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Investigating the *In vitro* Regeneration Potentiality of Three High Yielding *Indica* Rice (*Oryza sativa* L.) Varieties

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MRH and NA has contributed equally to this work. They conducted the lab work and prepared the manuscript. Authors NA and LH conceptualized and designed the experiment. Author RCD helped in analyzing the data, interpreting the results and author UM helped in preparing the tables, images and graphs and manuscript editing. Author LH supervised the entire work. All authors read and approved the final manuscript.

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ABSTRACT

Aims: An efficient and reproducible *In vitro* regeneration protocol is vital for varietal improvement research. The current research was conducted to optimize the callus induction, shoot and root regeneration of three *indica* rice varieties.
Place, Duration and Design of Study: The experiment was conducted in the tissue culture and biotechnology laboratory of the department of Genetics and Plant Breeding of Bangladesh

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Agricultural University using completely randomized experimental design.
Methodology: Dehusked mature seeds of three *indica* rice varieties namely, BRRI dhan28, BRRI dhan29 and BINA dhan6 were cultured *In vitro* in MS medium supplemented with different concentrations and combinations of phytohormones.
Results: The callus induction ranged from 14 - 84% which showed a general increasing trend with the increase in the concentration of 2,4-Dichlorophenoxyacetic acid (2,4-D) starting from 1.0 mg L^{-1} till 2.5 mg L^{-1}. A further increase in the concentration of 2,4-D to 2.5 mg L^{-1}, however, decreased the percentage of callus induction in all three varieties. MS medium supplemented with 2.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} 6-Benzylaminopurine (BAP) was better than any other composition for callus induction. For size of callus and nature of callus, however, MS medium supplemented with 2.0 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP was found to perfume best. The highest percentage of callus induction was observed in the variety BRRI dhan29 (84%) followed by BRRI dhan28 (74%). Almost all the varieties produced yellowish and compact calli except BINA dhan6 which produced creamy and friable calli. The desiccation treatment has shown to increase size but decrease the compactness of the callus. The differences are, however, not statistically significant. MS medium supplemented with 0.6 mg L^{-1} 1-Naphthaleneacetic acid (NAA) and 6 mg L^{-1} Kinetin (Kn) showed highest shoot regeneration in BRRI dhan29 (85%) followed by BRRI dhan28 (60%). Higher frequency of root formation was observed in all three varieties using Indole-3-butyric acid (IBA). The survival rate of the plantlet in acclimatization chamber (96%) and in field condition (93.33%) was higher for BRRI dhan29. BINA dhan6 has shown the least regeneration potentiality for all the aforementioned traits.
Conclusion: Of the three varieties, BRRI dhan29 and BRRI dhan28 has shown higher regeneration potentiality. This optimized protocol will thus be useful in genetic improvement of these varieties using biotechnological manipulations.

Keywords: Embryogenic callus; Oryza sativa L.; In vitro regeneration; culture medium; phytohormone; explants and indica varieties.

1. INTRODUCTION

Rice, one of the world's oldest domesticated grain crop, is grown in 9% of the world's arable land which accounts for third highest worldwide production of food, after maize and wheat [1] With the dwindling land and water resources and increasing global human population, it is predicted that rice consumption will be increased by 40% by the year 2025, indicating the increased production of rice as a crucial aspect to achieve long-term global food security [2-3]. In Bangladesh, a country with more than 170 million people, rice is the synonym for food providing 27% of per capita energy and 20% of per capital protein. To ensure the long term food security in this overpopulated deltaic country, which is under serious threat of climate change related adversities for crop production, the constant effort to increase rice production is of paramount importance [4-5].

The increase in rice production depends upon the improvement of rice varieties, which have higher yield potentials, excellent grain quality, increased protein content and wider resistance to biotic and abiotic stresses. But these important steps of genetic improvement require modern biotechnological interventions besides classical breeding [6-7]. And tissue culture plays a pivotal role as a biotechnological tool especially in the genetic manipulation of cereals as the production of callus and its subsequent regeneration are the prime steps in crop plants to be manipulated by biotechnological means [8]. In Bangladesh, any new variety especially that of resistant to biotic and abiotic stresses that are going to be released has to be improved to a such extent that it can beat the existing high yielding varieties in terms of yield and quality to get farmers acceptance. That's why many recent new varieties are aiming to introgress novel resistant genes in the background of currently popular and widely cultivated rice varieties. An optimized regeneration protocol for the popular high yielding varieties will be of great use for such genetic improvement programs. The present study was thus designed and materialized with the objectives to observe the regeneration potentiality of three popular high yielding *indica* rice genotypes namely, BRRI dhan28, BRRI dhan29 and BINA dhan6 in a way to optimize the plant regeneration protocol for these widely cultivated varieties using the mature embryos. This may serve as the basis for any future genetic improvement programs for rice in Bangladesh or elsewhere.

