J. Patuakhali Sci. and Tech. Uni. 2018, 9 (1&2): 89-96 ISSN 1996-4501

DEVELOPMENT OF *IN VITRO* REGENERATION SYSTEM OF SOME BRRI RELEASED RICE VARIETIES

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Abstract

The study was carried out to investigate the callus induction and plant regeneration ability of ten BRRI released rice varieties under in vitro condition and to evaluate the effect of different gelling agents added to the media. The Murashige and Skoog (MS), Chu's (N6), and Nitsch and Nitsch media containing 2mgL^{-1} 2,4-D were used for callus induction. While for plant regeneration, the Murashige and Skoog (MS), Chu's (N6), and Nitsch and Nitsch media supplemented with 100mg L-1NAA, 100 mgL^{-1} Kinetin, and 100mg L^{-1} BAP were used. Depending on the genotypes, BRRI dhan48 (81.67%) performed better followed by BR5 (70.04%) when cultured on MS medium with agar for callus induction. The callus induction was found lowest in BRRI dhan47 (12.04%) followed by BRRI dhan50 and BRRI dhan39, respectively, when cultured in N6 medium with agar. On average, BRRI dhan48 (73.33%) showed average highest plant regeneration frequency followed by BR5. Maximum mean of plant regeneration frequency was found on MS medium, however, the lowest plant regeneration was found in BRRI dhan39 (13.33%) followed by that in BRRI dhan50 (), BRRI dhan49 (), respectively, when cultured onN6 medium with agar. The result revealed large genotypic variations in callus induction and plant regeneration. Thus, it can be concluded that this *in vitro* protocol could be as a model for efficient plant regeneration of rice and future gene transformation study.

Key Words: Rice (Oryza sativa L.), *In vitro*, Regeneration, Callus, Gelling agents.

Introduction

Since the prehistoric period, rice has been the staple food for the largest number of people on the Earth. Rice is second to wheat and corn with respect to area of cultivation and production, respectively. More than 90% of the world's rice is produced and consumed in Asia, which contains 60% of the world's population (BBS, 2017). Bangladesh ranked fourth in both rice area and production. In Bangladesh, cultivable land is decreasing but population is increasing day by day. Therefore, rice production needs to be increased to feed the upcoming growing population and to increase rice production; one of the options is to increase the area under rice cultivation which is getting harder as more farm areas are being converted to residential and industrial areas. The most viable option, therefore, is to increase productivity by using different biotechnological tools (Bajaj & Mohanty, 2005). During the last decade, the techniques of tissue culture, like anther culture, protoplast fusion, root culture and seed culture are being employed in rice breeding to exploit somaclonal variation for the creation of new varieties (Ram & Singh, 1998).

In vitro techniques constitute an important component of biotechnology and have the potential not only to improve the existing cultivars, but also for the synthesis of novel

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plants and early release of high yielding rice varieties resistant to various biotic and abiotic stresses. Success of cell and tissue culture research depends upon reliable callus culture and plant regeneration procedures. The frequencies of callus induction and plant regeneration in rice tissue culture are influenced by many factors like composition of culture medium, genotype, types of explants and environment (Torbert *et al*., 1998). Among them, the genotype and nutrient composition are regarded to be the major sources of variation (Khanna & Raina, 1998). The genotype is a strong determinant for the in vitro regeneration ability in seed culture and this character is under genetic control (Henry *et al.,* 1994). It has been demonstrated in several cases that when higher plant tissues undergo a process of differentiation and cell proliferation *in vitro*, wide range of mutations occur at a frequency much higher than expected (Brar & Khush, 1994). Soma clones provide a novel and valuable source of genetic variability, which can be exploited for crop improvement particularly, for the development of stress tolerance rice cultivars (Lutts *et al.,* 1999). High frequency of callus induction and subsequent green plant regeneration is a pre-requisite for the utilization of indica rice soma clones in breeding programs. In this context, the evaluation of new factors and their manipulation for efficient callusing and green plant regeneration from mature seeds in indica rice is still challenging (Niroula *et al.*, 2005).

