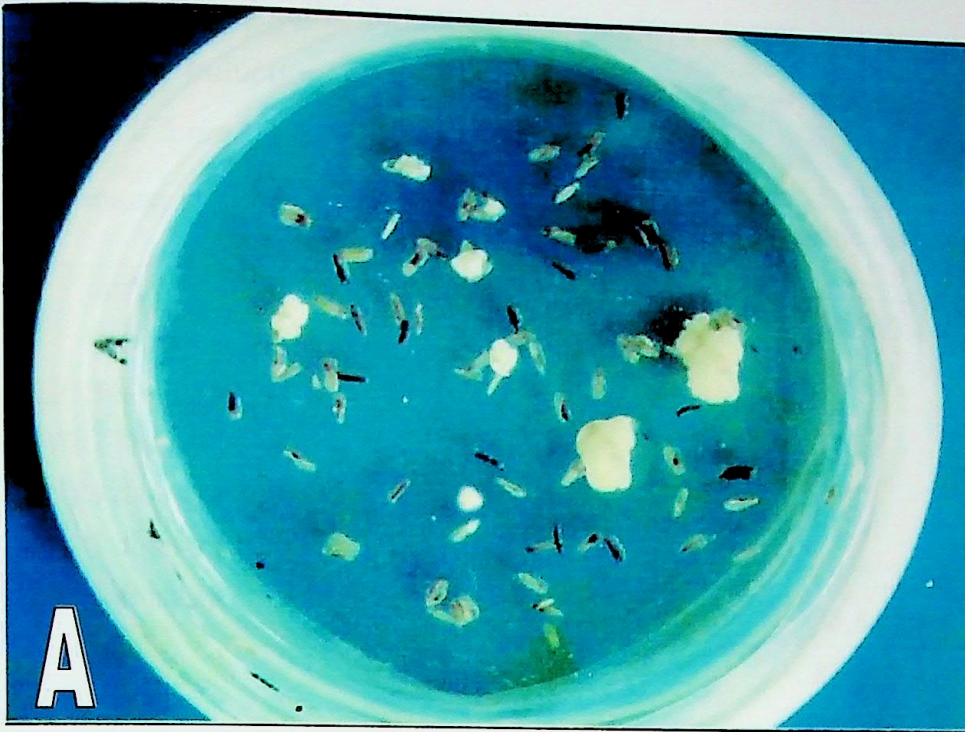


Fig. 4.1: Different types of anther derived calli.

A. Initiation of anther derived calli.

B. Anther derived calli.

Table 4.2. Frequency of callus induction from anthers of different rice varieties in three induction media supplemented by 2.5 mg/l NAA+0.5 mg/l Kin + 0.5 mg/l 2,4-D.

Media	Genotype	Callus formation (% $\pm$ S.E.)	Embryogenic response (% $\pm$ S.E.)
N6	BR-5	15.5 $\pm$ 1.2	28.3 $\pm$ 3.1
	BR-10	—	—
	BRRI dhan-29	—	—
	BRRI dhan-31	—	—
	BRRI dhan-34	—	—
	BRRI dhan-37	—	—
	BRRI dhan-38	22.1 $\pm$ 2.3	35.8 $\pm$ 2.7
Z2	BR-5	35.6 $\pm$ 3.4	83.6 $\pm$ 2.9
	BR-10	—	—
	BRRI dhan-29	—	—
	BRRI dhan-31	27.7 $\pm$ 2.7	77.5 $\pm$ 2.7
	BRRI dhan-34	36.2 $\pm$ 2.8	79.8 $\pm$ 1.8
	BRRI dhan-37	50.3 $\pm$ 1.8	91.1 $\pm$ 1.8
	BRRI dhan-38	53.4 $\pm$ 1.6	93.2 $\pm$ 1.0
R2	BR-5	—	—
	BR-10	—	—
	BRRI dhan-29	—	—
	BRRI dhan-31	—	—
	BRRI dhan-34	—	—
	BRRI dhan-37	—	—
	BRRI dhan-38	16.4 $\pm$ 1.8	28.8 $\pm$ 2.2

*Calculated on the basis of number of anthers (minimum 1000) inoculated.

The production of haploid plants from anthers offers plenty of scope for rapid development of homozygous lines for early release of new crop varieties. Besides, it allows the use of haploid cells or protoplasts for induction and selection of recessive mutations. Scientists working with rice have successfully produced

callus and plants from planted ovaries, anthers and from isolated pollen (Niiuzeki and Oono, 1968; Chu, 1975; Oono, 1977; Hu, 1978; Chen *et al.*, 1980 and Kurchenko, 1980). By application of anther and pollen culture techniques it is possible to improve our rice varieties through production of double haploid plants. Isolated anther of *Datura innoxia* was able to form haploid embryos *in vitro* was reported by Guha and Maheshwari (1964, 1966). Subsequently, Bourgin and Nitsch (1967) obtained the first haploid plants from isolated anther of *Nicotiana*.

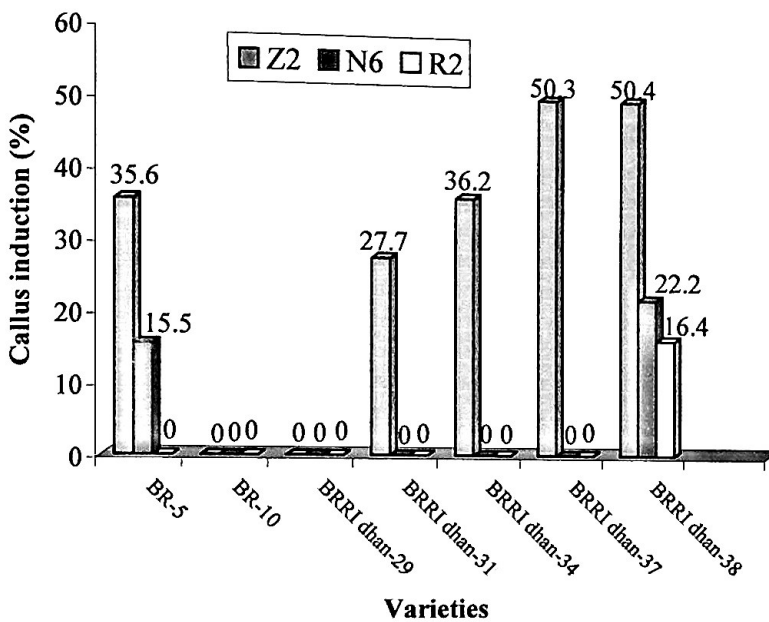


Fig. 4.2: Callus induction efficiency of Z2, N6, and R2 media in seven rice varieties.

During the last decade much progress have been made in anther culture of rice, wheat, maize, *brassica* and other crop species. Androgenesis of anthers and microspore culture is an important tool for the determination of a shot term breeding values of a species. But the success of anther culture mostly depends on the genotype of the anther donor material and other factors.

As shown in Fig. 4.2, the induction medium Z2 supplemented with 2,4-D 0.5 mg/l, NAA 2.5 mg/l and kinetin 0.5 mg/l was found suitable for inducing callus compared to other hormonal concentrations of Z2 and N6 and R2. Moreover, it was observed that BRRI dhan-38 responded the highest mean callus induction frequency (53.4%). This result well agrees with the result of Hakim (1991) and Sandhu *et al.*, (1993).

There was a great variation in morphological nature of callus and percentage of callus formation. Rapid callus initiation was observed when the culture was supplemented with 2,4-D in combination with NAA or kinetin than 2,4-D alone. Cold treatment of panicles at 6°C for 1-6 days enhanced the culture response supported by Lee *et al.* (1989). They were also reported the beneficial effect of cold pretreatment in enhancing cultural response. Initiation of callus occurred within 25-28 days and the frequency of callus induction ranged from 27.7% to 53.4% depending on the genotype. Taking this view in consideration, a lower concentration of sucrose (3% w/w) was used for callus induction and comparatively better result was found. Because, sucrose has been used as the major carbohydrate source in the induction media in cereals androgenics. Bajaj (1983) also reported that pretreatment of cold appears to inhibit spindle formation and increase the frequency of androgenics.

4.4.2 Plantlet Regeneration

For plantlet regeneration calli were transferred to two regeneration media (RM) of RM1 and RM2 and the results are summarized in **Table 4.3** and **Fig. 4.4**. In both media the percentage of regeneration of calli varied from 7 to 34 % and the highest regeneration was found in BRRI dhan-37 with the addition of Kinetin (0.5 mg/l), BAP (2.0 mg/l) and NAA (1.0 mg/l). When compared between two, medium with the addition of the former supplement stated above (RM1) gave excellent regeneration of plantlet in anther calli. Upon transfer to RM1 medium among five genotypes the highest percentage of green plants (33.2%) and albino plants (11.1%) were produced in BRRI dhan-37 followed by 28.1% green and 10.5% albino plants in BRRI dhan-38 (**Fig. 4.3**). While in RM2 medium, the highest percentage of green (30.5%) and albino plants (10.1%) was recorded in BRRI dhan-38 followed by 25.7% green and 11.5 % albino plants in BRRI dhan-34. Oono (1975) reported the differentiation of primarily green plants from microspore derived callus of the japonica variety Minehikari.

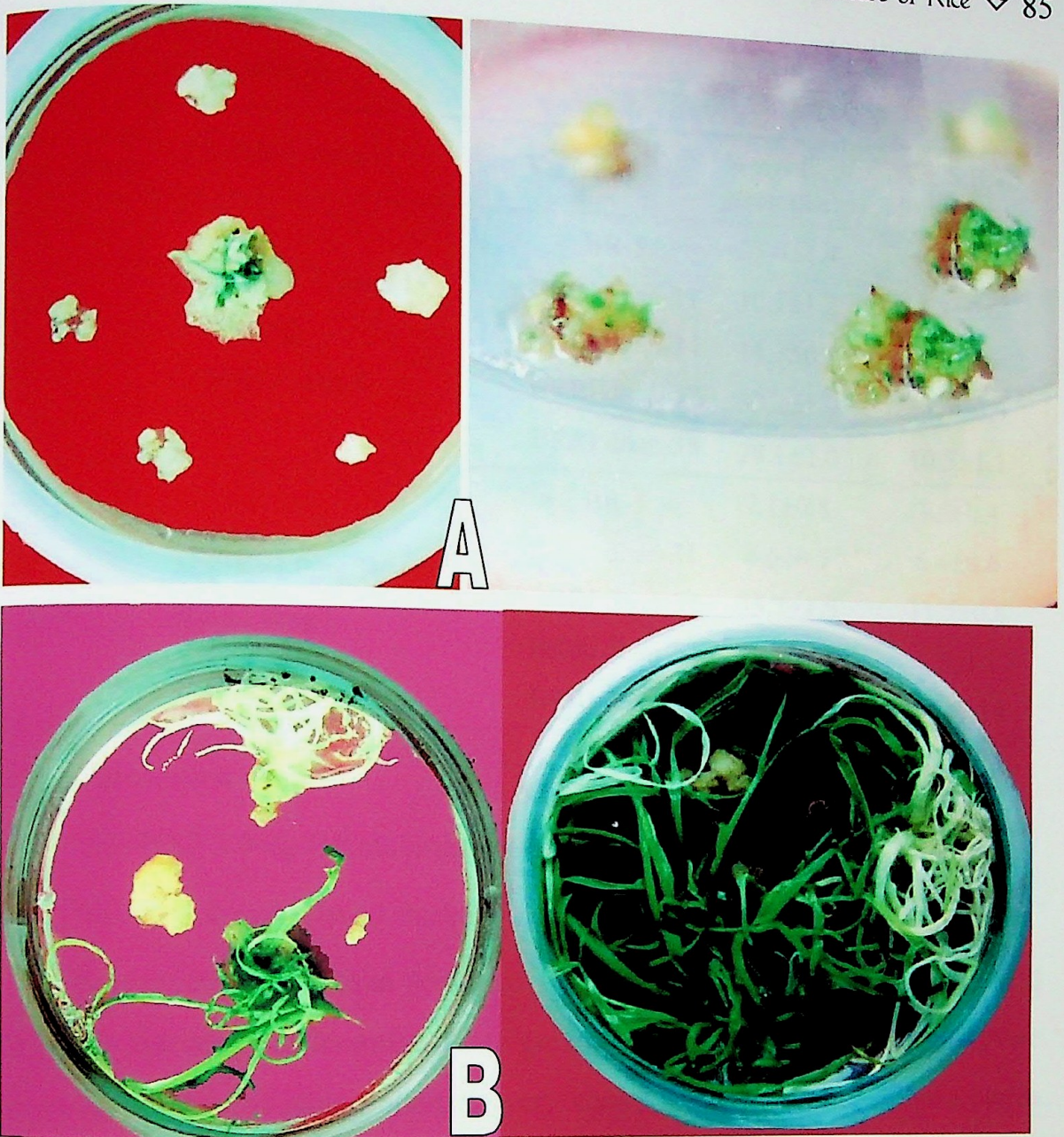


Fig. 4.3: Production of plants via regenerable callus.