2. MATERIALS AND METHODS

2.1 Plant Materials

Mature dehusked seeds of three *indica* rice varieties namely, BRRI dhan29, BRRI dhan28 and BINA dhan6 were used as explants for callus induction and subsequently plants regeneration.

2.2 Sterilization of Seeds and Establishment of Aseptic Culture

The thoroughly pre-washed dehusked seeds were disinfected using 70% ethanol for three minutes and 0.1% $HgCl_2$ and one drop of Tween-20 for 15 minutes followed by sufficient rinsing with double distilled water to remove any traces of sterilant.

2.3 Induction and Maintenance of Callus

MS medium [9] supplemented with five different concentrations of 2,4-D (1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1}) was used for callus induction and subsequent sub-culturing. The cultured explants were incubated under fluorescent light, controlled temperature (22±2℃), photoperiod (16 hrs) and relative humidity (75%).

2.4 Partial Desiccation Treatment

Desiccation treatment was imposed by incubating the twelve days old calli to sterile petridishes containing sterile Whatman no.1 filter paper at 25±1℃ in dark for 48 hrs.

2.5 Regeneration of Shoot and Root

Three weeks after inoculation of explants, the calli were transferred to MS medium supplemented with 2, 4, 6 and 8 mg L^{-1} Kinetin (Kn) with a constant concentration of 0.6 mg L^{-1} Naphthalene acetic acid (NAA) for shoot regeneration. Once regenerated more light intensity was used for shoot elongation. Repeated subcultures were done at an interval of 15 days. The regenerated shoots (2-3 cm in length) were then aseptically transferred to MS medium supplemented with 0.4, 0.5 and 0.6 mg L^{-1} IBA for root induction. The vials were checked daily and the contaminated ones were discarded immediately.

2.6 Acclimatization and Field Establishment of Regenerated Plantlets

The regenerants (5-8 cm in length with sufficient root system) were transplanted to pots containing garden soil (25%), sand (50%) and cowdung (25%) in the acclimatization chamber. The plants along with the pots were covered with moist polythene bag which sprayed with distilled water at every 24 hrs to maintain higher humidity around the plantlets. The plantlets were also nourished with Hoagland's solution before transferring it to the field after 12-15 days of hardening.

2.7 Recording and Analysis of Data

The experiments were conducted with Completely Randomized Design (CRD) and data were recorded for different callus, shoot and root parameters. The analyses of variances for different parameters were performed and means were compared by the Duncan's Multiple Range Test (DMRT). The different stages of *In vitro* development of shooting and rooting regenerants and *In vivo* establishment of the plantlets were photographed.

3. RESULTS

Mature seeds of three *indica* rice varieties namely, BRRI dhan28, BRRI dhan29 and BINA dhan6 were tested for their callus induction, shoot and root regeneration capabilities under different concentrations and combinations of phytohormones in a way to optimize the protocol of *In vitro* plant regeneration. Investigations on *In vitro* regeneration of *indica* rice genotypes were accomplished by callus induction, partial desiccation (maintenance of calli), organogenesis via calli and finally plantlet regeneration and subsequent establishment in field conditions.

3.1 Variability between Genotypes, Treatments and their Interactions

Significant differential response was observed between the varieties, hormonal combinations and most of the possible interactions between these sources of variations for the *In vitro* regeneration parameters such as percent callus induction, size of callus, number of callus producing shoot etc. as revealed by Analysis of variance (ANOVA). The detailed results are shown in Table 1.

3.2 Effect of Variety, Desiccation and Phytohormones on Callus Parameters

The callus induction potentiality was measured as percent callus induction and days required for callus induction in MS medium supplemented with five different concentrations of 2,4-D *viz*., 1.0, 1.5,

2.0, 2.5 and 3 mg L^{-} with a fixed concentration of BAP (0.5 mg L^{-1}). The percent callus induction, with few exceptions, increased with the increase in the concentrations of 2,4-D in all three genotypes starting from 1.0 mg L^{-1} till 2.5 mg L^{-1}. The highest callus induction was observed in MS medium supplemented with 2.5 mg L^{-1} 2,4-D for each of the varieties such as BRRI dhan29 (84%), followed by BRRI dhan28 (74%) and BINA dhan6 (Fig. 1). Surprisingly, a further increase in the concentration of 2,4-D to 3.0 mg L^{-1} didn't show an increase in the percentage of callus induction, instead had shown a sharp decrease in all three varieties (Fig. 1).