Various types of explants were used for callus induction and subsequent plant regeneration in rice tissue culture, among them mature embryo has a great advantage as it is available throughout the year (Hoque and Mansfield, 2004). Many experiments have been conducted to optimize the techniques as well as composition of culture medium for callus induction in rice seed culture (Islam *et al*., 2004. *In vitro* plant regeneration in rice depends on genotypes (Hoque and Mansfield, 2004) as well as on the component of the culture medium (Khanna & Raina, 1998). Therefore, the identification of useful cultivars for embryogenic callus formation and subsequent plant regeneration, in vitro are the key steps in rice genetic improvement program through application of biotechnology (Hoque & Mansfield, 2004).

As above, a study was undertaken to find out the suitable media and gelling agent for embryogenic callus induction and subsequent green plant regeneration from selected modern rice varieties of Bangladesh. Therefore, objectives of the study were as follows:

1. Investigating the callus induction and plant regeneration ability of ten BRRI released rice varieties under in vitro condition.
2. Evaluating the effect of different gelling agents.

Materials and Methods

The 10 BRRI released rice varieties viz., BR5, BR11, BRRI dhan28, BRRI dhan29, BRRI dhan39, BRRI dhan46, BRRI dhan47, BRRI dhan48, BRRI dhan49, and BRRI dhan50 obtained from Tissue Culture Laboratory of Biotechnology Division, Bangladesh Rice Research Institute (BRRI), Joydebpur, Gazipur were used as experimental materials in the present investigation. Healthy and dehusked embryos were used as explants. Mainly two types of media were used. One was for callus induction and another was for plant regeneration. Three types of callus induction medium were used e.g., (a) MS medium (Murashige and Skoog, 1962) supplemented with 2mgL-1 2,4-D, 30gm L^{-1} sucrose gelled with 8gm L-1 agar or 3gmL-1 phytagel or 3gm L^{-1} gelrite and pH 5.8, b) N6 medium supplemented with

2mg L^{-1} 2,4-D, 40g L^{-1} sucrose gelled with 8g L^{-1} agar or 3g L^{-1} phytagel or 3gL^{-1} gelrite pH 5.8 and (c) Nitsch and Nitsch medium (Nitsch and Nitsch, 1969) supplemented with 2mg L-12,4-D, 40gmL-1 sucrose gelled with 8g L^{-1} agar or 3g L^{-1} phytagel or 3g L^{-1} gelrite pH 5.5. Again, Three types of plant regeneration medium were used e.g., (a) MS medium supplemented with 100mg L^{-1}NAA+100mg L^{-1} Kinetin+100mg L^{-1}BAP, 40g L^{-1} sucrose gelled with 8g L^{-1} agar or 3g L^{-1} phytagel or 3g L^{-1} gelrite pH 5.8, (b) N6 medium supplemented with 100mg L^{-1}NAA+100mg L^{-1} Kinetin+100mg L^{-1}BAP, 40gmL^{-1} sucrose gelled with 8g L^{-1} agar or 3g L^{-1} phytagel or 3g L^{-1} gelrite pH 5.8 and (c) Nitsch and Nitsch medium (Nitsch and Nitsch, 1969) supplemented with 100mg L^{-1} NAA+100mg L^{-1} Kinetin+100mg L^{-1} BAP, 20g L^{-1} sucrose gelled with 8g L^{-1} agar or 3g L^{-1} phytagel or 3g L^{-1} gelrite pH 5.5. All types of media were prepared accordingly with the help of stock solutions which were prepared prior to media according to formulated rules (Islam *et al.*, 2004) and then sterilized. All the utensils, culture room and transfer area were also taken under sterilization according to corresponding sterilization techniques. For the preparation of explants, healthy seeds were separated out and dehusked. Then they were rinsed in sterile water treated with two drops of Tween-20 for 10 minutes followed by washing with sterile water for three times and after that, they were dipped in 70% ethanol for three minutes followed by washing with sterile water for three times. Then they were rinsed with 0.1% Mercuric chloride (HgCl2) for 20 minutes and washed with sterile distilled water for 3-4 times. The seeds were then placed on sterile filter paper to absorb excess water. The entire activities were performed in Laminar Air Flow cabinet. The sterilized seeds were directly cultured on callus induction media (MS, N6, Nitsch, and Nitsch medium) as per treatment. The explants were placed horizontally on callus induction medium in Petri dish. The culture was then inoculated with the treated seeds in the dark at 25±2°C for callus induction. After 2-3 weeks of inoculation, seeds of the responsive varieties started to produce callus. Callus induction frequency was calculated on the basis of the number of seeds producing callus. Subsequent observation was carried out to note the response. Calli with a size of at least 2 mm were transferred into the regeneration medium (MS/N6/Nitsch and Nitsch medium) and were incubated in a temperature controlled growth room at 25±2°C under 12 hours light photoperiod with a light intensity of about 2000-3000 lux provided for plant regeneration. Observation was carried out in the following days to note the response. Regenerated plants were counted on the basis of the number of callus producing plantlets. The experiments were conducted in Completely Randomized Design (CRD). The data were statistically analyzed wherever applicable. The analysis of variance for the test characters was performed and means were compared by the Duncan's Multiple Range Test (DMRT).

