



Callus Induction, Regeneration and Establishment of Rice Plant from Mature Embryo

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Plant growth regulators were used to test callus induction and *in vitro* regeneration in six rice genotypes (RM-AC-2, BRRI dhan89, BRRI dhan88, Nipponbare, Koshihikari and Zenshan97). Four different concentrations (1, 2, 3 and 4 mg/L) of 2,4-D for callus induction and three different concentrations (1,2 and 3 mg/L) of NAA with three doses (5,10 and 15 μ /L) of kinetin for callus regeneration were used to test the effect of plant growth regulators. This study found a high callus induction on MS medium enriched with 2 mg/L 2, 4-D. In cases of RM-AC-2, BRRI dhan89, BRRI dhan88, Nipponbare, Koshihikari and Zenshan97, callus induction frequencies were 92.7%, 87.8%, 84.6%, 82.9%, 86.2% and 62.9%, respectively. In the regeneration, it was found that an MS medium enriched with 2 g/L Kinetin and 10 μ m/L NAA has the ability to induce increased regeneration of different rice varieties (RM-AC-2 (72.4%), BRRI dhan89 (66.9%), BRRI dhan88 (62.5%), Nipponbare (63.3%), Koshihikari (48%) and Zenshan97 (39.6%). From the regenerated plants, one plant of the RM-AC-2 genotype availed to complete its life cycle and generated 32 effective tillers and yielded 89g. This rice plant is very promising for high yielding rice variety development program in Bangladesh. The improved callus development and regeneration ability of this genotype might be helpful for future rice variety development and genetic transformation program.

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1. INTRODUCTION

Rice is a major cereal crop consumed as a staple food by over half of the world's population. Consumption of rice is very high in developing countries and nations in Asia. Almost 95% of the rice production is done in Asian countries and about half of the world's population consumes it [1]. Among the two major subspecies of rice (*indica* and *japonica*), the tropically grown, long grained indica rice accounts for 80% of cultivated rice [2]. Establishment of a highly efficient and widely used tissue culture system for rice will accelerate the rice variety development program and successful application in genetic transformation technology to improvement of cultivars especially against biotic stress.

In vitro culture system is an attractive research area due to the high potential for regeneration of fertile rice plants from various explants [3,2]. For genetic manipulation towards varietal improvement high percentage of re-generable embryogenic calli is a prerequisite [3]. Generally, frequency of callus formation depends on the genotype and developmental stages of rice seeds. Many indica rice genotypes are recalcitrant to *in vitro* manipulations due to poor callus induction, proliferation and regeneration abilities [4,5,6,7]. Efforts have been made to identify suitable rice explants to induce embryogenic callus under appropriate *in vitro* culture conditions [8,4,9,10].

Meristematic organs, such as mature seeds [10,9], mature and immature embryos [11,12,8,6,9] have been reported to be suitable explants for the production of embryogenic callus in rice. Immature embryo was the first explant used to induce embryogenic callus in rice transformation work due to their highly responsive nature in tissue culture systems [13]. However, production of a large number of immature rice embryos is laborious and has seasonal limitations. Although mature seeds contain many actively dividing cells compared to immature tissues, they are not suitable for embryogenic callus induction, especially for recalcitrant rice genotypes [14,8,6,9,7]. However, it has distinct advantages in practical experiments since it is readily available throughout the year and can be conveniently stored for an extended period.

Histological observations of rice embryogenic callus at different developmental stages are

essential for the identification of internal cellular organization. Vega *et. al.*, 2009 reported on two distinguishable clusters of embryogenic and non-embryogenic cells which could be observed during the induction of somatic embryogenesis in rice cultivar. Therefore, by proper manipulations of the right type of explant, culture medium and conditions, it is possible to obtain embryogenic callus with high capacity for efficient regeneration which is an essential prerequisite for genetic improvement of rice through conventional breeding as well as genetic transformation purpose. The objectives of the present study were to evaluate six rice genotypes (*Indica* & *japonica*) for callus induction and regeneration, which results used for future rice genetic transformation.