For the trait, days required for callus initiation, all the genotypes showed similar pattern of response to that for percent callus induction. In general, the callus initiation was observed to become quicker with the increase in the concentration of 2, 4-D up to 2.5 mg L^{-1} starting from 1.0 mg L^{-1}. BRRI dhan29 induced callus more quickly (4 days) followed by BRRI dhan28 (6 days) and BINA dhan6 (8 days) in MS medium supplemented with 2.5 mg L^{-1} 2, 4-D. With further increase in the concentration of 2, 4-D to 3.0 mg L^{-1} caused further delay in the days required for callus initiation in all the three varieties (Fig. 1).

Table 1. Mean squares values of genotypes, treatments and their interactions for different *in vitro* regeneration parameters

Sources of variation	Mean of square					
	% Callus induction (a)	Size of callus (b)	No. of callus producing shoot (c)	No. of shoot per callus (d)	No. of shoot per plant (e)	No. of root per plant (f)
Variety (V)	112.00**	15.84**	16.19**	60.77**	2.70**	54.73**
Treatment (T)	26.85**	5.78**	1.72*	1.72**	0.15**	316.09**
V x T	2.07*	0.63**	0.16**	0.16**	0.37^{NS}	6.20*
Desiccation (D)	-	0.04^{NS}	-	-	-	-
D × V	-	0.23*	-	-	-	-
D × T	-	0.03**	-	-	-	-
D × V × T	-	0.07^{NS}	-	-	-	-
Error (E)	0.83	0.04	0.79	0.28	5.33	2.58

*Degrees of freedom (df) for a = 2(V), 4(T), 8(VxT) & 66(E); for b = 2(V), 4(T), 8(VxT), D(1), DxV(2), DxT(4) & DxVxT(8) & E(120); for for c & d is 2(V), 4(T), 6(VxT) & 36(E) and for for e & f is 2(V), 2(T), 4(VxT) & 18(E). * & ** indicates significant at 5% & 1% level of probability, respectively and NS indicates non significant.*

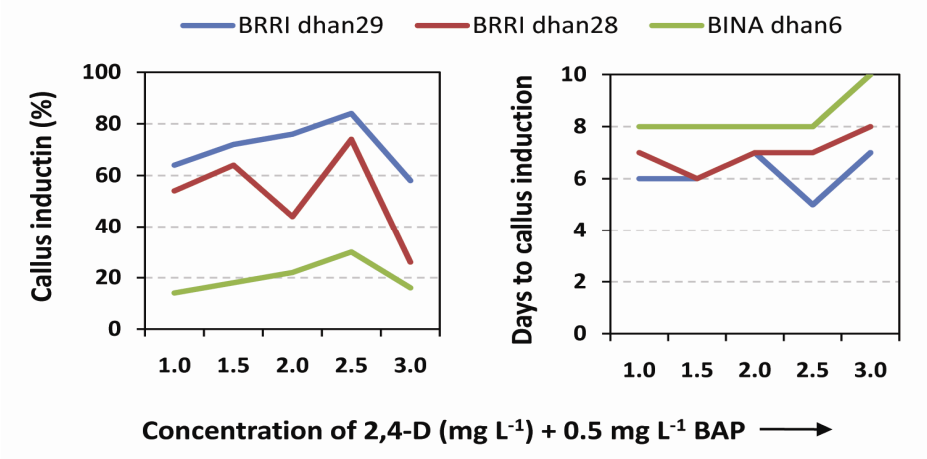

Fig. 1. Figures showing the varietal response in terms of percent callus induction and days required for callus induction in three rice varieties namely, BRRI dhan29, BRRI dhan28 and BINA dhan6 when the mature embryos were cultured on MS medium supplemented with five different concentrations such as 1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1} of 2,4-D and a fixed concentration (0.5 mg L^{-1}) of BAP. Data recorded within 12 days of inoculation and presented as mean of 10 replicates

For the trait, the size of callus also, an increasing trend was observed with the subsequent increase in the concentration of 2, 4-D in the culture media (MS + 0.5 mg L^{-1} BAP) starting from 1.0 mg L^{-1} up to 2.0 mg L^{-1} which decreased with further increase in the concentration of 2,4-D (Table 2). Unlike the previous two traits namely, the percent of callus induction and days required to callus initiation which showed best performance at MS medium supplemented with 2.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP, the size of the callus was found to be the best in MS medium supplemented with 2.0 mg L^{-1} 2,4-D. Moreover, it was also observed that the desiccation treatment has shown to increase the size of the callus compared to that of non-desiccation treatment. For example, the desiccation treatment produced largest calli in BRRI dhan29 (5.8 cm) followed by BRRI dhan28 (5.4 cm) and BINA dhan6 (4.6 cm) at MS medium supplemented with 2.0 mg L^{-1} 2,4-D whereas the size of the non-dessicated calli in respective varieties were slightly smaller (Table 2).