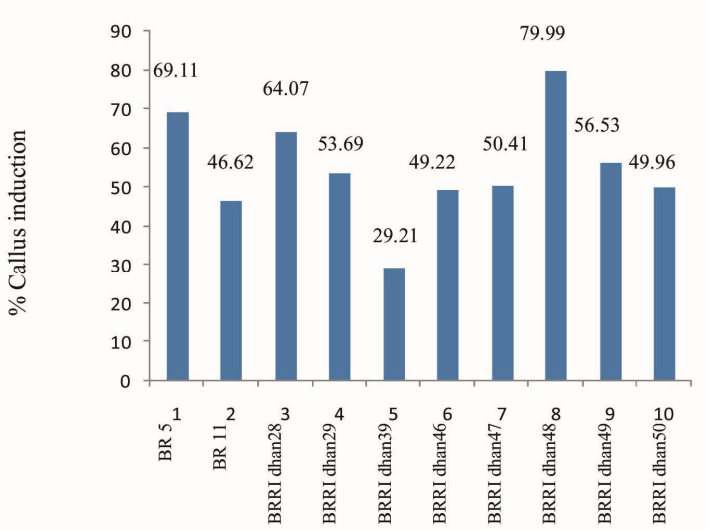
Results and Discussion

Plant biotechnology offers a number of opportunities to scientists solve certain breeding problems through molecular manipulations. In this study, ten varieties were used to evaluate their performance of callus induction and plant regeneration. The response of seed culture widely varied depending upon the test genotypes. Significant variation was observed in all test parameters among the varieties. Callus initiation began 10-15 days after inoculation of seed in the callus induction media. Calli were mostly yellowish white and some were creamy white in color. BRRI dhan48 had largest size of callus but BR5 produced smallest size of callus. The texture of calli were variable. Some calli were either compact or loose and others

were either watery or friable. The average highest percentage of callus induction was found in BRRI dhan48 (66.59%) followed by of BR5 (56.53%) and the lowest percentage of callus induction was found in BRRI dhan39 (22.65%) compared to that in other varieties. On the other hand, average callus induction was highest in MS medium (54.88%) followed by that of Nitsch and Nitsch and N6 (Table 5). It is perhaps due to the genotypic effect on different culture media. These results are similar to the findings of Khatun *et al.* (2003) and many other reports. Panddy *et al*. 1994 and Islam *et al*. 2004 also found similar results. They also reported that the success of in vitro cultures largely depend on nutritional constituents and growth regulators. The difference in the composition of culture medium could result in variation of callus induction (Torbert *et al.,* 1998). Furthermore, genotypic variation also plays its role (Khanna and Raina, 1998; Abeyaratne *et al.,* 2004). The concentrations of hormones also affect the callus induction ability of the genotypes. In the present study, 2,4-D was used at the rate of $2mgL^{-1.}$ Previous studies also showed that callus could be induced better in 2mg L^{-1} concentration of 2,4-D (Mosavi *et al.,* 2001, and Sikder *et al.,* 2006).