A. Plantlets developed from the embryoids.

B. Anther derived plants.

Table 4.3. Frequency of plantlet regeneration from anther derived calli of different rice varieties.

Regeneration media (RM)	Treatment (mg/l)	Varieties	% $\pm$ S.E. of green plants	% $\pm$ S.E. of albino plants
RM1	MS+BAP 2.0mg/l+ Kn0.5mg/ l+NAA1.0mg/l	BR-5	25.6 $\pm$ 1.4	8.5 $\pm$ 0.7
		BRRI dhan-31	10.1 $\pm$ 1.2	4.5 $\pm$ 0.6
		BRRI dhan-34	11.3 $\pm$ 0.5	5.2 $\pm$ 0.6
		BRRI dhan-37	33.2 $\pm$ 1.9	11.1 $\pm$ 0.9
		BRRI dhan-38	28.1 $\pm$ 2.0	10.5 $\pm$ 1.1
RM2	MS+Kn1.0mg/l+ NAA1.0mg/l	BR-5	7.1 $\pm$ 0.8	3.5 $\pm$ 0.4
		BRRI dhan-31	8.6 $\pm$ 0.7	6.4 $\pm$ 0.6
		BRRI dhan-34	25.7 $\pm$ 1.5	11.5 $\pm$ 0.8
		BRRI dhan-37	23.3 $\pm$ 1.4	9.2 $\pm$ 1.1
		BRRI dhan-38	30.5 $\pm$ 2.1	10.1 $\pm$ 0.6

RM= Regeneration media

RM1= MS+BAP 2.0mg/l+Kn0.5mg/l+NAA1.0mg/l

RM2= MS+Kn1.0mg/l+NAA1.0mg/l

In regeneration medium some calli lost their ability to produce plants and died while the others differentiated into green and albino plants. In anther culture of rice appearance of albino plants is very common phenomenon. Albino plants are morphological similar to the green plant except for chlorophyll deficiency. The frequency of albinos is variable, ranging from 5 to 80% (Chen *et al.*, 1980). Several workers have suggested the possible causes of albino plant formation in callus culture. Chen (1986) reported that the production of albino plants was influenced by many factors, e.g. genotype, medium composition, incubating conditions and physiological status of donor plants. Wang *et al.* (1977) stated that different genotypes showed variation induction of albino plant ranging from 10%-

70%. High sucrose concentration (above 6%) in the induction medium increased the proportion of albino plants (Wang *et al.*, 1987). Calli derived from barley and wheat pollen cultured in a medium containing 12% sucrose produced more albinos. Among physical factors, high temperature at the initial stage of incubation also resulted in increased albino production (Wang *et al.*, 1987). The low content of chlorophylls may be one causes of albino formation. The production of a large number of albinos appears to be a serious problem in anther culture in rice.

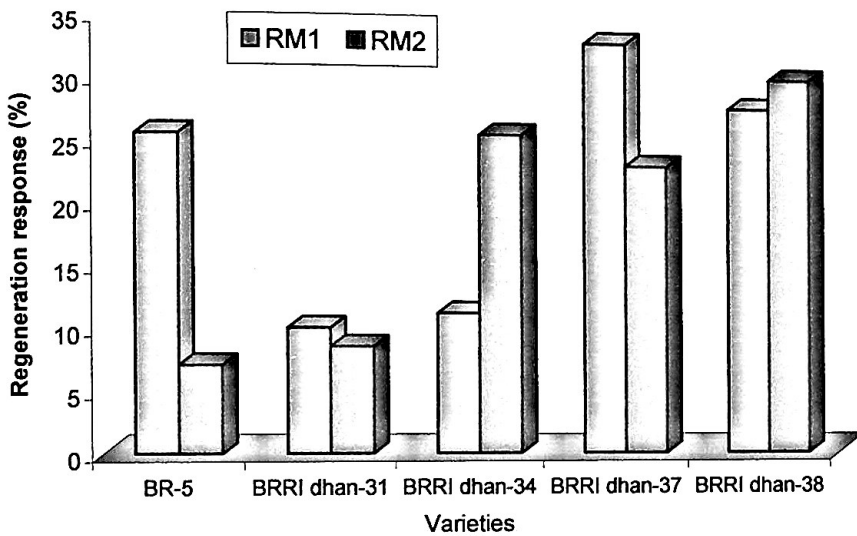


Fig. 4.4: Comparative regeneration response of different varieties on to different media (RM)

Results from several laboratories have indicated that the proportion of regenerated albino plants was genetically controlled (Oono, 1975 and Chen and Lin, 1976). Present study indicates that the ability of callus formation from anther of varieties are largely dependent on culture medium in addition choice of variety would be the critical factor to ensure the recovery of a high frequency of green plants. Factors that influence the differentiation of anther callus are i) age of the anther callus, ii) sucrose concentration and iii) ratio of auxin to cytokinin in the regeneration medium (Chu, 1982). Stimulation of shoot bud formation and plant regeneration by two step culture method and plant regeneration as described by Inone and Macda, 1981. So it is possible to suggest that to obtain the homozygous diploid plants need to culture the pollen, besides genotypes must seek ways to improve the cultural conditions and the media to resolve the albino problem.

4.4.3 Optimization of Cold Pretreatment Duration

Anthers of cold pretreated panicles were cultured in Z2 and regeneration medium (RM) and mean values of callus induction and their regeneration were recorded and presented in **Table 4.4**. The highest embryo production were recorded in treatment-3 (T_3) and they were 83.6%, 77.5%, 79.8%, 91.1% and 93.2% in the genotypes of BR-5, BRRI dhan-31, BRRI dhan-34, BRRI dhan-37 and BRRI dhan-38, respectively while the efficiencies of green plant regeneration were 27.3%, 15.6%, 17.7%, 30.61% and 33.0% respectively. In all treatments comparatively higher values both of embryo production and green plant regeneration were found than that of control (**Table 4.4**). However, T_3 provided the most effective condition for embryo induction and regeneration and its performances were remarkably differed from other treatments and control.

In regard to explant, anther culture is more delicate, sensitive and simple and variation in physical condition also offers positive causes to improve induction and regeneration efficiency. In the present investigation, cold treatment was applied for improving quality of anther culture of rice. For cold treatment, duration is another aspect of variation in culture response. Bajaj (1983) reported that cold treatment increase the frequency of androgenesis and also mentioned that it appears to inhibit spindle formation and some cases may cause an abnormal first mitosis. The present experiment was conducted to sort out the optimum duration of cold treatment subjected to the panicles before culture. Under verified optimum conditions in T₃ and T₄ the embryo 7-10 fold and 4-10 fold increased formation and regeneration of green plants over the values of control culture.

Table 4.4. Varietal performance in anther induction and regeneration under different durations of cold treatment of panicle 6⁰ C.

Cold pretreatment(%)	Genotype	Anther response (%)	Frequency of embryo induction (%)	Frequency of embryo regeneration (%)	Frequency of green plants (%)	Frequency of albino(%)
Control	BR-5	2.03	7.10	3.10	2.50	1.25
	BRR1 dhan-31	1.89	5.90	2.30	1.20	1.15
	BRR1 dhan-34	2.52	5.40	1.50	1.00	1.00
	BRR1 dhan-37	3.9+0	7.58	3.62	2.65	1.48
	BRR1 dhan-38	4.01	8.90	4.31	2.20	2.50
T ₁	BR-5	10.05	30.20	13.20	10.20	6.40
	BRR1 dhan-31	9.35	27.36	15.80	7.00	8.12
	BRR1 dhan-34	11.20	32.00	17.01	10.10	10.00
	BRR1 dhan-37	14.26	33.92	19.40	15.70	11.20
	BRR1 dhan-38	15.12	35.25	20.20	17.01	12.50
T ₂	BR-5	11.04	31.24	14.00	10.50	7.04
	BRR1 dhan-31	9.78	28.01	11.75	8.10	6.01
	BRR1 dhan-34	14.21	35.00	21.02	12.20	12.20
	BRR1 dhan-37	16.53	39.50	25.60	17.82	11.50
	BRR1 dhan-38	17.69	41.20	29.10	18.01	13.60
T ₃	BR-5	35.45	83.6	40.02	27.30	19.50
	BRR1 dhan-31	27.79	77.5	37.23	15.60	17.90
	BRR1 dhan-34	36.35	79.8	37.50	17.65	18.00
	BRR1 dhan-37	50.20	91.1	41.20	30.61	22.30
	BRR1 dhan-38	53.23	93.2	43.50	33.00	24.60
T ₄	BR-5	30.10	74.54	35.60	23.01	16.89
	BRR1 dhan-31	24.36	65.90	30.40	13.90	15.00
	BRR1 dhan-34	32.23	76.98	36.80	15.00	18.75
	BRR1 dhan-37	45.62	82.65	38.01	26.35	19.02
	BRR1 dhan-38	48.45	86.50	39.05	29.03	21.05
T ₅	BR-5	13.30	32.02	13.05	9.97	6.38
	BRR1 dhan-31	12.60	29.09	11.50	7.56	4.52
	BRR1 dhan-34	18.25	42.50	15.30	11.06	10.21
	BRR1 dhan-37	20.64	47.95	16.32	12.75	11.45
	BRR1 dhan-38	21.13	51.03	18.06	14.78	12.30
T ₆	BR-5	6.10	11.00	4.00	3.04	3.75
	BRR1 dhan-31	5.25	9.50	3.02	2.25	1.75
	BRR1 dhan-34	8.90	18.20	7.10	3.45	4.75
	BRR1 dhan-37	10.39	23.01	9.50	5.00	6.05
	BRR1 dhan-38	11.00	25.30	10.90	8.10	7.10

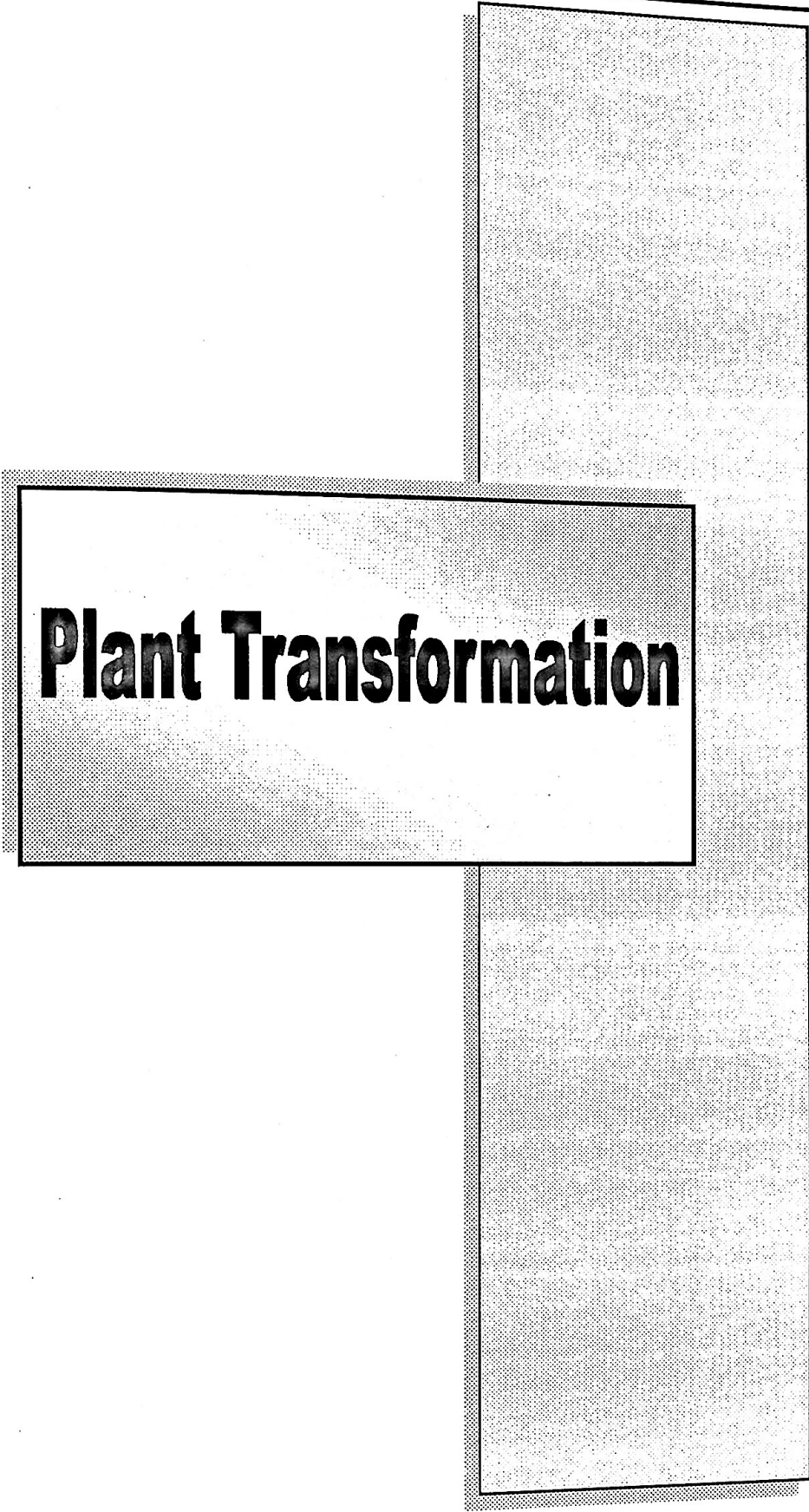
Control: Anthers are directly cultured without any cold pretreatment of panicle.

