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Mature Embryo-Based *in vitro* Regeneration of Indica Rice Cultivars for High Frequency Plantlets Production

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ABSTRACT

The study was conducted at Biotechnology Division of Bangladesh Rice Research Institute (BRRI) to investigate the effects of plant growing medium and plant growth regulator (PGR) for the callus induction and high frequency plantlets regeneration of indica rice. Ten indica rice varieties viz. BR5, BR11, BRRI dhan28, BRRI dhan29, BRRI dhan33, BRRI dhan41, BRRI dhan47, BRRI dhan48, BRRI dhan49 and BRRI dhan50 were cultured on MS, N6 and LS media. The MS medium was found better for callus induction as compared to N6 and LS media. Among the tested varieties BRRI dhan48 induced the highest percent and best quality callus. Interaction effects of BRRI dhan48 to MS medium yielded 71.55% callus induction. The regeneration efficiency of BRRI dhan48 was tested on MS medium supplemented with different combination of NAA plus BAP and NAA plus kinetin. MS medium supplemented with 2.0 mg L^{-1} NAA and 2.0 mg L^{-1} Kn was found the best in respect of percent regenerated (76.67%) plantlet as well as for the growth of plantlets *in vitro*.

Key words: Indica rice (*Oryza sativa* L.), growing media, growth regulators, callus induction, regeneration

INTRODUCTION

Rice is the staple food of around 30-40% of the world population. Over 90% of rice is cultivated in Asia. Due to rapid climatic changes rice cultivation facing a threat of biotic (diseases and insects) and abiotic (saline, drought, submerse, cold, heat) stresses. Different research approaches like conventional breeding, somaclonal variation, marker assisted selection were carried out during last decades to develop tolerant rice varieties against above mentioned stresses. Though some remarkable progresses are achieved, that is not up to the mark. Genetic transformation is the important biotechnological tool for developing stresses tolerant rice varieties. However, the success of genetic transformation depends on several factors like genotypes, media, light, hormonal effect etc. Genetic transformation of rice with *Agrobacterium* requires suitable regeneration system from a transformed callus and ironically, shoot regeneration represents a major bottleneck in this endeavour. Most of the indica rice genotypes, the world's most cultivated rice types, still remain less amenable to genetic transformation due to their poor regeneration potential (Sahoo *et al.*, 2011) Although reporting is abundant on callus induction, regeneration and also transformation in japonica rice, it is limited in indica rice. In this context, the evaluation of new factors and their manipulation for efficient callusing and green plant regeneration from mature embryo in indica rice is still challenging (Niroula *et al.*, 2005). That is why, a detail study has long been demanding in callus induction and plantlets regeneration in indica rice. Therefore the present study was undertaken to select the best media for selected genotype.

MATERIALS AND METHODS

Plant materials, medium, layout and design

The experiment was conducted at Biotechnology

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Division of BRRI, Gazipur, Bangladesh. Ten BRRI released indica rice varieties viz BR5, BR11, BRRI dhan28, BRRI dhan29, BRRI dhan33, BRRI dhan41, BRRI dhan47, BRRI dhan48, BRRI dhan49 and BRRI dhan50 were used. Three culture media viz MS (Murashige and Skoog, 1962), N6 (Chu *et al.*, 1975) and LS (Linsmaier and Skoog, 1965) were evaluated to select the best medium for callus induction and regeneration. The experiment was conducted in 18 mm × 1.2 mm test tubes and laid out following a completely randomized design (CRD) with five replications.

Callus induction

To induce callus, dehusked mature embryo were cultured on MS, N6 and LS media and incubated in growth room at 25±2°C under 12 hr. photoperiod of 50 µmol $m^{-2}s^{-1}$ provided by florescent tubes. The basal media were supplemented with 30.0 g L^{-1} sucrose, 0.3 g L^{-1} casamino acid and 2.0 mg L^{-1} 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and solidified with 7.0 g L^{-1} agar. The pH of the media was adjusted to 5.8 prior to autoclave at 121°C with 1.16 kg cm^{-2} pressure for 20 minutes.