Nature of callus was determined by visually observing the level of compactness of the induced calli. It was observed that the desiccation treatment, even though it produced larger calli, had reduced the compactness of the induced calli compared to that of

Table 2. Effect of desiccation and response of three *indica* rice (*Oryza sativa* L.) varieties on different callus parameters when cultured in MS medium supplemented with five different concentrations of 2, 4-D with constant concentration of BAP (0.5 mg L^{-1})

Desiccation × Variety × Treatment			Size of callus (mm)	Nature of callus	Color of callus
Desiccation status	**Name of variety**	**2, 4-D + BAP (mg L^{-1}) (mg L^{-1})**			
With desiccation	**BRRI dhan29**	1.0 + 0.5 (T_1)	4.00 k	2.44 f-i	Dark yellow
		1.5 + 0.5 (T_2)	4.60 hi	2.48 b-h	Dark yellow
		2.0 + 0.5 (T_3)	5.80 a	2.53 a-d	Dark yellow
		2.5 + 0.5 (T_4)	5.10 de	2.52 a-f	Dark yellow
		3.0 + 0.5 (T_5)	4.80 fgh	2.44 e-i	Dark yellow
	BRRI dhan28	1.0 + 0.5 (T_1)	4.94 efg	2.35 kl	Light yellow
		1.5 + 0.5 (T_2)	5.10 de	2.42 g-k	Light yellow
		2.0 + 0.5 (T_3)	5.40 bc	2.50 b-g	Dark yellow
		2.5 + 0.5 (T_4)	5.20 cde	2.52 a-f	Light yellow
		3.0 + 0.5 (T_5)	4.40 ij	2.47 c-h	Light yellow
	BINA dhan6	1.0 + 0.5 (T_1)	3.48 m	2.32 l	Dark creamy
		1.5 + 0.5 (T_2)	3.70 lm	2.36 kl	Dark creamy
		2.0 + 0.5 (T_3)	4.60 hi	2.45 e-i	Dark creamy
		2.5 + 0.5 (T_4)	3.94 kl	2.41 h-k	Dark creamy
		3.0 + 0.5 (T_5)	3.70 lm	2.37 i-l	Dark creamy
Without desiccation	**BRRI dhan29**	1.0 + 0.5 (T_1)	4.20 jk	2.46 d-h	Light yellow
		1.5 + 0.5 (T_2)	4.80 fgh	2.49 b-h	Dark yellow
		2.0 + 0.5 (T_3)	5.60 ab	2.58 a	Dark yellow
		2.5 + 0.5 (T_4)	5.00 ef	2.56 ab	Dark yellow
		3.0 + 0.5 (T_5)	4.80 fgh	2.52 a-e	Dark yellow
	BRRI dhan28	1.0 + 0.5 (T_1)	4.70 gh	2.36 jkl	Light yellow
		1.5 + 0.5 (T_2)	4.80 fgh	2.44 f-j	Light yellow
		2.0 + 0.5 (T_3)	5.30 cd	2.54 abc	Light yellow
		2.5 + 0.5 (T_4)	5.10 de	2.47 c-h	Light yellow
		3.0 + 0.5 (T_5)	4.20 jk	2.45 e-i	Light yellow
	BINA dhan6	1.0 + 0.5 (T_1)	3.50 m	2.35 kl	creamy
		1.5 + 0.5 (T_2)	3.70 lm	2.37 i-l	Dark creamy
		2.0 + 0.5 (T_3)	4.64 hi	2.46 c-h	Light yellow
		2.5 + 0.5 (T_4)	4.30 j	2.42 g-k	creamy
		3.0 + 0.5 (T_5)	3.62 m	2.37 i-l	Dark creamy
LSD value			**0.25**	**0.06**	**-**

Data recorded at 14-21 days after inoculation and presented as mean of five replicates. Nature of callus was recorded based on a grade of 1-3, with '1' being graded for compact, '2' for friable and '3' for loose callus. Values followed by same letter(s) in a column are not statistically significant ($p<0.05$) as determined by the Duncan's Multiple Range Test (DMRT)