For the treatment T₁, T₂, T₃, T₄, T₅ and T₆ panicles were pretreated by cold at 6⁰C chambers for 1,2,3,4,5 and 6 days, respectively. For all treatments cultures were transfer to 28⁰C chamber in dark for embryo formation.

In the present experiment the duration varied from 1 to 6 under six treatments days and the duration range 3-4 days was proved to be most suitable for

induction and regeneration in rice anther culture in both T₃ and T₄. The results presented in the **Table 4.4** also indicated that cold treatment before culture is a necessary step for inducing androgenesis. On the other hand, the efficiency of induction and regeneration of anthers gradually decreased while the duration extending from 5 to 6 days. If the duration further increased the viability of the anther has been totally lost and the similar result has been reported in wheat where low temperature for a long time decreased the callus induction.

Cold pretreatment is very much effective in enchanting the callus formation and regeneration in rice (Croughan and Chu, 1991, Genovesi and Maggill, 1979 and Wang *et al.*, 1974), wheat (Amssa *et al.*, 1980), maize Genovesi, 1990 and *Datura* (Nitsch and Norreel, 1973). Lazar *et al* (1985) reported that variation of intensity and duration of cold pretreatment increased the embryo yield. In the present findings, all varieties showed lower percentages of embryos and regeneration when spikes were pretreated for a long time (5-10 days) at 6°C. This observation further strengthened the statement that the increased duration drastically affects the callus induction.



Plant Transformation

CHAPTER-V

5. Plant Transformation

5.1. Introduction

For millennia, cultivators have selectively bred plants in an attempt to induce or enhance a desired trait. Early farmers who settled the banks of the Euphrates learned to better their crops by selecting specific seeds from their harvest for use in the next seasons planting. Although this was an effective practice, the process usually took hundreds of years and was strictly limited to the alleles present in the same species, or closely related species populations. This need to control and predict the amount of plant food has been a fundamental motivator in developing new plant technologies. Since the advent of recombinant DNA technology in the 1970's, the recent years have shown remarkable advances in plant genetics and the rise of plant transformation. These new advances, both biological and mechanical have given researchers the ability to express foreign genes, in desired plant hosts, essentially breaking the plant species barrier. This powerful technology is of great importance to many aspects of human society. It has opened the door to molecular farming, a novel system for genetic research and new crops adapted to our civilization. The ability to feed our vastly increasing population may very well rest in the hands of plant geneticists. Currently, the practical success of plant genetic manipulation can be seen in agricultural products such as Bt cotton, resistant to insect, the Newleaf™ potato, which expresses a natural insecticide and the FlavrSavr™ tomato, who's ripening is inhibited until exposure to ethylene. With many more products on the way, it is clear that plant transformation will continue to have a profound impact on plant genetics and global agriculture.

Plant transformation has been defined as the stable incorporation and expression of foreign genes into plants. In 1983, Robert Fraley and colleagues reported the successful incorporation of a kanamycin resistance gene into the tobacco genome. Within a year other researchers reported similar accomplishments (De Block *et al.*, 1984; Horsch *et al.*, 1984; Paszkowski *et al.*, 1984), marking the beginning of plant transformation. Since then, successful transformations have been described for over 120 species in 35 different families (Birch, 1997) and the number is increasing every year. The process of plant transformation is currently a long complex multi stage procedure involving three general phases. Phase 1 involves the selection and application of a delivery system, which incorporates the target DNA of interest into a viable host cells. Phase 2 involves integration, expression and selection of inserted DNA indicating proper integration and expression of gene product. Finally, phase three involves the recovery of viable transgenic plant.

In true sense, plant transformation celebrates the culmination of several plant biochemical, physiological and genetic technologies. Currently, plant transformation technologies come in many different forms and are a long and expensive process. However, continued research is devoted to improving these techniques and in time will become more available and affordable. These technologies are now sufficiently developed, allowing the commencement of novel genetic research and the development of novel transgenic plants expressing a variety of phenotypes. Plant transformation in the tradition of science, is changing the face of plant genetics by challenging and improving upon previous work. It is clear that the implications of these advancements are and will become a direct and indirect integral part of our society. With this new technology comes a

new responsibility to produce and release transgenic plants in safe and responsible manner. Finally, as the production of transgenic plants is now a reality, scientists must extend their knowledge beyond the laboratory and learn to deal with the economic, social and legal implications of plant transformation.

5.1.1. Genetic Transformation

The ability to move DNA into an organism and thereby alter its genotype or genetic makeup is central to both basic and applied molecular biology. Genes derived from unrelated species and even other kingdoms, such as bacteria, fungi, plants, animals, that would otherwise be inaccessible to an organism, can be combined in the lab using genetic transformation techniques. Common plant transformation methods are: PEG-mediated gene transfer, Electroporation-mediated gene transfer, Microprojectile bombardment-mediated gene transfer, *Agrobacterium*-mediated gene transfer etc.

PEG-mediated gene transfer

In 1986, Uchimiya *et al.* first obtained transgenic calli after polyethylene glycol (PEG)-induced DNA uptake of the *nptII* gene into root-derived protoplasts, followed by selection on kanamycin. The first transgenic rice plants were recovered by Zhang *et al.* (1988). Datta *et al.* (1990) published the first report of the recovery of transgenic indica rice plants from cultivar Chinsurah Boro II by PEG method.

Electroporation-mediated gene transfer

Electroporation has also been widely used to introduce naked DNA into protoplasts. Toriyama *et al.* (1988) first used this method for the production of Yamahoushi cultivar transgenic rice via anther culture-derived protoplasts with the

aminoglycoside phosphotransferase II (*aph(3')II*) gene, conferring resistance to the antibiotics kanamycin and G418.

Microprojectile bombardment-mediated gene transfer

Microprojectile bombardment also called the biolistic method or the particle gun method has been used in many laboratories. The concept has been described in detail by Sanford (1988). The ability to deliver foreign DNA into regenerable cells, tissues or organs appears to provide the best method for achieving genotype-independent transformation bypassing *Agrobacterium* host specificity. In 1991, Christou *et al.* bombarded the scutellar region of rice embryo and bombarded tissues were plated on regenerated media supplemented with appropriate selective agents.

***Agrobacterium*-mediated gene transfer**

The transformation of dicotyledons by *Agrobacterium* is well established. But in the case of monocots it is not the general process. In the past monocots, particularly graminaceous crop plants including important cereals like rice and wheat were considered to be recalcitrant to this technology and they were outside the *Agrobacterium* host range. However, transformation methods based on the use of *Agrobacterium* are still preferred in many instances because of the following properties: (i) easy to handle, (ii) higher efficiency, (iii) more predictable pattern of foreign DNA integration, and (iv) low copy number of integration. Remembering the advantages of *Agrobacterium*-mediated transformation many researchers continued their efforts to develop its suitable protocol in rice transformation. Raineri *et al.* (1990) first described the production of transformed japonica cultivar by co-cultivation of mature embryos with *Agrobacterium*.

5.1.2 *Agrobacterium* –Mediated Transformation

The search for microorganisms that could be used as vehicles for DNA, lead researchers to the genus of *Agrobacterium*. Two species of *Agrobacterium* that could be used as a transformation delivery system, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* both had the unique ability to transfer portions of their DNA into the genomes of plants (Zambryski *et al.*, 1989). The mechanism by which they transfer DNA is the same, however the differences in the resulting phenotypes are attributed to the presence of a Ti plasmid in *Agrobacterium tumefaciens* and the Ri plasmid in *Agrobacterium rhizogenes*. The Ti plasmid DNA induces host plants to grow tumourous masses whereas the Ri plasmid DNA leads to the abundant proliferation of roots. *Agrobacterium tumefaciens* is capable of infecting almost any plant tissue whereas *Agrobacterium rhizogenes* can only infect roots. In its uniqueness, *Agrobacterium tumefaciens* has become the primary choice for many plant transformation experiments.

The transfer of foreign DNA to plants was first reported in 1983 in tobacco and petunia calli mediated by *Agrobacterium*. Transgenic rice plants were successfully produced in 1988 by PEG and electroporation mediated DNA delivery (Toriyama *et al.*, 1988; Zhang *et al.*, 1988) into protoplasts. *Agrobacterium* mediated transformation is a favorable tool in dicot transformation while considered difficult in monocot especially in cereals (Christou and Timmins., 1992). Some groups have reported *Agrobacterium* mediated rice transformation (Raineri *et al.*, 1990 and Li *et al.*, 1992) but failed to produce transgenic rice plants. However, Chan *et al.* (1993) and Hiei *et al.* (1994) were able to regenerate transgenic rice plants by *Agrobacterium* mediated transformation.

Over the last decade the direct DNA delivery into intact tissues by biolistic gun has become much popular and routinely used method for rice transformation. The biolistic approach has overcome the limitation of genotype specificity, which was faced in *Agrobacterium* mediated transformation. It has successfully been used in many crops including soybean, cotton and wheat (McCabe *et al.*, 1988 and Vasil *et al.*, 1992). In rice, Paul Christou group (Christou *et al.*, 1991; Christou, 1992 and Li *et al.*, 1993) has successfully developed transformation system by biolistic gun. This method is currently the only successful method for chloroplast transformation in green algae *Chlamydomonas* (Blowers *et al.*, 1989) and mitochondrial transformation in yeast (Butow and Fox, 1990).

Presently the most commonly used methods for foreign gene transfer to plants are either mechanical or biological. The biological methods are evolved from *Agrobacterium*-mediated gene transfer. For this research the purpose was to develop and improve the practical application of *A. tumefaciens* for monocot transformation since this was once theorized as not being possible for monocot transformation (Dong *et al.*, 1996; Rashid *et al.*, 1996). This recently, however, has become the method of choice because it had previously proven to be simple, offered more precise results in gene transfer, results in permanent genetic change, and is less likely to have a high occurrence of somaclonal variation. To alleviate these problems with *Agrobacterium* transformation in monocots, researchers sought ways to make substantial improvements. One such improvement was the use of the rice shoot apical meristem instead of immature embryos or other rice explants. This decision soon proved to be a more successful avenue since the rice shoot apex succumbs to *Agrobacterium*-mediated infection (Hiei *et al.*, 1997; Park *et al.*, 1996 and Rashid *et al.*, 1996). Additionally, the apical meristem gave rise to

plants that have both the identical genotypic and phenotypic make-up of the original plant intact.