Plant regeneration

Three weeks old embryogenic calli of BRRI dhan48 and MS medium supplemented with different combinations of Naphthaleneacetic acid (NAA) + 6-Benzylaminopurine (BAP) and NAA + Kinetin (Kn) were used for plant regeneration. The pH of the regeneration media were adjusted to 5.8.

Data collection

The data on callus induction and plantlet regeneration were collected on 21 and 50 days after culture. Callus induction, regeneration, number of plantlets $callus^{-1}$, average plantlet height, number of root $plantlet^{-1}$, average root length etc were also collected. Data were analyzed to compare mean values to express treatments effects. The means were calculated and analysis of variance of all the characters was performed by F-test. Significance of the difference between the pair of means was evaluated by Duncan's Multiple Range Test (Gomez and Gomez, 1984) at the 5% level of significance using MSTAT-C computer programmed (Russel, 1986).

RESULTS

Callus induction

Callus initiation in varieties. The highest percentage of callus induction was 59.60% found in BRRI dhan48 followed by 53.27% in BRRI dhan28 and the lowest was 26.43% in BRRI dhan49 (Table 1). Thus BRRI dhan48 showed comparatively better potential in callus induction compared to other varieties. At the beginning scutelum callus was compact almost in all the varieties but turned into friable after two weeks of culture. Calli of BRRI dhan48 were bright yellowish, glassy and looks most healthy compared to other varieties. Colour of

Table 1. Mean callus induction percent of rice varieties tested

Name of the varieties	Callus induction (%)
BR5	42.18 c
BR11	37.82 cd
BRRI dhan28	53.27 b
BRRI dhan29	28.22 gh
BRRI dhan33	41.07 cd
BRRI dhan41	36.67 de
BRRI dhan47	31.48 fg
BRRI dhan48	59.60 a
BRRI dhan49	26.43 h
BRRI dhan50	33.11 ef

Same letter in the column did not differ significantly at the 5% level of probability.

Table 2. Effect of media on mean callus induction percent of rice varieties tested

Media	Callus induction (%)
MS	47.00 a
N6	36.08 b
LS	33.88 b

Same letter in the column did not differ significantly at the 5% level of probability.

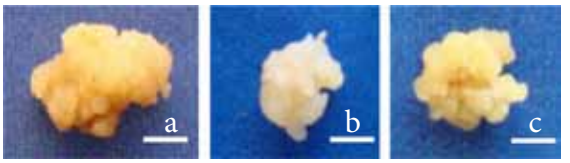

Fig. 1. Callus of a) BRRI dhan48 (yellowish), b) BRRI dhan28 (whitish) and c) BRRI dhan33 (light yellowish). Scale bar = 0.5 cm.

the callus was whitish in BRRI dhan28 and light yellowish in BRRI dhan33 (Fig. 1a, 1b and 1c).

Effect of medium on callusing. Significant variation was observed in callus induction due to the effect of different culture media. The mean callus induction ranged from 33.88% to 47.00% (Table 2). The highest and the lowest callus induction were 47.00% and 33.88% found in MS medium and LS medium respectively.

Interaction effects of variety to medium on callus induction. The interaction effect of variety to medium on callus induction was varied significantly. The highest callus induction 71.55% was recorded in BRRI dhan48 in MS medium followed by 63.47% in BRRI dhan28 in LS medium (Table 3). The lowest callus induction was 19.96% observed in BRRI dhan47 in N6 medium (Table 3).