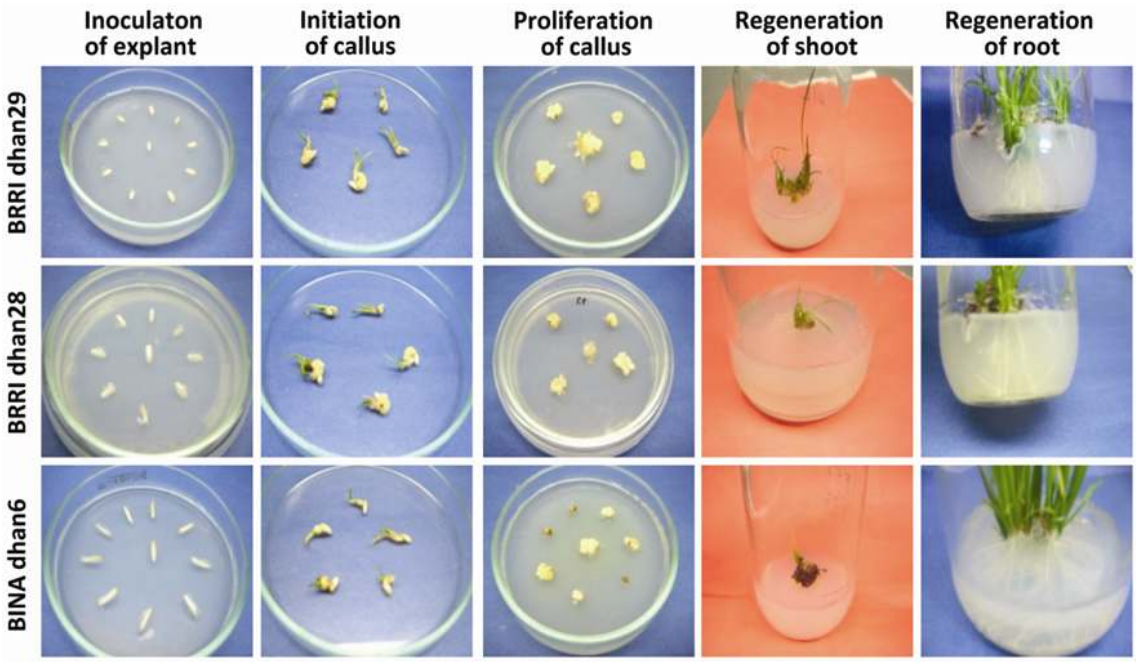

Fig. 2. Figures showing the mature embryos inoculated as explants, initiation of callus within 6-10 days of inoculation and proliferation of callus at 21 days after inoculation on MS medium supplemented with 2,4-D and BAP, regeneration of shoots on MS medium supplemented with NAA and Kinetin and regeneration of roots on MS medium supplemented with IBA of three *indica* rice varities namely, BRRI dhan29, BRRI dhan28 and BINA dhan6

non-desiccated calli (Table 2). For example, the more compactness was observed in the calli of BRRI dhan29 (2.58) in MS medium supplemented with 2.0 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP without any desiccation treatment whereas the desiccation treatment slightly reduced the compactness of the calli. BINA dhan6 had shown more loose calli compared to other varieties for both desiccated and non-desiccated calli (Table 2).

Colour of callus was recorded based on visual inspection as four color grades namely, dark yellow, light yellow, dark creamy and creamy. In general, the calli of the variety BRRI dhan29 was dark yellow colored across all the five media compositions whereas, with few exceptions, the calli of the variety BRRI dhan28 and BINA dhan6 were generally light yellow and dark creamy, respectively (Table 2). The desiccation treatment is found to show very little effect on color of callus.

3.3 Effect of Variety and Treatment in Shoot Regeneration

The shoot regeneration potentiality of the undifferentiated calli of each of the three *indica* rice varieties namely, BRRI dhan29, BRRI dhan28 and BINA dhan6 was determined as percentage of shoot regeneration, days required for shoot regeneration and number of shoots produced per callus in MS medium supplemented with four different concentration of kinetin *viz*., 2, 4, 6 and 8 mg L^{-1} with a fixed concentration (0.6 mg L^{-1}) of NAA.

The percent shoot regeneration was found to be increased with the increase in the concentration of Kinetin from 2 mg L^{-1} till 6 mg L^{-1} which then showed a decreasing with further increase in the concentration of kinetin to 8.0 mg L^{-1} in all three varieties used in the experiment (Table 3). The highest percentage of shoot regeneration was observed in BRRI dhan29 (85%) followed by BRRI dhan28 (60%) and BINA dhan6 (40%) in MS medium supplemented with 0.6 mg L^{-1} NAA + 6.0 mg L^{-1} Kinetin.

Days required for shoot regeneration didn't however showed clear cut increasing or decreasing trend instead has shown differential varietal response and differential effect of media composition. The variety BRRI dhan29, which performed best for the previous traits, required lowest number of days for shoot regeneration at

MS medium supplemented with 0.6 mg L^{-1} NAA and 6.0 mg L^{-1} kinetin (Table 3). Surprisingly, BINA dhan6, which performed poorly for the previous traits, required even fewer number of days (12 days) for shoot regeneration in MS medium supplemented the minimum concentration of kinetin (2 mg L^{-1}). The variety BRRI dhan28 did not show any shoot regeneration before 20 days at any media compositions (Table 3).