5.1.3 Selectable Markers & Reporter Genes

Transformation vectors are constructed with a reporter gene or a selectable marker, which indicates successful incorporation and expression of the vector genes. The reporter gene or selectable marker gene is fused to the same plant promoter that is fused to the foreign gene of interest. Commonly, antibiotics and herbicides are used as selectable markers (Hinchee *et al.*, 1994). During the regeneration process of the plant transformation procedure, transformed host cell or tissues are propagated in the presence of a substance, which normally inhibits the growth of the plant cells. The expression of the selectable marker gene provides resistance to the inhibitory growth substance, allowing for preferential regeneration of only transformed cells. Reporter genes do not induce survival on a toxic medium but code for a gene product that has an easily detectable phenotype. The most widely used antibiotic marker is kanamycin and was used in the first transformation experiments (Fraley *et al.*, 1983). Streptomycin, rifampicin and hygromycin are also using in plant transformation as selectable markers.

5.1.3.1. Reporter Genes

Unlike selectable markers, reporter genes do not confer resistance to a drug but produce a gene product that has a distinct and easily detectable phenotype. In some transformation experiments, the gene of interest can also act as a reporter if the gene product has a detectable phenotype. The phenotype of the reporter gene should be quick and easy to detect. With most reporters successful transgene expression can be determined through a visual assay. Once a positive visual assay is observed, vector integration can be confirmed by Southern blot hybridization

analysis of the host genome. The reporter gene assay is an invaluable step as it eliminates many of the transformants to a selected number of possible successful integrations. The three most common reporter genes used in plant transformation are the β -glucuronidase gene (GUS), green fluorescent protein (GFP) and the luciferase gene (LUC). These three non-plant genes are fused to viral or plant promoters and incorporated into the delivery vector.

5.1.3.1.1. GUS (β -glucuronidase) Gene

The GUS reporter gene was isolated from *E. coli* and was shown an effective reporter for plant transformation (Jefferson *et al.*, 1986). The enzyme β -glucuronidase, popularly described as GUS, breaks down glucuronides giving a colored reaction, so that its presence can be detected in situ, i.e. inside the plant tissue, used either as thin sections or in any other form. Several glucuronides, which can be used as substrates, include p-nitro phenyl glucuronide (PNPG), 5-bromo, 4-chloro, 3-indolyl glucuronide (BCIG), naphthol AS-B1 glucuronide (NAG) and resorufin glucuronide (REG). Cleavage of the substrate by β -glucuronidase produces blue colored product. Therefore, any transformed tissue appears blue. One drawback is that some tissues express a basal level of GUS, which can lead to background. The major-advantage of using this reporter gene lies in its assay, which requires no DNA extraction, electrophoresis or autoradiography.

The GUS (β -glucuronidase) reporter gene system has been using as a powerful tool for the assessment of gene activity in transgenic plants (Jefferson 1989). Since the development of the GUS system, many thousands of transgenic plants have been generated expressing GUS. In such plants, the spatial distribution of gene activity can be visualized in the absence of any background signal. GUS

offers a promising route for transformation, monitoring and molecular genetic analysis of the numerous important epi- and endophytic organisms that are not readily cultured, such as oomycetes and mycorrhizal fungi. In addition GUS fusions can be used in many laboratory model systems lacking endogenous activity, including *Saccharomyces*, *Schizosaccharomyces*, *Dictyostelium*, some strains of *Caenorhabditis elegans* and *Drosophila*.

5.1.3.1.2. GFP (Green Fluorescent Protein)

The green fluorescent protein is normally produced within photocytes of the jellyfish *Aequorea victoria*, and must undergo a series of post-translational maturation steps to produce the fluorescent form of the protein. There are two major uses for green fluorescence protein (GFP) in plant: monitoring gene expression and protein localization at high resolution, and providing an easily scored genetic marker in living plants. GFP can be used as a replacement for β -glucuronidase, which is commonly used as a reporter for genetic fusions in plants, it allows direct imaging of the fluorescent gene product in living cells without the need for prolonged and lethal histochemical staining procedures (Chalfie, *et al.*, 1994). In addition, GFP expression can be scored easily using a long-wave UV lamp if high levels of fluorescence intensity can be maintained in transformed plants. An assay for gene expression using fluorescence in vivo would be a very useful tool for plant transformation and breeding experiments.

To employ GFP successfully in plants, three major steps need to be taken:

- (1) The GFP apoprotein must be produced in suitable amounts in the plant cells.
- (2) The apoprotein must undergo efficient post-translational cyclization and oxidation to produce the mature GFP (Heim, *et al.*, 1994).
- (3) The fluorescent protein may need to be suitably targeted within the cell, to allow efficient post-

translational processing, safe accumulation to high levels, or to facilitate detection of expressing cells.

5.2. Gene Transformation in Rice

Rice (*Oryza sativa* L.) is one of the most important cereal crops, providing a staple diet for almost half of the world's population. However, rice yield and quality are affected by pests and diseases, as well as by environmental stress. Plant genetic engineering provides an opportunity to incorporate novel resistance traits into rice. The method for plant transformation, either through direct DNA delivery by particle bombardment or *Agrobacterium*-mediated transformation is well established for dicotyledonous species. In recent years, the successful transformation of rice by *Agrobacterium* had been reported, although monocotyledonous plants were considered to be outside the host range of *A. tumefaciens* (Gould *et al.*, 1991; Raineri *et al.*, 1990). Transformed rice callus was first reported by Chan *et al.* (1992) but no transgenic plants were obtained. Chan *et al.* (1993) reported the generation of first transgenic japonica rice plants by inoculating immature embryos with *Agrobacterium*. Park *et al.* (1996) described the transfer of gene coding for glufosinate-ammonium (PPT) resistance into japonica rice by using *Agrobacterium*. They confirmed the inheritance of the transferred genes to the progeny by Southern blot analysis. Insertion of the reporter gene glucuronidase (GUS) gene and the selectable marker, hygromycin phosphotransferase (*hptII*) gene, into several cultivars of indica rice using *Agrobacterium*-mediated transformation was also subsequently demonstrated (Rashid *et al.*, 1996). Cheng *et al.* (1998) used *Agrobacterium*, which harbored the *cryIA(b)* and *cryIA(c)* genes to introduce stem borer resistance into a japonica rice. Recently, Datta *et al.* (2000) introduced the chimeric chitinase gene in rice cultivars by using *Agrobacterium*. The transgenic plants exhibited resistance to

sheath blight pathogens. Stable integration, inheritance and expression of the chitinase gene were demonstrated by Southern blot and Western blot analysis of transformants.

Early selection and screening of putative transformants for stable transformation is essential and GUS expression could be used as a rapid early screening method (Hu *et al.*, 1990). The success of producing transgenic plants depends upon transmission of functional foreign genes from primary transformants to their progenies (Hayashimoto *et al.*, 1990).

The present research work was set up to verify a transformation system using *Agrobacterium*-mediated transformation into indica rice. The rice cultivar used for the transformation was BRRI dhan -30, which is a glutinous rice widely grown in Bangladesh. The successful production of transgenic plants of this cultivars will open the way for introducing agronomically useful traits into any commercially grown cultivars, providing the best opportunity for maximizing yields. Most of the rice transformation work reported was based on japonica varieties. However very little work had been done on improving Bangladeshi local rice varieties using modern biotechnological methods. There is an urgent need to undertake such studies to produce local transgenic rice varieties with beneficial agronomically important genes. Under the present investigation efforts are intensified in this purview of transformation was to establish an efficient protocol for gene transformation in rice by *Agrobacterium*-mediated method. Here two reporter genes GUS and GFP were considered as the easily detectable target genes towards establishing at least an efficient protocol for *Agrobacterium*-mediated gene transfer method in rice and this was the first attempt in Rajshahi University in the field of gene transfer technology in cereal crop by biological methods.

5.3 *Agrobacterium*-Mediated Transformation of Rice

5.3.1 Plant Materials and Methods

BRRI dhan-30, a popular indica type long grain rice (*Oryza sativa* L.) was used in the present work. Seeds of this variety were collected from Regional Rice Research Institute, Rajshahi, Bangladesh. Three week old scutellum derived calli of rice cv. BRRI dhan-30 was used as the explant for transformation in this experiment.

5.3.2 *Agrobacterium* Strains and Culture

The *Agrobacterium* strain LBA4404 (pCL3) and LBA4404 (pCL4) harboring with GUS and GFP respectively were constructed and provided by Prof. P. Nick, Friburg University, Germany. *Agrobacterium* strains LBA4404 (pCL3) and LBA4404 (pCL4) were grown in 50 $\mu\text{g mL}^{-1}$ kanamycin and 50 $\mu\text{g mL}^{-1}$ spectinomycin containing LB medium (Tryptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L) solidified with 12 g of agar at pH = 7 for 3 days at 28° C. Two days prior to inoculation, sterile wooden sticks were used to select single colonies of pCL3 and pCL4 and allowed to grow overnight in 50 ml of LB media with 50 $\mu\text{g mL}^{-1}$ kanamycin and 50 $\mu\text{g mL}^{-1}$ spectinomycin on a 200 rpm shaker at 28° C (Fig. 5.1). After 24 hours, 0.5 ml of the overnight culture was resuspended in 50 ml of LB without antibiotics and placed on a 200 rpm shaker at 28°C to be incubated overnight. The optical density (OD) of the bacterial solution was measured with a spectrophotometer at 600 nm. An OD value of 0.5-0.7 was taken as the optimal concentration. The restriction maps of the T-DNA region of pCL3 and pCL4 are given in Fig. 5.2 and 5.3 respectively. *A. tumefaciens* LBA4404 has its T-DNA deleted but contains a functional *vir* region that is capable of transferring T-DNA into plant cells. The effect of the concentration of the *Agrobacterium* and the optimal OD reading were evaluated in one set of experiments.

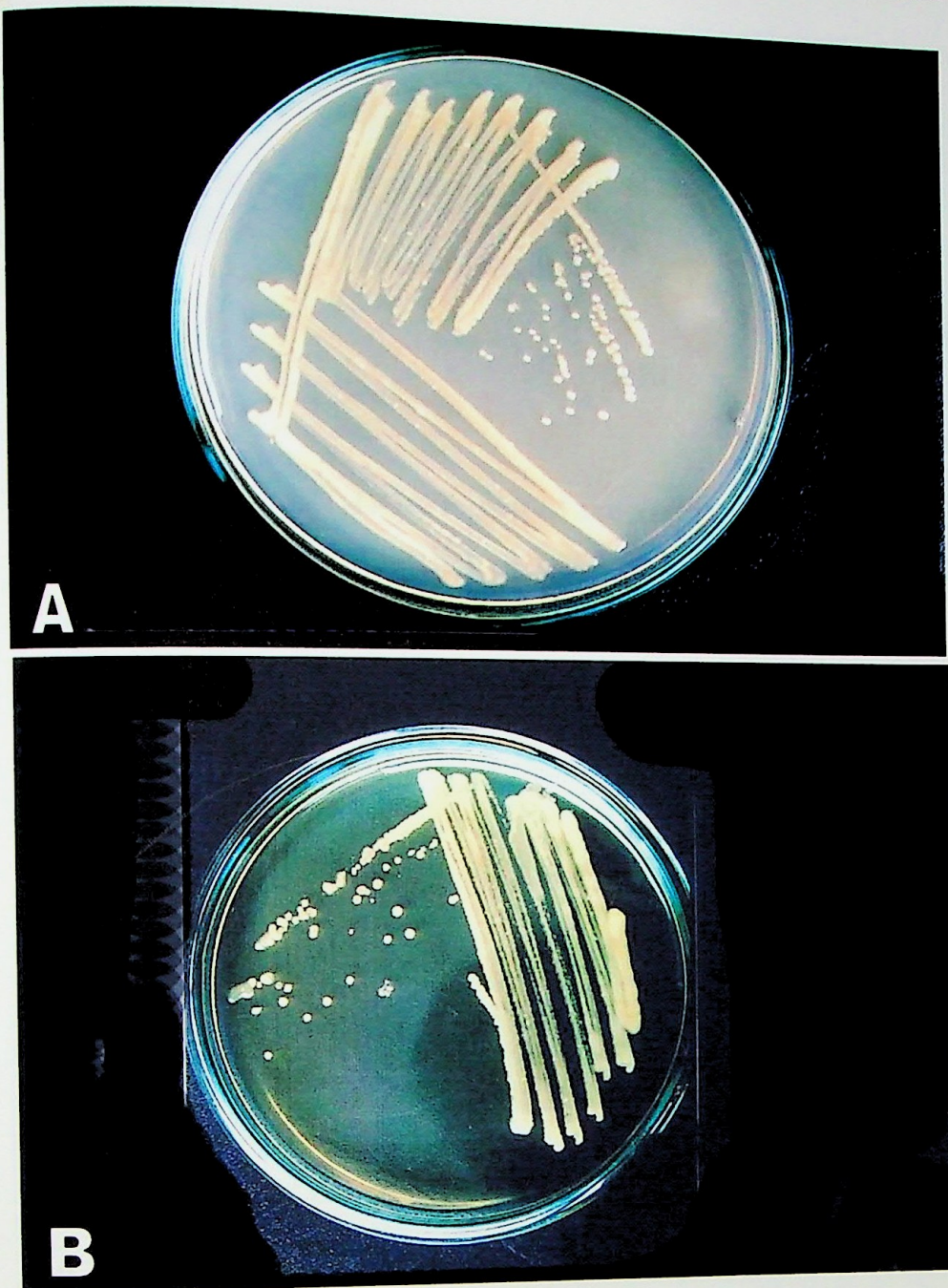


Fig. 5.1: *Agrobacterium* stains LBA4404 of single colonies.