After transferring the calli into regeneration media, green spots became visible on the surface of the calli within 5-7 days and after 27-30 days, fully rooted shoots were developed. A number of factors, such as genotype, developmental stage of cells of the explants, hormonal composition in the medium, carbohydrates source, water and salt stress inducing treatments and media modification have been reported to improve the frequency of plant regeneration in rice (Jain, 1997). In present study, plantlet regeneration occurred in all genotypes with most of media tested. Lee *et al.* (2002) reported that MS media is best for plant regeneration. However in present study, Nitsch and Nitsch media showed best result (38.11%) than MS and N6 (24.42%), respectively (Table 6). In this study, plantlet regeneration was higher in BRRI dhan48 when cultured on MS media supplemented with NAA, BAP, and Kinetin. The results were similar to the findings of Rashid *et al.* (2004) who reported higher frequencies of plant regeneration obtained in MS media supplemented with NAA and BAP. Khanna and Raina (1998) found that MS media supplemented with NAA, BAP, and Kinetin gave the best results. From the above discussion, it could be concluded that Cytokinin was more responsive for calli differentiation.

Tan *et al.* (2000) reported that types of gelling agent might enhance the frequency of green plantlet differentiation. In the study, different gelling agents were used for solidifying the media and it was found that agars performed better than gelrite and phytagel (Table 9) in terms of callus induction (44.57%) as well as plant regeneration (34.56%). Lee *et al.* (2002) observed that agar concentration in the regeneration medium was also critical for the shoot induction. Huang *et al.* (1995) reported that tissue cultures on properly solidified Gelrite media generally performed superior for shoot proliferation. But this is contrary to the findings of Meneses *et al.* (2005) who reported that higher frequencies of callus were obtained using the MS medium. Variety and media interaction was found highly significant for both callus induction and plant regeneration. The frequency of callus induction (79.99%) and plant regeneration (64.44%) was highest in BRRI dhan48 with MS medium (Figure 1 and Figure 2).



Figore 1. Callus induction of ten BRRI released rice varieties cultured on MS medium supplemented with 2,4-D.

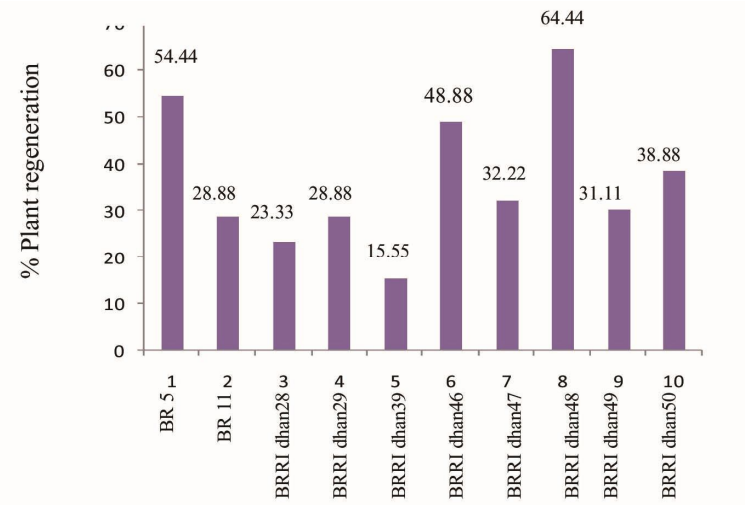


Figure 2. Plant regeneration of ten BRRI released rice varieties cultured on MS medium supplemented with NAA, BAP, and Kinetin.

Variety and gelling agent interactions were also highly significant for callus induction and plant regeneration. The highest percentage of callus induction (69.61%) was recorded in BRRI dhan48 when culture media solidified with gelrite than agar and phytagel (Table 11). On the other hand, lowest percentage of callus induction was recorded in BRRI dhan39 with phytagel (21.58%) than agar and gelrite (Table 11).

The highest percentage of plant regeneration was recorded for BRRI dhan48 in culture media solidified with agar (61.11%) than gelrite and phytagel, respectively, and the lowest percentage of plant regeneration was recorded in BRRI dhan39 with agar (13.33%) than phytagel and gelrite (Table 12).

Variety, media and gelling agent interactions was significant at 5% level for callus induction and plant regeneration. In BRRI dhan48 MS media with agar gave highest result (81.67% callus induction and 73.33% plant regeneration) which was followed by the same variety in MS media with gelrite for callus induction (79.49%) and with Nitsch and Nitsch medium with agar for plant regeneration (66.67%). In BR5, MS media with phytagel also gave better result. Lowest result was found in BRRI dhan47 for callus induction when cultured in N6 medium with agar. The pictorial view of callus induction and plant regeneration has been presented in Figure 3.