MS medium supplemented with 0.6 mg L^{-1} NAA and 6.0 mg L^{-1} kinetin produced maximum number of shoot per callus in all the three varieties. BRRI dhan29 showed highest number of shoot per callus (5.25) followed by BRRI dhan28 (4.25). Shoot regenerated from the callus of BINA dhan6 was very low compared to that of other varieties in all media compositions (Table 3).

3.4 Effect of Variety and Treatment in Root Induction

The root induction potentiality was measured as percent root induction and number of root per plant in MS medium supplemented with three different concentration of IBA *viz.*, 0.4, 0.5 and 0.6 mg L^{-1} in three *indica* rice varieties namely, BRRI dhan29, BRRI dhan28 and BINA dhan6.

MS medium supplemented with 0.5 mg L^{-1} IBA induces highest percentage of root in all the three varieties such as in BRRI dhan29 (90%) and BRRI dhan28 (90%) followed by BINA dhan6 (80%). Regeneration of root seemed not to be a problem as the regeneration of root was, in fact, never below 70% in BRRI dhan29 and BRRI dhan28 and it was around 50% in BINA dhan6 (Table 4).

The number of roots produced per plant ranged from 15-23 in the three *indica* rice varieties studied. Unlike percent root regeneration, the variety BINA dhan6, after BRRI dhan29 (23), has produced higher number of roots per plant (22) in MS medium supplemented with 0.6 mg L^{-1} IBA which was followed by BRRI dhan28 (20). However, the rest genotypes produced higher number of roots per plant in MS medium supplemented with 0.5 mg L^{-1} IBA (Table 4).

3.5 Plant Establishment in Field Condition

Rooted plantlets were transplanted into tiny plastic pots containing soil mixture, covered with polythene bags and placed into acclimatization room for proper hardening which were then transplanted to earthen pots in a way to establish in field condition (Fig. 3). The variety BRRI dhan29 showed the highest percentage of survival in both pot (96%) and soil (93%) and BINA dhan6 showed the lowest percentage of survival in both pot (67%) and soil (62%) as shown in Table 5.

Table 3. Differential response of three *indica* varieties namely, BRRI dhan29, BRRI dhan28 and BINA dhan6 for various shoot regeneration parameters at MS medium supplement with four different concentrations of Kinetin such as 2.0, 4.0, 6.0 and 8.0 mg L^{-1} Kinetin and a fixed concentration (0.6 mg L^{-1}) of NAA

Variety × Treatment		Percent shoot regeneration	Days to shoot regeneration	Number of shoot per callus
Variety	NAA + Kinetin (mg L^{-1}) (mg L^{-1})			
BRRI dhan29	0.6 + 2 (T_1)	35	19	4.25 b
	0.6 + 4 (T_2)	60	18	4.50 ab
	0.6 + 6 (T_3)	85	15	5.25 a
	0.6 + 8 (T_4)	65	16	4.25 b
BRRI dhan28	0.6 + 2 (T_1)	45	22	3.25 c
	0.6 + 4 (T_2)	55	21	3.75 bc
	0.6 + 6 (T_3)	60	22	4.25 b
	0.6 + 8 (T_4)	40	20	4.00 bc
BINA dhan6	0.6 + 2 (T_1)	15	12	0.50 d
	0.6 + 4 (T_2)	25	16	0.75 d
	0.6 + 6 (T_3)	40	18	1.25 d
	0.6 + 8 (T_4)	30	18	1.00 d

Values followed by same letter(s) in a column are not statistically significant ($p<0.05$) as determined by the Duncan's Multiple Range Test (DMRT)

Table 4. Differential response of three *indica* varieties namely, BRRI dhan29, BRRI dhan28 and BINA dhan6 for various root regeneration parameters at MS medium supplement with three different concentrations such as 0.4, 0.5, 0.6 mg L^{-1} of IBA

Variety × Treatment		Percent root induction	Number of root per plant
Variety	**IBA (mg L^{-1})**		
BRRI dhan29	0.4 (T_1)	80	21 bc
	0.5 (T_2)	90	23 a
	0.6 (T_3)	70	19 de
BRRI dhan28	0.4 (T_1)	70	18 ef
	0.5 (T_2)	90	20 cd
	0.6 (T_3)	80	16 gh
BINA dhan6	0.4 (T_1)	50	15 h
	0.5 (T_2)	80	17 fg
	0.6 (T_3)	60	22 ab

Values followed by same letter(s) in a column are not statistically significant ($p<0.05$) as determined by the Duncan's Multiple Range Test (DMRT)

Table 5. Survival rate of regenerants of three *indica* rice verities namely, BRRI dhan29, BRRI dhan28 and BINA dhan6 after transferring to pot in acclimatization chamber and then to soil (earthen pot) for subsequent plant establishment in field