A. *Agrobacterium* stain LBA4404 (pCL₃).

B. *Agrobacterium* stain LBA4404 (pCL₄).

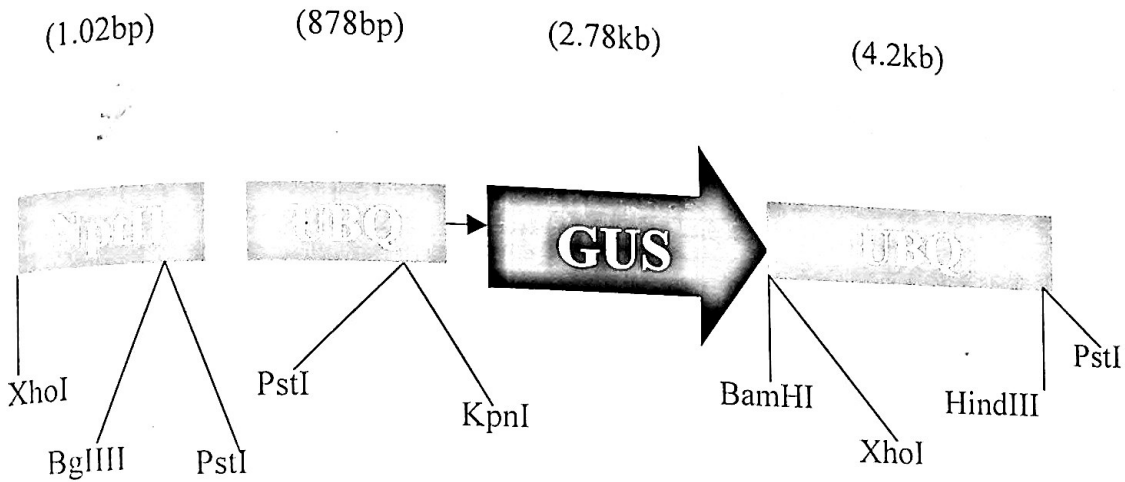


Fig. 5.2. Restriction map of T-DNA region of the vector LBA4404 (pCL3). The arrow indicates the direction of transcription and sizes of individual fragments are placed in parentheses. NptII-neomycin phosphotransferase gene, UBQ-maize ubiquitin promoter, GUS- intron containing GUS gene and restriction enzymes- XhoI, BglIII, PstI, KpnI, BamHI, HindIII.

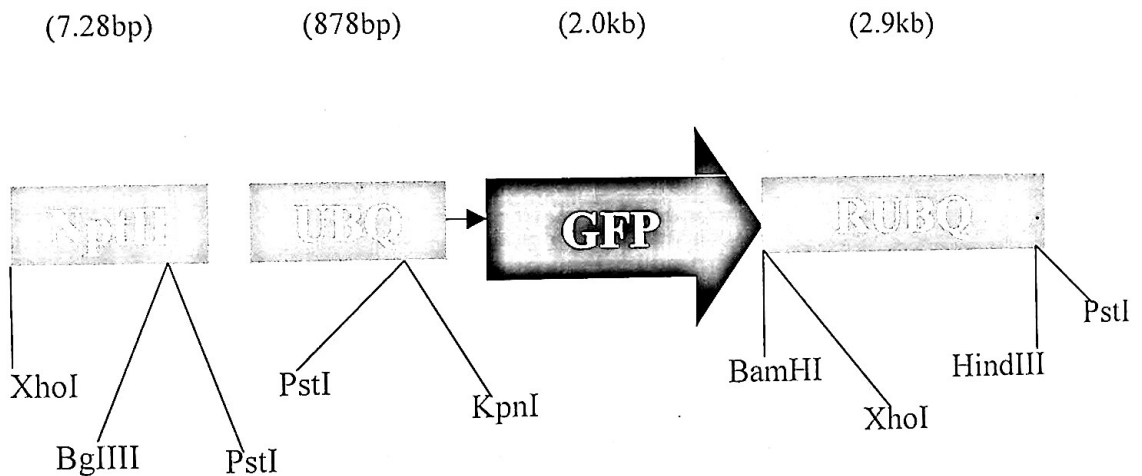


Fig. 5.3. Restriction map of T-DNA region of the vector LBA4404 (pCL4). The arrow indicates the direction of transcription and sizes of individual fragments are placed in parentheses. NptII-neomycin phosphotransferase gene, UBQ-maize ubiquitin promoter, RUBQ- rice ubiquitin promoter, GFP- intron containing GFP gene and restriction enzymes- XhoI, BglIII, PstI, KpnI, BamHI, HindIII.

5.3.2.1 Inoculation and Co-cultivation

Actively generative embryogenic calli of BRRI dhan-30 rice were selected for co-cultivation. Immediately the calli were inoculated by placing them in 5ml of the *Agrobacterium* suspensions of GUS and GFP strains in LB medium. The *Agrobacterium* suspensions were rocked for 5 minutes at 5 rpm at room temperature with a desktop rocker. The inoculated calli were vacuums infiltrated for 5 minutes at 20 Hg. After co-cultivation in these suspensions, the calli were blotted dry on sterile filter paper, to remove excess moisture and then plated on culture plates containing 4.3 g/L Murashige and Skoog (MS) salts, 0.1 g/L myo-inositol, 0.002 g/L glycine, 25g/L sucrose, 0.50 mg/L kinetin, 0.050 g/L arginine, 1 mL B5 vitamins, 100 ppm acetosyringone and 1.75 g/L phtyagel. Plated calli were then co-cultivated for 2-3 days in the dark at 25° C. After 3 days the explants were removed from the co-cultivation media, and rinsed 2-3 times sterile water, before being assayed for GUS activity. The effect of acetosyringone to the co-cultivation media was evaluated in this research, to have an effect on calli-mediated DNA transformation.

5.4. Assay of GUS Activity

The co-cultivated rice calli after necessary washing were used for the test of GUS assay. For GUS assay test the calli were taken in a falcon tube with 1 ml GUS substrate buffer (5mM potassium ferrocyanide, 0.1M sodium phosphate buffer pH 7.0, 20µl of 0.3% v/v Triton X-100) and incubated at 37°C for 25 minutes. After incubation the treated calli were placed in a petri plate and subjected to vacuum infiltration for 5 minutes at 20 Hg. The chemical X-gluc was then added to the substrate buffer and again the materials were put under vacuum infiltration for 7 minutes at 20 Hg. The explants were removed from the vacuum and placed in 37°C for 24-38 hours. Within 24 hours explants were examined under microscope for (GUS) gene expression, observed as blue spots.

5.5 Results and Discussion

5.5.1 Culturing of the *Agrobacterium* Strains

Timing and temperature greatly affected virulence of the strains LBA4404 (pCL3) and LBA4404 (pCL4). These strains had to be promptly removed from culture within 48 hours. When these strains were left for a more extended period of time, the ability of the bacteria to cause transformation within the shoot calli decreased. Temperatures higher than 28°C also proved to be detrimental. Making fresh *Agrobacterium* suspensions for each experiment was routinely done since it aided in maintaining competency of the *Agrobacterium* suspension and increased chances of successful transformation.

The highly regenerative embryogenic rice calli were co-cultivated with *Agrobacterium* harboring with GUS gene. During co-cultivation the *Agrobacterium* was supposed to transfer its T₁ plasmid along with GUS gene to the rice callus. After co-cultivation the callus tissue were subjected to GUS assay in order to confirm the GUS gene integration into the rice genome. Histochemical assay was performed on the co-cultivated rice calli according to the method described by Jefferson (1987) with some modifications. Following histochemical assay a number of blue spots were appeared in the body of the rice calli, which can be seen by naked eye (**Fig. 5.4 A**). For microscopic observation treated rice calli were pressed on a slide covering by cover slide and observed under microscope (**Fig. 5.4B**).

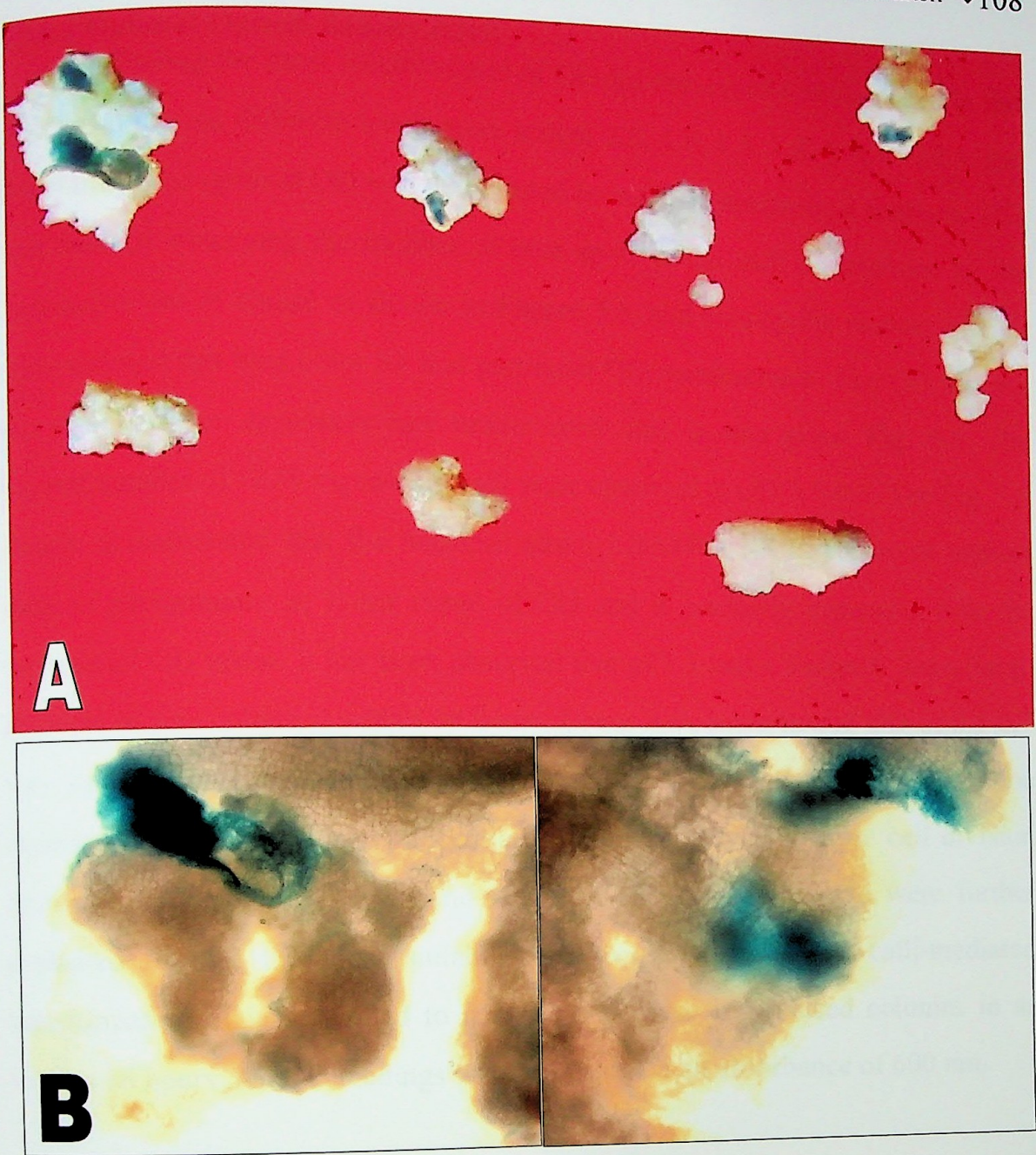


Fig. 5.4: Visual detection of histochemical staining for GUS activity in the calli of rice cv. BRRI dhan-30 following *Agrobacterium*-mediated transformation.