Plantlet regeneration

Effects of NAA and BAP on plantlet regeneration. The plantlet regeneration from callus was found 2-3 weeks after transferred in the regeneration medium. All parameters showed significant variations depending on NAA and BAP supplement into MS medium. The highest percent (70.00%) regenerated plantlet was observed in medium supplemented with 2.0 mg L^{-1} NAA and 1.0 mg L^{-1} BAP followed by 63.33% at NAA 2.0 mg L^{-1} and BAP 2.0 mg L^{-1}. The lowest percent (16.67%) regenerated plantlet was in medium supplemented with 0.1 mg L^{-1} NAA and 0.5 mg L^{-1} BAP (Table 4). The highest number of plantlets was 12.3 per callus in medium supplemented with 2.0 mg L^{-1} NAA and 1.0 mg L^{-1} BAP followed by 9.17 plantlets at NAA 2.0 mg L^{-1} and BAP 2.0 mg L^{-1} (Table 4). The highest average plant height was 10.18 cm in medium supplemented with 2.0 mg L^{-1} of NAA and 1.0 mg L^{-1} BAP followed by 6.00 cm at NAA 1.0 mg L^{-1} and BAP 1.0 mg L^{-1} (Table 4). The highest average root length 7.33 mm was found in medium supplemented with 2.0 mg L^{-1} NAA and 1.0 mg L^{-1} BAP followed by 4.50 mm at NAA 2.0 mg L^{-1} and BAP 2.0 mg L^{-1} (Table 4). The maximum number of root plantlet^{-1} was

Table 3. Interaction effects of variety to medium on callus induction in rice.

Variety ×Medium	Callus induction (%)
BR5 × MS	53.44 d
BR11× MS	47.38 e
BRRI dhan28 × MS	48.70 e
BRRI dhan29 × MS	30.95 jk
BRRI dhan33 × MS	49.26 de
BRRI dhan41 × MS	47.52 e
BRRI dhan47 × MS	38.21 fgh
BRRI dhan48 × MS	71.55 a
BRRI dhan49 × MS	31.20 jk
BRRI dhan50 × MS	51.73 de
BR5 × N6	31.43 jk
BR11 × N6	34.08 hij
BRRI dhan28 × N6	47.64 e
BRRI dhan29 × N6	29.58 jkl
BRRI dhan33 × MS	49.52 de
BRRI dhan41 × N6	39.60 fg
BRRI dhan47 × N6	19.96 o
BRRI dhan48 × N6	58.68 c
BRRI dhan49 × N6	22.66 no
BRRI dhan50 × N6	27.59 klm
BR5 × LS	41.67 f
BR11 × LS	32.00 ijk
BRRI dhan28 × LS	63.47 b
BRRI dhan29 × LS	24.11 mno
BRRI dhan33 × LS	24.42 mno
BRRI dhan41 × LS	22.89 no
BRRI dhan47 × LS	36.25 ghi
BRRI dhan48 × LS	48.57 e
BRRI dhan49 × LS	25.42 lmn
BRRI dhan50 × LS	20.00 o

Same letter in the column did not differ significantly at the 5% level of probability.

Table 4. Effects of NAA combined to BAP on callus-derived plantlet regeneration.

MS medium with		Percent regenerated plantlet	Number of plantlets callus^{-1}	Average plantlet height (cm)	Average root length (mm)	Number of root plantlet^{-1}
NAA	BAP					
mg L^{-1}	mg L^{-1}					
0.1	0.5	16.67 l	3.17 i	3.00 ef	2.83 cdef	2.92 fgh
0.1	1.0	20.00 kl	4.50 ghi	2.83 efg	2.67 def	3.71 cdef
0.1	2.0	26.67 ijk	7.02 cde	3.67 cde	3.83 bc	4.06 cde
0.1	4.0	23.33 jkl	4.50 ghi	2.83 efg	3.83 bc	3.63 cdef
0.25	0.5	26.67 ijk	4.17 hi	3.33 def	3.83 bc	3.26 efg
0.25	1.0	30.00 hij	6.83 def	5.50 b	3.33 cde	3.99 cde
0.25	2.0	33.33 ghi	7.83 bcd	4.00 cd	3.50 bcd	3.97 cde
0.25	4.0	23.33 jkl	6.83 def	3.40 def	3.50 bcd	4.87 b
0.5	0.5	33.33 ghi	3.50 i	3.50 def	2.83 cdef	3.41 defg
0.5	1.0	40.00 fg	8.17 bcd	4.17 cd	3.17 cdef	4.34 bc
0.5	2.0	36.67 gh	8.50 bc	4.33 c	2.67 def	2.95 fgh
0.5	4.0	30.00 hij	4.67 ghi	2.17 g	2.33 ef	3.00 fgh
1.0	0.5	46.67 ef	8.83 b	5.50 b	2.83 cdef	4.38 bc
1.0	1.0	56.67 bcd	8.50 bc	6.00 ab	2.50 def	4.05 cde
1.0	2.0	60.00 bc	8.50 bc	5.50 b	2.50 def	4.18 bcd
1.0	4.0	50.00 de	5.50 fgh	3.50 def	2.17 f	2.79 gh
2.0	0.5	56.67 bcd	5.83 efg	2.77 fg	2.16 f	2.25 h
2.0	1.0	70.00 a	12.33 a	10.18 a	7.33 a	5.90 a
2.0	2.0	63.33 ab	9.17 b	3.33 def	4.50 b	4.00 cde
2.0	4.0	53.33 cde	7.17 cde	3.50 cdef	2.17 f	3.45 defg