Planting condition	Variety	No. of plantlets transplanted	No. of plants survived	Survival rate (%)
In pot	BRRI dhan29	25	24	96.00
	BRRI dhan28	20	17	85.00
	BINA dhan6	12	8	67.00
In soil	BRRI dhan29	15	14	93.33
	BRRI dhan28	20	18	90.00
	BINA dhan6	8	5	62.50

Fig. 3. Figures showing the regenerated plantlets of BRRI dhan29 in plastic pot covered with polythene bag (A), acclimatized plantlets of BRRI dhan29 in plastic pot (B) and established plantlets of BRRI dhan29 in earthen pot (C) as derived by culturing mature embryos of rice on callus induction, shoot and root regeneration media supplemented with various plant growth hormones

4. DISCUSSION

A reproducible plant regeneration protocol was optimized in three *indica* rice varieties of Bangladesh. These varieties used in this study are very popular among farmers and are widely cultivated in Bangladesh due to higher yield and quality. Any new variety that are being developed in Bangladesh either with desirable agronomic traits or with increased resistance to biotic and abiotics stresses is expected to perform better than these popular varieties in order to get farmers' acceptance. This prompts the use of the genetic background of these popular varieties for most of the current rice improvement programs in Bangladesh. This is the reason why these three

varieties were chosen particularly for optimizing *In vitro* regeneration protocol.

4.1 Varietal Difference in Callus Induction and Plantlet Regeneration

Callus induction and plant regeneration potential is reported to be greatly influenced by genotype [10] and significant genotypic variation has been reported in rice [11-13]. Among the three genotypes studied, BRRI dhan29, which showed better performance in terms of various callus induction, shoot and root regeneration parameters in this study, was extensively studied for investigating it's *In vitro* regeneration potentiality compared to the other two genotypes [14-16]. Our observation of more than 90% callus induction from mature seeds of BRRI dhan29 is in agreement with that of Hoque and Mansfield [15] who have found 88% callus induction from 3 days old root explants. Kabir et al. [16] and Hoque et al. [14] have found 69.45% and 61.9% of callus induction in this genotype which is still a high frequency compared to very low percentage of callus induction in other crop species. These indicate the higher potentiality of this genotype in term of callus induction. However, unlike our observation where BRRI dhan28 showed lower percentage of callus induction (70%) compared to that of BRRI dhan29, Kabir et al. [16] observed completely the opposite trend where BRRI dhan28 showed comparatively higher percentage of callus induction (86.11%). This reason behind this contrasting trend of results is not clear, the variation in source of theses genotypes (and hence, of actual accessions) used by these two experiments.

In our study, the callus was found to be initiated in BRRI dhan29 within 5-7 days of culture in all the media compositions followed by BRRI dhan28 (6-8 days) and BINA dhan6 (8-10 days). It was also reported to be within 7-10 days all in *indica* and genotypes [17,11] and in *indica*, *japonica* and their hybrids [18] using mature seeds as explants and cultured on MS medium supplemented with a variety of plant growth hormones.

In this study, plant regeneration was observed to vary from 25% (in BINA dhan6) to 85% (in BRRI dhan29). Kabir et al. [16] has reported around 85% of shoot initiation in BRRI dhan28 and around 67% in BRRI dhan29 whereas only 35.2% plant regeneration was reported by Hoque et al. [14]. Regeneration of root was not a problem as it was, in fact, never below 70% in BRRI dhan29 and BRRI dhan28 indicating the callus induction and shoot regeneration as the main steps for plant regeneration in rice.

For all the callus induction, shoot and root regeneration traits, the variety BINA dhan6 has shown poor performance. The reason behind the poor response of BINA dhan 6 is not clear, but it can be due to its genetic background which makes it less responsive towards callus induction, shoot and root regeneration.

4.2 Mature Seeds of Rice as Explants

One of the important prerequisite for any rice improvement program using biotechnological manipulations is the induction of embryogenic calli that can offer high frequency plantlet regeneration [19]. Efforts have thus been made to identify suitable explants using a variety of plant parts such as immature embryo [20], leaf base and roots [21-22], leaf blade [23]; node and tips, mesocotyl [24], radicle and coleoptile of seedlings [18], thin cell layer of apical meristematic tissue [25] and anther etc. The callus induction ability was also reported to be varied depending on orientation, age [15] and physiological state [26]. The mature seeds were reported to be the best explants in *In vitro* regeneration [27] and best starting material for *Agrobacterium* mediated transformation studies of rice [28-29]. Besides the high callogenic property, the seeds are also more frequently used for *In vitro* regeneration in rice for their year round availability and comparative ease in sterilization [14, 30]. The mature seeds were thus used in this study. Compared to our 85% of shoot regeneration in BRRI dhan29, the highest percentage of plant regeneration in this variety obtained by Hoque and Mansfield [15] was only 20.8% and Shahnewaz and Bari [12] observed only 65% of plant regeneration using anther of BRRI dhan29 as explant. This variation can be due to the fact that they have used root as explants compared to our mature dehusked seeds.