- A. Rice calli with blue spot after co-cultivation with LBA4404 (pCL3).
- B. Calli was transformed with GUS. Calli showed the specific competent tissue transformed with GUS, seen under microscope.

5.5.1.1 Optimal Optical Density (OD)

In finding the most suitable level of OD for the *Agrobacterium* used in this experiment, seven different levels were tested; 2.0, 1.8, 1.5, 1.3, 1.0, 0.7, 0.5, and 0 (control). The optimal OD was determined by using histochemical analysis. This was done by observing the number of blue spots in the calli transformed by the *Agrobacterium* of choice as shown in **Figure 5.5**, and also by the number of calli that exhibited this blue color for that particular OD measurement. The OD was not the same for the *Agrobacterium* strains used: this is also given **Fig. 5.5**. The highest number of GUS expressing calli were observed at OD 0.5-0.7. The Fig. 3.8 clearly demonstrated that the bacterial strain LBA4404 (pCL3) showed highest peak of performance in lower range of OD and it gradually decreases with the increase of OD taken. It has been observed that pCL3 had a bacterial lawn (large amounts of tiny colonies) within 36 hours. Using *Agrobacterium* at OD of 0.5-0.7 was coincidental with the fact that most *Agrobacterium* strains would be experiencing the early log (exponential) phase during which active cell division occurs at 0.4-0.8 OD (Janssen and Gardner, 1989). These results were further evaluated to find the most suitable *Agrobacterium* strain for calli-mediated transformation. It was decided to use pCL3 because it produced colonies in as little as 36 hours. All OD readings were measured at an absorbance of 600 nm.

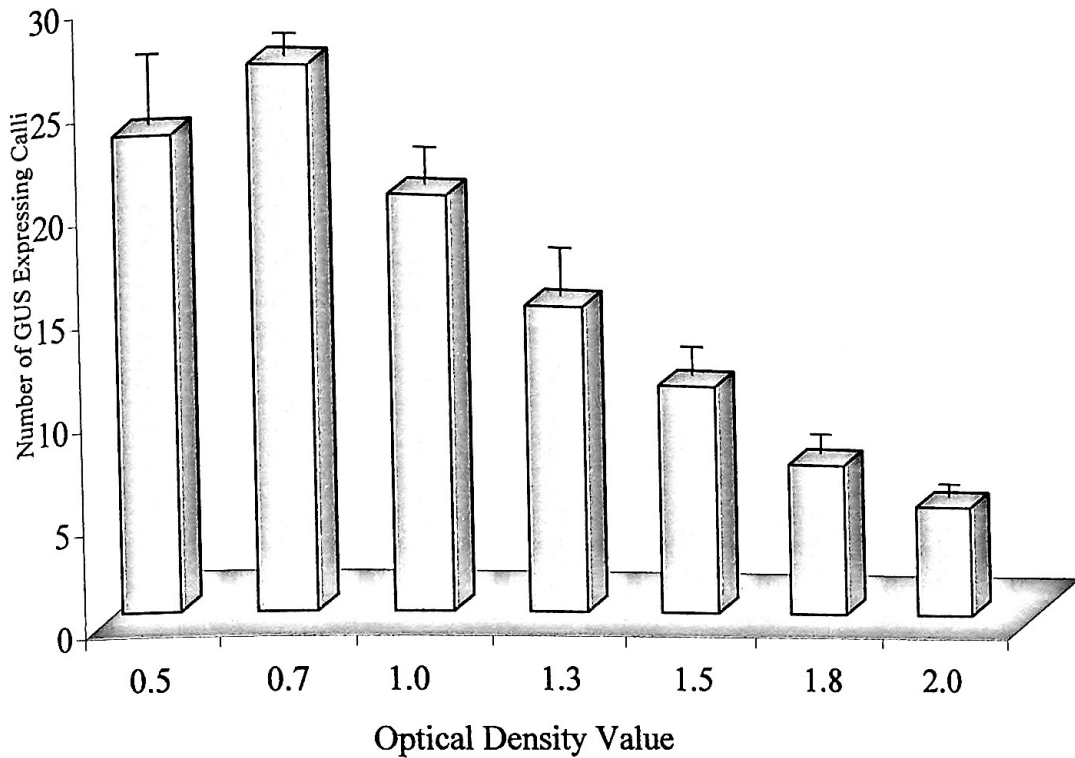


Fig. 5.5: Optimal optical density of pCL3 *Agrobacterium* engineered strains as measured by the number of GUS expressing calli. Bars represent standard error of the means. Optical density (OD) was measured at 600nm.

5.5.2. Effect of Acetosyringone during Co-cultivation

Various studies (Khanna and Raina, 1999; Gould, 1997) examined the importance of acetosyringone to *Agrobacterium* co-cultivation procedures. These studies stated that without it, there would be a complete absence of GUS expression, the quickest and most visual proof of transformation. However, the problem was with the level of acetosyringone to use since concentrations to use ranged from 30 ppm – 1000 ppm from different studies. Therefore, five different concentrations were tested; 100 ppm, 200 ppm, 400 ppm, 600 ppm and 1000 ppm using calli in each concentration. The effectiveness of the concentration of

acetosyringone was observed by the presence of blue color from GUS assays performed on the calli. The results are presented in Fig. 5.6. The results show that as the concentration of acetosyringone increased, the number of blue GUS spots/calli also increased. However, between 400 ppm and 1000 ppm the results (i.e. the number of blue spots) were not significantly different. Therefore, using acetosyringone at 1000 ppm was decided upon for the use of co-cultivation medium.

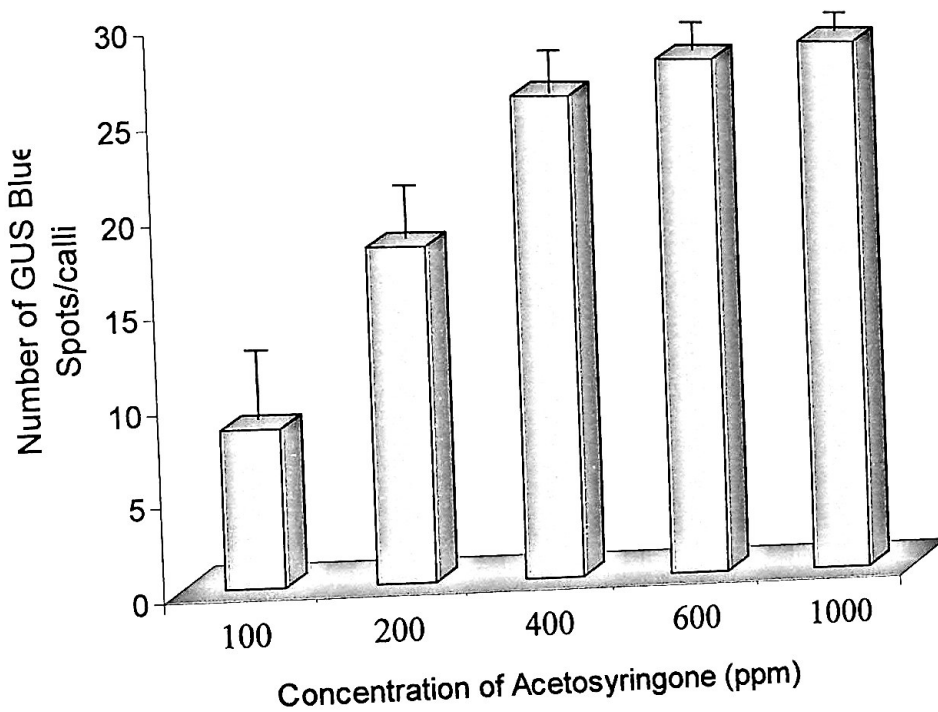


Fig. 5.6: Acetosyringone effect on GUS activity as measured by the number of blue spots in the calli. Bars represent the standard error of the means.

5.6 GFP Expression

Visualization of GFP does not require to kill cells or subjected to any staining assay the fluorescence can be used to study the gene expression in vivo. Rice calli was transformed with LBA4404 (pCl₄) and transformed tissue were washed and the calli were presented by cover slide and visualized under fluorescence microscope. Images were taken under GFP filter and **Fig. 5.7** showed several examplly photos.

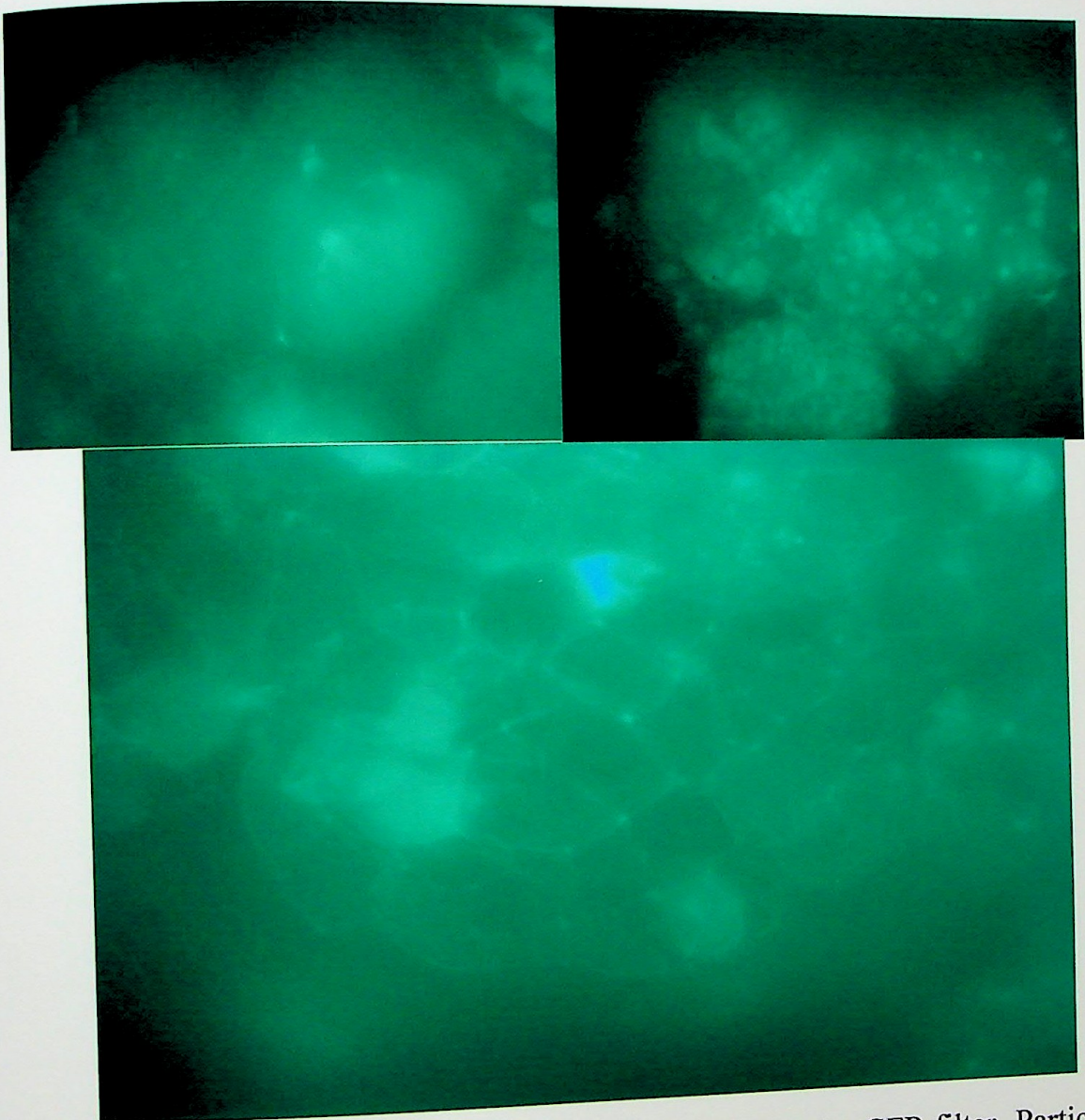
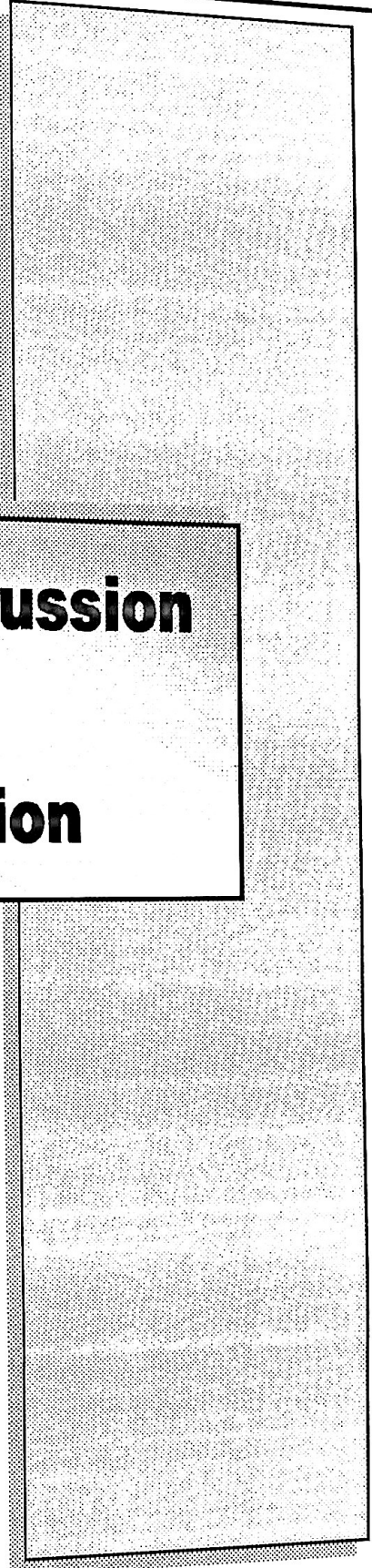