2. MATERIALS AND METHODS

2.1 Plant Material

Three *indica* and three *japonica* rice cultivars seed grain were collected to conduct this experiment. Details about the cultivars used in this study are given below:

2.2 Preparation of Explant

Healthy and disease-free grain was selected for the callus induction. Fresh seed grain was taken from every genotype and kept at 45° C for 12 hours. Then the seeds were dehulled. Intact and white seeds were selected. About 150-200 seeds were taken into a 150 mL sterile falcon tube. Seeds were rinsed with sterile distilled water for 3 times. Then 25 mL of 70% ethanol was added to the tube and shaken gently for 1 minute. The alcohol was decanted and washed with sterile distilled water. 30 mL of 3% sodium hypochlorite solution with one drop of tween 20 per tube was taken and shaken using a rotator for 30 minutes. The sodium hypochlorite solution was decanted and washed 6 times with sterile distilled water. The tube was gently shaken for 1-2 minutes between washings. After the last wash, the seeds were bolt-dried onto a sterile What man filter paper.

2.3 Preparation of Callus Induction Media and Callus Initiation

Media for callus initiation and maintenance was Murashige and Skoog [15,16] complete media from MS media L-proline (2880 mg/L), casamino

acid (300 mg/L), sucrose (30000 mg/L) and different doses (0,1,2 & 3 mg/L) of 2,4-D were used for callus initiations and media was gelled; with gelatin 4 g/L. The media were freshly prepared, autoclaved, maintained the pH of 5.8, and dispensed in 9 cm diameter Petri dishes as 25 mL aliquots.

2.4 Callus initiation from mature embryo

The embryo of sterile seed was put upwards above the medium and seed endosperm deeply embedded in the medium. Before the seed was placed in the plate, sterile tweezers pierced holes in the medium. One layer of parafilm was used to seal the plate. For ten days, the culture plate was incubated at 28° C under continuous light. Data was collected ten days later. Ten mature embryos were plated in each Petri dish and two Petri dishes were used per replication for each treatment in each genotype. The ability of each genotype to induce callus was calculated as follows:

$$\text{Callus induction ability (\%)} = \frac{\text{Total number of embryo produce callus}}{\text{Total number of embryo cultured}} \times 100$$

All data collected were statistically analyzed separately using Statistix10 software.

2.5 Preparation of regeneration media

Media for regeneration were based on the formulation of Murashige and Skoog [15,16], Casamino acid (2000 mg/L), sucrose (30000 mg/L), sorbitol (30000 mg/L), kinetin (0,1,2 and 3 mg/L), NAA (0,5,10 and 15 µg/L) and were gelled; with gelatin 4g/L. The media were freshly prepared adjust pH to 5.8 autoclaved dispensed in 9 cm diameter Petri dishes approximately 25 mL aliquots.

2.6 Plant Regeneration

Ten-day old embryogenic calli were placed in regeneration media which had been prepared before. Cultures were kept at 28°C with an 18-hour photoperiod. Those embryogenic calli were

kept in the regeneration media for 30 days, and the media was changed every 10 days. Data on callus regenerated ability was collected after 21 days. Ten calli were plated in each petri dish and two petri dishes were used per replication for each treatment in each genotype. The callus regeneration ability of each genotype was calculated as follows:

$$\text{Callus regeneration ability (\%)} = \frac{\text{Total number of callus regenerated}}{\text{Total number of callus cultured in regenerated media}} \times 100$$

All data collected were statistically analyzed separately using Statistix10 software.

2.7 Preparation of Rooting Media

Rooting media was prepared according to the formulation of Murashige and Skoog [15,16], gelled; with sucrose (3000 mg/L). The media was adjusted pH to 5.8 and, autoclaved and dispensed as 50 mL aliquots into test tube.

2.8 Root Initiation

The regenerated plantlets were transferred and grown on rooting media for 30 days at 25° C under an 18-hour photoperiod regime. The media was changed every 10 days.

Acclimatization: After 40 days, the plantlets were taken out of the media and washed thoroughly with fresh water to remove the media from the root. Then those plantlets were transferred in plastic pots filled with vermiculite and covered with polyethene to protect from desiccation. Then the plantlet was kept in a growth chamber with eight hours dark and 16 hours light for two weeks. After that polyethene used for covering the seedling was removed from the plantlet. The plant was then transferred to the growth room at 25°C having 16 hours light and eight hours dark in a pot having 50% soil and 50% vermiculite. Well growth plantlet was then transferred to the shaded area at field conditions in pot having 10 kg field soil. After one week then plantlet was then transferred to the normal sunlight at field conditions at natural conditions until maturity.