4.3 Desiccation Treatment Influences Callus Parameters

Desiccation is one of the factors that can enhance embryogensis and plant regeneration in rice [31-32]. The optimal desiccation period, however, varied depending on genotype such as 72 & 48 h desiccation treatment increased plant regeneration by 5 and 2-fold in rice variety MR232 and MR232, respectively [33], 48 h

treatment increased 1.2-5.6 fold in HKR-46 and HKR-126 cultivars [17], 24h desiccation treatment increased shoot regeneration of cell suspension derived calli of japonica rice by 5-47% [34] and that of *indica* rice by 3 folds [35]. In our experiment, desiccation treatment (for 48 h) has shown increased size, decreased compactness of callus with no conspicuous effect on color of callus (Table 2). Siddique et al. [36] observed 2-3 folds increase in plant regeneration in BRRI dhan32 and higher tolerance of callus of BRRI dhan47 to NaCl stress. Desiccation treatment enhances plant regeneration as it reduces the hyperhydricity (water content) of the calli, a condition which is believed to increase oxygen supply to the embryogenic cells in a way to promote embryogenesis [36-37].

4.4 Culture Medium Influences Regeneration Potential

The developmental processes of somatic embryos involve multiple phases including de-differentiation and re- differentiation which requires activation and/or suppression of a number of metabolic pathways [38]. The chemical composition of the culture medium and plant growth regulators largely determines the nature and the state of cell membrane permeability of callus which ultimately influences callus induction, somatic embryogenesis and eventual plant regeneration responses [19, 39]. The chemical composition of the culture medium is thus considered as one of the important factors [40-41]. Efforts have been made to improve the regeneration rate in rice by manipulating the sources of carbohydrate [20], nitrogen [42] and amino acids [43] etc. of culture medium.

A number of media has been used for *In vitro* regeneration in rice such as modified MS, N6, B5, LS, R, NBKNB, GH, PP and Sk medium [11,13,19,39,42,44-45]. Of which, N6 was reported to be the best media for japonica rice anther culture; SK-1 is for *indica* rice anther culture; B5 and AA2 for suspension and protoplast cultures as reviewed by Khanna and Raina [39]. MS media has been proved to be the best for rice somatic cultures [11,44-46]. We have thus used MS media for *In vitro* regeneration of *indica* rice varieties and observed higher success rate in terms of percent callus induction (84%), shoot regeneration (85%) and root regeneration (90%) in the variety BRRI dhan29. Asaduzzaman et al. [47], on a different variety, BRRI dhan30, observed even higher callus induction (97.3%) and shoot regeneration (88.2%) using MS as basal culture media while Hoque and Mansfield [15] didn't find any plant regenerated from BRRI dhan29 when modified LS medium was used as culture media compared to 20.8% using modified MS medium.

4.5 Phytohormone Influences Tissue Culture Response

Both exogenous and endogenous levels of plant growth regulators (PGRs) such as auxins and cytokinins play important role in *In vitro* regeneration of plants [48-50]. In auxin/cytokinin ratio in the culture medium, an elevated auxin usually favours initiation of embryogenic calli whereas higher cytokinin favours shoot induction [51-52], a phenomenon that prompted the manipulation of their respective concentration to improve overall success in plant regeneration [41,53-54]. Among auxins, 2,4-D plays vital role not only in induction and proliferation but also in embryogenesis of the callus [19]. However, addition of other PGRs such as Kinetin, NAA and IAA has shown to improve embryogenesis and quality of the callus [55-57]. Our results, using 2,4-D and BAP, has shown higher percentage of callus induction which are embryogenic, large, compact and dark yellow in color. Kabir et al. [16] has also observed similar trend of result using either kinetin or BAP with 2,4-D. We have also observed higher regeneration of shoot using kinetin and NAA and higher regeneration of root using IBA as PGRs in basal MS medium.

5. CONCLUSION

High frequency callus induction, plantlet regeneration protocol is thus optimized for three high yielding popular *indica* varieties of Bangladesh. The maneuverability of tissue culture techniques is further demonstrated by the difference observed for the varieties, desiccation treatments and phytohormone combinations. Of the three varieties studied, BRRI dhan29 followed by BRRI dhan28 performed better in terms of its regenerability using mature seed as explants. The best phytohormone combinations were also identified which will be helpful in any varietal improvement programs by biotechnological manipulations and molecular breeding in these three varieties.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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