Fig. 5.7: Calli transformed with GFP and visualized under GFP filter. Particular sector showing the GFP fluorescence.

The experimental results stated under transformation chapter clearly demonstrated that in Bangladesh condition we have developed the method for gene transformation in rice by *Agrobacterium*-mediated method. Efficient transformation of two reporter genes, GUS and GFP by *Agrobacterium*-mediated methods opens the new door for the successful accomplishment of stable transformation of other agronomically important genes in rice.



General Discussion and Conclusion

CHAPTER V

6. General Discussion and Conclusion

The aim of the present study was to establish an efficient tissue culture regeneration technology and the protocol of DNA transfer of reporter GUS gene transformation system in the local varieties of rice in Bangladesh as mentioned in general introduction. At the advent of modern plant biotechnology a number of novel tools have been developed to improve the target crop plant by directly introducing different beneficial genes against devastating diseases, pests biotic and abiotic stresses. The fundamental requirement of genetic transformation towards developing a crop plant is to establish an efficient regeneration protocol for the target plant. Under the present investigation a screening has been made to find out the most responsive rice varieties in Bangladesh for tissue culture and regeneration. The most widely cultivated six nice varieties were tested for tissue culture response and three varieties were found most responsive in tissue culture. Efforts have been intensified towards developing the most efficient regeneration protocols for these three highly responsive rice varieties. For our ultimate target to establish gene transfer technology the variety BRRI dhan-30 was selected for transformation of two reporter genes GUS and GFP. The results obtained have been presented and discussed in the appropriate sections, in this chapter only the most important aspects are discussed in detail and the areas which require more work are pointed out on the perspectives of practical applications.

6.1 Tissue Culture Response of Indica Rice Varieties

With the aim of improvement of rice varieties, commonly cultivated in Bangladesh, six indica varieties, BR -22, BR - 23, BRRI dhan-28, BRRI dhan-29, BRRI dhan-30, BRRI dhan-31 were collected and their *in vitro* tissue culture

response was studied. Before going to genetic transformation of rice it is an important step to investigate comparative tissue culture response of different varieties because the tissue culture regeneration protocol is an essential tool towards the genetic manipulation (Larkin and Scowcroft, 1981)

6.1.1 Somatic Embryogenesis

Somatic embryogenesis is the initiation and development of embryos from somatic cells. All the somatic cells of a plant are considered as totipotent but all of them do not readily produce somatic embryos in culture medium. The somatic cell in different organs of a plant body bears different levels of totipotency and depends upon the genotype. For this reason, a large number of rice varieties were selected to study their comparative embryogenic capability. The results clearly indicated that rice varieties used in this study hold different levels of embryogenic varieties response in different media from different tissue explants. Among six tested BRRI dhan-30, BRRI dhan-28 and BRRI dhan-31 showed greater embryogenic response in all tested media. The callus forming response of different rice varieties showed that indica varieties behaved differentially in callus formation from different tissue explants. Abe and Futsuhara (1984) also observed the varietal difference of rice *in vitro* tissue culture. The genotype variability for callus formation and plant regeneration in rice was also observed by Abe and Futsuhara (1986a and b). The variety BRRI dhan-30 gave the highest embryogenic callus formation (97.3 %) on N6 medium. The overall performance of six rice varieties showed that BRRI dhan-30, BRRI dhan-28, BRRI dhan-31 gave higher response and BRRI dhan-30, BRRI dhan-28, gave moderate response in embryogenesis. The varieties BR -22, BR-23, BRRI dhan-29 gave poor response in embryogenesis. It can be concluded from embryogenic study that varieties

BRRI dhan-30, BRRI dhan-28 and BRRI dhan-31 can be considered as suitable varieties for tissue culture studies and offer better opportunity for genetic manipulation. The poor responding varieties, on the other hand, need more research work on changing media composition with different concentrations of growth regulators for addressing their right potentialities in tissue culture.

For tissue culture study different tissue explants of rice (scutellum, leaf base, mesocotyl and root) were used for callus induction and regeneration. The tissue explants namely scutellum, leaf base, mesocotyl and root were also considered as suitable explants in rice tissue culture studies (Abdullah *et al.* 1986; Gupta *et al.* 1989 and Jain *et al.*, 1996). This leads to conduct experiments to study the comparative embryogenic potentiality of various explants of rice varieties subjecting to different callus inducing media. Among the tested explants scutellum produced greater callus irrespective of varietal difference. The explant mesocotyl showed the lowest performance in callus formation in all tested media. In the forgoing study scutellum proved to be the best explant for callus formation. Scutellum explant also had some added advantages, (a) it does not require the germination of seedlings in order to get explants (b) easy and simple method of callus formation in short time.

The success in plant tissue culture technology is greatly dependent on the choice of nutritional components and growth regulators in cultured media. In the present study three different media LS (supplemented with 2.5 mg/l 2-4 D), N6 (supplemented with 2.0 mg/l 2-4 D) and MS (supplemented with 2 mg/l 2-4 D) were used in order to select suitable culture medium for callus inducing from different indica rice varieties. These are commonly used media in rice tissue culture (Gupta *et al.*, 1989; Rueb *et al.*, 1994 and Jain *et al.*, 1996). The media LS

and N6 were found equally suitable for callus induction potentially. The LS medium was also proved to be the best medium for somatic embryogenesis in rice by Jain *et al.* (1996). It was observed in embryogenic study of rice that media supplemented with 2.0-2.5 mg/l of 2,4-D produced higher percentage of embryogenic calli. The lower concentration of 2,4-D did not produce better callus as observed in MS and addition with 2,4-D also reduced embryogenesis in rice. In present study medium MS was not proved to be good for rice embryogenesis. Earlier studies showed stimulatory effect of tryptophan on somatic embryogenesis in rice (Siriwardana and Nabors, 1983) and plant regeneration (Chowdhury *et al.*, 1993; Gupta *et al.*, 1989). In the present study MS medium combination with 2,4-D, the poor callus response on MS medium was may be due to their combined effect or varietal difference. The varieties BRRI dhan-30 and BRRI dhan-28, BRRI dhan-31 were selected of regeneration studies from somatic embryogenesis because of their better response in callus induction as well as economic importance.

6.2. Regeneration of Rice

Regeneration study was particularly needed for plant transformation, as it is an essential component and practical tool for transgenic technology. The regeneration ability of selected varieties (BRRI dhan-30 and BRRI dhan-28, BRRI dhan-31) was investigated in respect of their different tissue explants (scutellum, leaf base, and mesocotyl) on different regeneration media. A number of factors including genotype, developmental stage of the cell, nature of the explant, hormonal composition of the medium, carbohydrate source and osmotic stress had been studied to improve the frequency of plant regeneration in rice (Abe and Futsuhara, 1984; Lai and Liu, 1988; Datta *et al.*, 1990; Chowdhury *et al.*, 1993 and

Jain *et al.*, 1996). In the present study the varieties BRRI dhan-30, BRRI dhan-28, BRRI dhan-31 having different genotype and origin were cultured on different regeneration media supplemented with different concentrations of phytohormones. Scutellum of mature seed was proved to be the best explant for regeneration. The highest regeneration (88.2%) was found in rice variety BRRI dhan-30 in MS medium containing sucrose 3%, sorbitol 3% along with phytohormones NAA 0.05 mg/l, and BAP 2.0 mg/l and solidified with 0.8% agar. Sorbitol was considered as an osmoticum, when used in conjunction with sucrose, had beneficial effect on morphogenesis in rice (Kishore and Reddy, 1986). Sorbitol along with sucrose as a carbon source in regeneration medium may cause osmotic stress to rice tissue and there are now several reports suggesting the stimulatory effect of mild osmotic stress on plant regeneration (Jain *et al.*, 1996). A culture medium with higher mannitol and agar concentration improved plant regeneration frequencies in japonica and indica varieties (Lai and Liu, 1988 and Jain *et al.*, 1996) and inclusion of sorbitol increased plant regeneration in Taipei 309 (Rueb *et al.*, 1994). It was observed in the present study that the absence of sorbitol in other media did not give better regeneration response. In present study some albino plants were also obtained in regeneration from somatic embryogenesis. Albino plant is unwanted and their production greatly limits the objectives of tissue culture technique (Torrizo and Zapata, 1986). Albinism was known to be genotype dependent (Liang *et al.*, 1980) and influenced by culture conditions (Lee and Chen, 1982). Albinism may results from changes in chloroplast or nuclear DNA or both. A greater understanding still required to study the physiological, genetic and molecular basis of this phenomenon (Chu, 1982).

Efficient regeneration from scutellum explant offers a new venture for *Agrobacterium* mediated transformation in rice because scutellum induces *Agrobacterium* infection (Hiei *et al.*, 1994; Vijaya Chandra *et al.*, 1995). Results obtained from tissue culture study revealed that BRRI dhan-30 was the most suitable rice variety for regeneration study used in this investigation. The variety BRRI dhan-30 exhibiting higher regeneration capability proved itself as suitable rice genotype in Bangladesh for under taking research of genetic manipulation. Regeneration medium containing sorbitol proved to be the best medium for regeneration of local varieties.

6.3 Meristem Culture of Rice

In dicot plants meristem present at the apex portion of growing shoot but in monocot like rice meristem lies at the base of developing leaf. Meristem culture has appeared as distinct and independent technology in tissue culture for micropropagation and disease elimination from many crops. The meristem (leaf base) was cultured in selected rice varieties (BR-22, BR-23, BRRI dhan-29, BRRI dhan-28, BRRI dhan-30, BRRI dhan-31). Meristem portion was carefully excised from young seedlings of rice and cultured in different media. In order to find out the suitable seeding age for meristem culture an experiment was laid out with different age (4, 5 and 7 days old) seedlings. Meristem was isolated from 4 days old seedling gave higher percentage of regeneration in all tested varieties. It was observed that meristem of rice in leaf base existed for short duration and rapidly changing with the age of seedling. The meristem region of the leaf base quickly undergoes to tissue differentiation.

It was observed that hormones (NAA and BAP) in the culture medium adversely affect the regeneration efficiency of rice plant from meristem culture.

Similar efficient result of meristem regeneration on hormone free medium was also reported by other researchers (Simola, 1987; Anthony, 1992 and Bobak *et al.*, 1995). As clearly mentioned in the results that the plants obtained from meristem have less somaclonal variation. A healthy fast growing plant could be obtained from the leaf bases in about 8 weeks. The regeneration of whole plant from meristem of rice gave less somaclonal variation and by passed the long term *in vitro* somatic embryogenesis.