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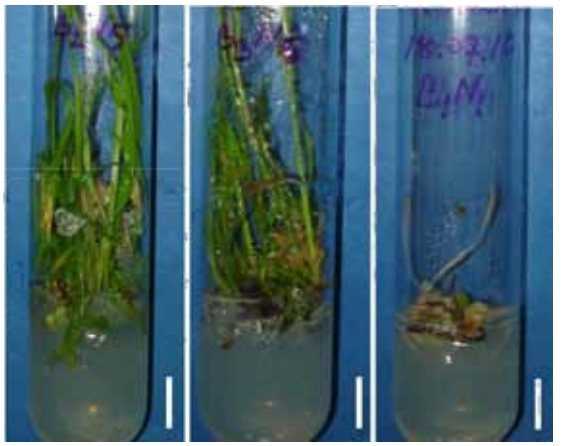
5.90, found in medium supplemented with 2.0 mg L^{-1} NAA and 1.0 mg L^{-1} BAP followed by 4.87 roots at NAA 0.25 mg L^{-1} and BAP 4.0 mg L^{-1}. The lowest number of root plantlet^{-1} was 2.25 found in medium supplemented with 2.0 mg L^{-1} NAA and 0.5 mg L^{-1} BAP (Table 4). The results revealed that data on percent regenerated plantlet, number of plantlets callus^{-1}, average plantlet height, average root length and number of root plantlet^{-1} were best in MS medium supplemented with 2.0 mg L^{-1} NAA and 1.0 mg L^{-1} BAP. Figures 2a, 2b and 2c show the regenerated plantlet in MS medium with different concentration of NAA and BAP.

Effects of NAA and Kinetin on plantlet regeneration. All parameters (Table 5) showed significant variations due to application of different levels of NAA and Kn in MS medium. The highest percent (76.67%) regenerated plantlet was found in medium supplemented with 2.0 mg L^{-1} NAA and 2.0 mg L^{-1} Kn followed by 66.67% at 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} Kn. The lowest plantlet (26.67%) regenerated in medium supplemented with 0.1 mg L^{-1} NAA and 0.5 mg L^{-1} Kn (Table 5). The highest number of plantlets (12.17) was obtained in medium supplemented with 2.0 mg L^{-1} NAA and 2.0 mg L^{-1} Kn followed by plantlets (10.0) at 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} Kn. The medium supplemented with 0.25 mg L^{-1} NAA and 0.5 mg L^{-1} Kn produced the lowest number of plantlets (2.0) (Table 5). The highest average plant height was 9.17 cm in medium supplemented with 2.0 mg L^{-1} NAA



a) 2.0 mg L^{-1} NAA + 1.0 mg L^{-1} BAP b) 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} BAP **c**) 0.1 mg L^{-1} NAA + 0.5 mg L^{-1} BAP