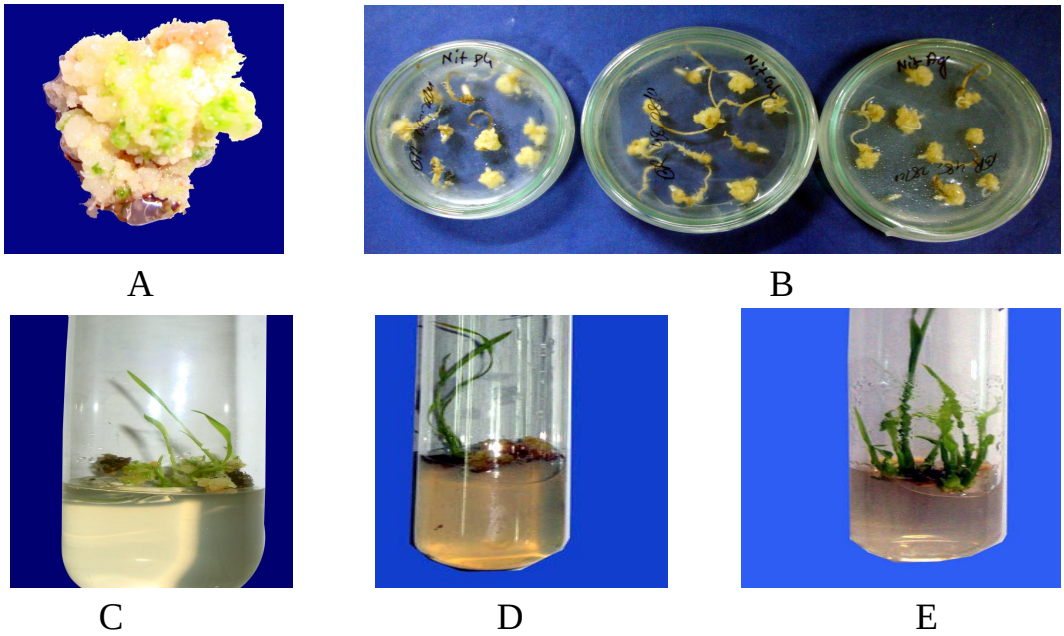


Figure 3: Pictorial view of callus induction and plant regeneration

Legend: A= Callus with green spots for BRRI dhan48 in MS medium with agar, B= Callus induction of BRRI dhan48 on Nitsch and Nitsch medium supplemented with 2,4-D. C= Plant regeneration of rice varieties cultured on MS medium supplemented with NAA, BAP and Kinetin. D, E= Plant regeneration of rice varieties cultured on Nitsch and Nitsch medium supplemented with NAA, BAP and Kinetin.

It may be summarized in a way that among the ten rice varieties tested, BRRI dhan48 was found best for future culture study. On the other hand, BR5 was found moderately good. Among the different media tested, MS media performed better compared to N6, and Nitsch and Nitsch media for callus induction. However, Nitsch and Nitsch medium showed best performance in plant regeneration. Among the variety and media interaction, BRRI dhan48 and BR5 performed better when cultured on MS medium. On the other hand, when different gelling agents are considered, agar performed better compared to gelrite and phytagel in both callus induction and plant regeneration. Among the varieties and gelling agent interaction, BRRI dhan48 and BR5 gave better performance when media solidified with gelrite and agar. In case of media and gelling agent interaction, MS with phytagel gave best result for callus induction while MS with agar showed best for plant regeneration. Finally, in case of varieties, media, and gelling agent interactions, BRRI dhan48 and BR5 performed better for callus induction and plant regeneration in MS medium with agar.

In this study, large genotypic variations in callus induction and plant regeneration were observed. It is also apparent from these studies that genotypic barriers could be overcome in many cases by manipulation of the culture media used for callus induction and plant regeneration. The identification and screening of useful genotypes, media, and gelling agents are the prerequisites for the application of tissue culture techniques to new breeding programs for rice genetic improvements. It can be concluded that the in vitro protocol described here could be used for efficient plant regeneration of rice cultivars followed by gene transformation.

Acknowledgement

It is acknowledged that the experiment was conducted during the period from August to December 2009 at the Tissue Culture Laboratory of Biotechnology Division, Bangladesh Rice Research Institute (BRRI), Joydebpur, Gazipur.

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