6.4 Anther Culture of Rice

Androgenesis refers to the development of haploid plants derived from anther culture and haploidization can be achieved by anther culture. Guha and Maheshwari (1964, 1966) first reported the haploid plant production in *Datura* through anther culture. Afterwards, haploid plant production has been reported in more than 200 species (Dunwell, 1986). *In vitro* androgenesis is the most convenient and useful technique for production of doubled haploid (DH) plants. The main advantage of using doubled haploids is the rapid homozygosity of the descendants, results in a time saving method for the development of new varieties. Anther and microspore culture provided a new venture in producing homozygous plants within a year as compared to the long inbreeding cycles (8-10 years) of conventional breeding methods.

Since, haploid plant formation through anther culture has been successfully applied in agricultural crops, such as *Brassica* sp., tobacco, potato, wheat, rice, maize and barley (Bajaj, 1983). First haploid plantlets were successfully obtained from the pollen callus developed from the anthers of *Oryza sativa* by Niiuzeki and Oono (1968). However, some factors are closely related to improve anther culture

of rice e.g. genotype, donor plant growth conditions, microspore condition, pretreatment and culture media.

In genetic and plant breeding programs, haploid and doubled haploid production through androgenesis is very much important. Under the present study, research endeavor has been made on three important factors influencing anther culture. Evaluating their androgenic response screened a number of rice varieties were screened. Different measures of pretreatment were introduced to improve the induction and regeneration efficiency. Investigation was made to find out suitable media composition to accomplish successful regeneration of anther culture. Intensified research is necessary under these three purviews (genotypic response, pretreatment and media compositions) to achieve successful results in anther culture. Screening of genotypes with a high androgenic potential is the basis for androgenic research. There remains an enormous scope of pretreatment for improving the conditions of efficient embryo induction. Media formulation for the appropriate culture is one of the most important factors to improve anther culture.

Another important factor in the success of anther culture is the donor genotype. Optimization of media and culture conditions for obtaining high androgenic response of low responding genotypes is still a challenge. One of the main problems in the success of androgenesis is the genotype response because androgenesis is strongly genotype dependent and is under genetic control (Bullock *et al.*, 1982; Lazar *et al.*, 1984). The growing conditions of the donor materials might have influence on the induction of androgenic microspore development (Hu *et al.*, 1983; Marburger, 1987). Under the present investigation some rice genotypes from Bangladesh were tested for their androgenic response. For anther

culture five of seven genotypes showed better performance on induction and regeneration.

The composition of media is one of the important factors in the success of androgenesis. The available literature does not allow recommendation of any anther culture medium for general application. Zhou *et al.* (1991) mentioned that albinism is a main problem in cereal crops for androgenic studies and it could be possibly solved by using a suitable medium. Addition of different amino acids and sugars in the medium along with other components may influence induction (Ho *et al.*, 1978; Schmid *et al.*, 1994). Sterilization method of media is also important because during sterilization some toxic components originated from certain components of media particularly sucrose, which may breakdown when subjected to high temperature (Orshinsky *et al.*, 1990). A comparative study was made on carbon source; sucrose was used and it was observed that sucrose containing media showed better performance on the induction. Similar type of results were obtained in wheat, maize, rye and potato anther culture (Deimling *et al.*, 1990; Batty and Dunwell, 1989). Trottier *et al.*, (1993) reported that in anther culture addition of higher concentrations of vitamins showed significant higher percentage of induction and regeneration of green plantlets.

In the present investigation, Z2 medium with higher concentrations of three vitamins (Nicotinic acid: 2.5 mg/l, Pyrodoxine HCl: 2.5 mg/l and Thiamin HCl: 2.5 mg/l) and sucrose were used in the induction medium resulting in higher embryo induction and regeneration.

A preliminary study on androgenic response was made by Bajaj (1983) using several physical factors e.g. radiation, photoperiod, temperature, supply of

water and nutrients, growth regulators and pesticides, and obtained significant results on induction. Physical pretreatment with cold could also influence the induction in rice. For androgenic response symmetrical division of microspores is very important and which is the main source of androgenic haploids. Zheng and Ouyang (1980) found that panicle pretreated with low temperature might influence symmetrically dividing microspores. Lazar *et al.* (1985) found that variation in intensity and duration of cold pretreatment leads to significant difference in callus formation frequency. In the present investigation, cold pretreatment (6°C) of panicle at different days (1-6 days) was applied prior to culture. The cold pretreatment of 1 to 4 days showed significant improvement of the induction efficiency. Three to four days of stress was found to be most effective range of cold treatment. The benefits of cold pretreatment to donor panicle could be attributed to slow degradation of the tapetum and locular matrix (Powell, 1988). Croughan and Chu (1991) mentioned that cold pretreatment is very effective in rice, low temperature (5°C) at several days on immature inflorescence decreases the induction potential when panicles were pretreated for a long time. In the present investigation, cold pretreatment of panicle for a longer time found to reduce the embryo induction. The present findings agreed well with the above mentioned results. In barley anther culture, cold pretreatment (4°C) for 4 weeks was very effective on induction (Huang and Sunderland, 1982). However, long pretreatment may not be effective on rice, it is a little discrepancy from the findings of Huang and Sunderland, 1982. It might be attributed to the genotypic differences.

6.5 Transformation of BRRI dhan-30 Variety

It was the first attempt in Bangladesh to transform rice plant with target genes. As a new intervention deserves enormous efforts to develop the optimum conditions in order to accomplish stable transformation. In the beginning a number of experiments were conducted in this study to optimize the effective use of the experimental tools involved in gene transformation venture in plants. The ultimate objective of this study was to incorporate reporter gene (GUS) in rice plant. BRRI dhan-30 was selected as a target variety for transformation of reporter genes. It was revealed from the results of tissue culture experiments that BRRI dhan-30, showing best callus induction and regeneration performance, proved to be the most suitable variety for gene transformation. BRRI dhan-30 is also a cash crop of Bangladesh playing an important role in the crop economy of the country.

The GUS gene was used as convenient tool for necessary optimization of transformation conditions. The GUS coding sequence and reporter gene constructs were being used extensively in gene transfer technology of rice (Peng *et al.*, 1992) and considered as good marker for monitoring transformation efficiency (Jefferson *et al.*, 1987). The advantages of GUS over the reporter sequences include the high *in vitro* stability of the GUS enzyme and the simplicity of the assay for its expression in the transformed material. In the present study transient GUS expression was evaluated in *Agrobacterium* mediated transformation in rice.

Regeneration study demonstrated that BRRI dhan-30 had the highest regeneration potential and most suitable for transformation work. The method of transformation in indica rice BRRI dhan-30 was performed by using *Agrobacterium* transformation system. The result confirmed that calli of rice BRRI dhan-30 can be used as the target tissue for *Agrobacterium*-mediated

transformation. The highly regenerative embryogenic rice calli of BRRI dhan-30 were co-cultivated with *Agrobacterium* harboring with GUS and GFP genes. The calli were inoculated by *Agrobacterium* suspensions of GUS and GFP stains in LB medium. After co-cultivation the callus tissue were subjected to GUS assay in order to confirm the GUS integration into the rice genome. Histochemical assay was performed on the co-cultivated rice calli according to the method described by Jefferson (1987) with some modifications. Following histochemical assay when the infected calli were incubated with the substrate X-glue which was cleaved in GUS expressing cells resulting in deposition of a blue indigo dye a number of blue spots were appeared in the body of the rice calli. GUS expression indicated that the GUS gene harboured in bacterial cells has been transformed and integrated into the genome of rice calli, which confirmed the protocol of gene transformation by *Agrobacterium* co-cultivation.

In finding the most suitable level of OD for the *Agrobacterium* used in this experiment, seven different levels of OD were tested; 2.0, 1.8, 1.5, 1.3, 1.0, 0.7, 0.5 and 0 (control). The highest numbers of GUS expressing calli were observed at OD 0.5-0.7. The bacterial strain LBA4404 (pCL3) showed highest peak of performance in lower range of OD and it gradually decrease with the increase of OD taken. Using *Agrobacterium* at OD of 0.5-0.7 was coincidental with the fact that most *Agrobacterium* stains would be experiencing the early log (exponential) phase during which active cell division occurs at 0.4-0.8 OD (Janssen and Gardner, 1989). Acetosyringone enhances the efficient transformation in monocot by *Agrobacterium*-mediated transformation. The effective concentration of acetosyringone in co-cultivation medium is another important venture to be optimized for stable transformation of target gene in rice. Acetosyringone in five

different concentrations were tested; 100 ppm, 200 ppm, 400 ppm, 600 ppm and 1000 ppm used for the treatment of calli. The effectiveness of the concentration of acetosyringone was observed by the presence of blue color from GUS assays performed on the calli. The results show that as the concentration of acetosyringone increased, the number of blue GUS spots/calli also increased. However, between 400 ppm and 1000 ppm the results (i.e. the number of blue spots) were not significantly different. Therefore, using acetosyringone at 1000 ppm was decided upon for use of the co-cultivation medium. Various studies (Khanna and Raina, 1999; Gould, 1997) examined the importance of acetosyringone for the effective use in *Agrobacterium* co-cultivation procedures.

The GUS (β -Glucuronidase) gene fusion system is now being used extensively as a reporter gene in plant and agricultural molecular biology. The advantage of GUS over other reporter systems include the robustness of the enzyme, the simplicity of the assays and variety of substrates available. Since the development of the GUS system, many thousands of transgenic plants have been generated expressing GUS. GUS fusions are now widely used to study plant-pathogen and plant-symbiont interactions. GUS offers a promising route for transformation, monitoring and molecular genetic analysis of the numerous important epi- and endophytic organisms that are not readily cultured, such as *Oomycetes* and mycorrhizal fungi. In addition GUS fusions can be used in many laboratory model systems lacking endogenous activity, including *Saccharomyces*, *Schizosaccharomyces*, *Dictyostelium*, some strains of *Caenorhabditis elegans* and *Drosophila*.

An improved protocol of *Agrobacterium*-mediated transformation with GUS reporter gene in japonica rice was reported by Toki (1997). His improved

protocol for *Agrobacterium*-mediated transformation in rice was basically same as that of Rashid *et al.*, (1996), but with the following modifications: Casemino acids and proline were included in the medium for callus induction and in the medium for selecting transgenic calli and all cultures were incubated under light at 30°C, except during co-culture period. These modifications enabled him to regenerate transgenic plants in only two months, starting with callus induction from mature seeds. *Agrobacterium*-mediated transformation in indica rice was first reported by Rashid *et al.* (1996). They confirmed the insertion of the reporter gene glucuronidase (GUS) gene and the selectable marker, hygromycin phosphotransferase (hptII) gene into several cultivars of indica rice. Chan *et al.*, (1992), transformed rice callus with *Agrobacterium* but they could not able to regenerate the transgenic plant. But Chan *et al.*, in 1993 obtained first generation of transgenic japonica rice plants by inoculating immature embryos with *Agrobacterium*. In 1998, Cheng *et al.* used *Agrobacterium*, which harbored with the *cryIA (b)* and *cryIA(c)* genes to introduce stem borer resistance into japonica rice. Recently, Datta *et al.* (2000) introduced the chimeric chitinase gene in rice cultivars by using *Agrobacterium*. The transgenic plants exhibited resistance to sheath blight pathogen. Stable integration, inheritance and expression of the chitinase gene were demonstrated by Southern blot and Western blot analysis of transformants.

Sheath blight disease is now the burning issue and posing a deadly threat to the rice cultivation in Barind Tact with high yielding rice variety Kollani, most favorite variety in the Barind Tact. A research unit specially formulated for Barind Tact working under BARI has been making their best effort to control this sheath blights disease of the rice Kollani but they could not able to find an effective

measure to combat this disease. Our research finding establishing the protocol of gene transfer technology by *Agrobacterium*-mediated transformation in Bangladeshi indica rice variety has opened the new door for searching the possible remedy of this devastating sheath blight disease of rice like the work of Datta *et al.*, (2000).

Under the present study, these are the first successful experiments of their kind in the country both in the field of tissue culture and transformation of indica rice. Tissue culture protocols were developed working on large numbers of rice varieties using different tissue explants. Conditions for the indirect gene transfer technology by the *Agrobacterium*-mediated gene transfer method was established for rice improvement. Establishment of efficient transformation system was developed in this study has laid a foundation work for the future advancement of gene transfer technology in the country. It opens new horizons for incorporation of target genes for beneficial agronomic characters in rice as well as other commercial agricultural crops.

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CHAPTER-VII

7. References

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