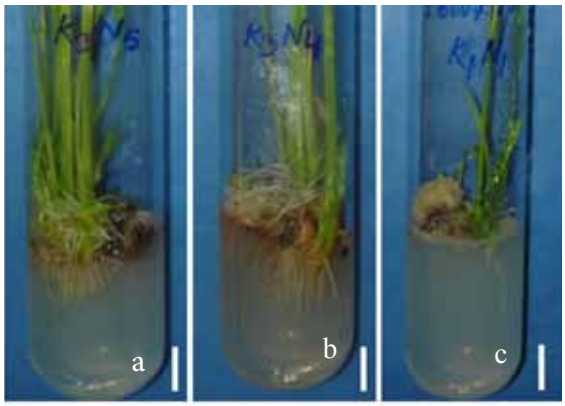
Fig. 2. Regenerated plantlet in MS medium with different concentration of NAA and BAP. Scale bar = 1.0 cm.

and 2.0 mg L^{-1} Kn followed by 7.67 cm at 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} Kn (Table 5). The highest average root length was 9.17 mm in medium supplemented with 2.0 mg L^{-1} NAA and 2.0 mg L^{-1} Kn followed by 7.17 mm at 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} Kn and the lowest was 2.17 mm in medium supplemented with 1.0 mg L^{-1} NAA and 0.5 mg L^{-1} Kn (Table 5). The maximum number (10.69) of roots were developed in medium supplemented with 2.0 mg L^{-1} NAA and 2.0 mg L^{-1} Kn and the lowest was 1.85 in medium supplemented with 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} Kn (Table 5). The results revealed that data on percent regenerated plantlet, number of plantlets $callus^{-1}$, average plantlet height, average root length and number of root $plantlet^{-1}$ were the best in MS medium supplemented with 2.0

Table 5. Effects of NAA combined to Kn on callus-derived plantlet regeneration.

MS medium with		Percent regenerated plantlet	Number of plantlets $callus^{-1}$	Average plantlet height (cm)	Average root length (mm)	Number of root $plantlet^{-1}$
NAA mg L^{-1}	Kn mg L^{-1}					
0.1	0.5	26.67 i	5.00 fg	4.50 efg	3.17 fgh	4.33 efgh
0.1	1.0	36.67 gh	6.50 de	6.17 cd	5.50 cd	5.27 de
0.1	2.0	66.67b	7.67 c	7.17 bc	6.00 c	5.79 d
0.1	4.0	56.67 cd	5.50 ef	5.50 de	4.50 de	5.38 de
0.25	0.5	43.33 fg	2.00 h	4.00 fgh	4.00 efg	7.11 bc
0.25	1.0	56.67 cd	4.00 g	3.17 hi	4.17 ef	4.09 fgh
0.25	2.0	63.33 bc	6.17 de	5.17 de	4.50 de	3.33 hij
0.25	4.0	46.67 ef	2.83 h	3.17 hi	4.33 e	4.08 ghi
0.5	0.5	33.33 hi	6.50 de	3.83 fgh	3.00 gh	1.85 k
0.5	1.0	36.67 gh	6.50 de	2.67 i	3.17 fgh	2.65 jk
0.5	2.0	40.00 fgh	4.500 fg	3.17 hi	3.17 fgh	3.16 ij
0.5	4.0	33.33 hi	7.33 cd	7.17 bc	3.17 fgh	2.49 jk
1.0	0.5	33.33 hi	6.37 de	6.83 bc	2.17 h	7.54 b
1.0	1.0	46.67 ef	7.33 cd	7.00 bc	2.33 h	7.35 bc
1.0	2.0	66.67 b	10.00 b	7.67 b	7.17 b	5.84 d
1.0	4.0	56.67 cd	4.30 g	3.50 ghi	4.50 de	5.40 de
2.0	0.5	43.33 fg	7.33 cd	6.17 cd	4.33 e	5.24 def
2.0	1.0	56.67 cd	6.50 de	6.67 bc	5.50 cd	6.24 cd
2.0	2.0	76.67 a	12.17 a	9.17 a	9.17 a	10.69 a
2.0	4.0	53.33 de	5.50 ef	4.83 ef	5.50 cd	5.13 defg

Same letter in a column did not differ significantly at the 5% level of probability.



a) 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} Kn b) 1.0 mg L^{-1} NAA + 2.0 mg L^{-1} Kn c) 0.1 mg L^{-1} NAA + 0.5 mg L^{-1} Kn

Fig. 3. Regenerated plantlet in MS medium with different concentrations of NAA and Kn. Scale bar = 1.0 cm.

mg L^{-1} NAA and 2.0 mg L^{-1} Kn. Figures 3a, 3b and 3c show the regenerated plantlet in MS medium with different concentration of NAA and Kn.

DISCUSSION

Callus induction

There are many factors influence callus induction in rice like genotype potentiality, pH of the media, plant growth regulators (PGRs) supplement, solidification of culture medium, light intensity, etc. Ge *et al.,* 2006; Khaleda and Al-Forkan (2006) reported that mature dehusked rice seeds were good for callus induction because of callus initiated from scutellum of mature rice seeds have high embryogenic potentiality. Other researchers (Rashid *et al.,* 2003; Cho *et al.,* 2004; Ge *et al.,* 2006) also reported that embryogenic calli were the excellent material for transformation of rice by using *Agrobacterium.* Khalequzzaman *et al.* (2005) also reported embryogenic calli obtained from mature rice seeds have high regeneration capacity. MS, LS and N6 are the most commonly used basal media for calli induction and regeneration (Pandey *et al.,* 1994). BRRI dhan48 in MS medium gave better callus induction as compared to LS and N6 media respectively. This findings were similar to the report by Khana and Raina (1998) who indicated that genotype was one of the major determinants in embryogenic callus induction. Al-Forkan *et al.* (2005), Gul *et al.* (2000) and Lee *et al.* (2000) also reported that embryogenic callus formation and plantlet regeneration were influenced by culture medium and genotype. The concentrations of plant growth regulator also affect the callusing of the genotypes. In this study, 2,4-D was used at the rate of 2 mg L^{-1}. Previous studies also showed that callus could be induced better in 2 mg L^{-1} 2,4-D (Mosavi *et al.,* 2001; Sikder *et al.,* 2006). These results are confirmatory to the finding of other researchers (Niroula *et al.,* 2005; Islam *et al.,* 2004; Azria and Bhalla, 2000). Thus present investigation revealed that both genotype and media composition and their interaction largely affect on callus induction.

Regeneration

In-vitro plant regeneration was investigated on MS medium supplemented with different combinations of NAA + BAP and NAA + kinetin. A number of factors, such as genotype, developmental stage of cells in the explants, PGR composition in the medium, carbohydrates source, have been reported to improve the frequency of plantlet regeneration in rice. After transferring the calli into regeneration medium, green spots became visible on the surface of the calli within 5-7 days and after 30-35 days fully rooted shoots were developed. MS medium supplemented with 2.0 mg L^{-1} NAA and 2.0 mg L^{-1} Kn was found the best in respect to percent regenerated plantlet (76.67%) as well as for the growth of plantlets *in vitro*. The addition of small amount of Kn has been reported to improve embryogenic calli and shoot formation efficiency (Nhut *et al.,* 2000; Afrasiab and Jafar, 2011) in indica rice. Present finding agree with the result of Lee *et al.* (2002) where it was reported that Kn was found to be more effective for plantlets regeneration compared with

BAP. Combinations of auxin and cytokinin along with the effect of basal salts played an important role for plant regeneration (Prodhan *et al*., 2001; Lee *et al*., 2002).

CONCLUSIONS

In present study MS medium gave better callus induction as compared to LS and N6 media respectively. Percent regenerated plantlet was found highest in BRRI dhan48 when cultured in MS medium supplemented with NAA and Kn compared to MS medium supplemented with NAA and BAP. Thus this finding revealed that Kn was more effective than BAP for regeneration and organogenesis. The present result might be helpful in future research on genetic transformation as well as in selection of stress tolerant cultivar development programme. From the result it might be concluded that MS medium for callus induction and MS medium supplemented with 2.0 mg L^{-1} NAA and 2.0 mg L^{-1} Kn for plantlet regeneration may be recommended for future research work.

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