Sl. No.	Name of the cultivar	Sub-species	Source
1.	RM-AC-2	<i>Indica</i>	Biotechnology division, BINA
2.	BRR1 dhan89	<i>Indica</i>	BRR1
3.	BRR1 dhan88	<i>Indica</i>	BRR1
4.	Nipponbare	<i>Japonica</i>	BRR1
5.	Koshihikari	<i>Japonica</i>	BRR1
6.	Zenshan97	<i>Japonica</i>	BRR1

Abbreviations: BINA: Bangladesh Institute of Nuclear Agriculture, BRR1: Bangladesh Rice Research Institute

3. RESULTS AND DISCUSSION

3.1 Callus Induction

Using MS medium supplemented with various amounts of 2,4-D (1.0, 2.0, 3.0 and 4.0 mg/L) and without 2,4-D as a control, *in vitro* callus induction was examined from mature embryos of six rice (*Oryza sativa* L.) genotypes (Fig. 1a & 1b). The callus induction ability (%) in different 2, 4-D concentrations showed significant differences. For different concentrations of 2, 4-D, the callus induction ability of different rice genotypes ranged from 50.1–92.7 %. (Table 1). The control without any 2, 4-D application failed to induce any callus growth. All genotypes recorded the highest callus induction ability at T₂ (2 mg/L 2,4-D). The genotype RM-AC-2 had the highest callus induction ability (92.7%), followed by BRR1 dhan89 (87.8%) and BRR1 dhan88 (84.6%) at T₂ (2.0 mg/L, 2, 4-D). The lowest callus induction ability was observed for the genotype Zenshan97 (50.1%) at T₄ (4 mg/L 2, 4-D).

It was observed that the frequency of callus induction decreased when the concentration of 2, 4-D exceeded 2.0 mg/L. Different concentrations of 2, 4-D were found to produce various morphology of callus. A high concentration of 2, 4-D (3.0 and 4.0 mg/L) resulted in yellowish, small or short, compact and fragile calli. Calli generated on MS medium supplemented with 1.0 and 2.0 mg/L 2, 4-D was creamy to whitish in colour and globular in shape.

The optimization of medium components, as well as the type and concentration of plant growth regulators, has been shown to improve embryogenic calli produced from rice scutellum via somatic embryogenesis. The production and quality of embryogenic calli has been shown to be influenced by interactions between genotypes and culture conditions [17]. Several studies have

reported that plant growth regulator 2, 4-D is the most suitable auxin for callus induction in rice tissue culture [18,19,20,21]. In this study, the effectiveness of different concentrations of 2, 4-D alone was evaluated for callus induction from dehusked seeds of six rice genotypes. Different concentrations of 2, 4-D were observed to produce various morphologies of callus. The present study revealed that callus induction was enhanced from the presence of 2 mg/L 2, 4-D and showed different callus percentages for all the genotypes that are studied. This finding was in agreement with previous research suggesting that the use of 2, 4-D gave successful callus induction in indica rice. The results of the present study were in agreement with those of Pandey *et. al.* 1994, Thadavong *et. al.* 2002 and Abeyaratne *et. al.* 2004, which showed that 2.0 mg/L 2, 4-D was the most optimum concentration for callus induction from mature rice seeds. Moreover, based on ten rice genotypes used, calli produced on semi-solid MS media supplemented with 2.0 mg/L 2, 4-D produced the most desired features [22].

3.2 Regeneration

Calli produced on MS media supplemented with 2.0 mg/L 2, 4-D were assessed for their plant regeneration ability. Significant differences were recorded for plant regeneration ability at different kinetin (0, 1, 2 and 3 mL/L) and NAA (0, 5, 10 and 15 µg/L) concentrations (Table 2). All treatments were found to be significant in the regeneration of plants rather than control. T5 (10 µ/L NAA + 2 mg/L Kinetin) was observed to have the highest regeneration ability for all the genotypes. The highest regeneration ability was observed for the genotype RM-AC-2 (72.4%) followed by BRR1 dhan89 (66.9%) and BRR1 dhan88 (62.5%) at T5 (10 µ/L NAA + 2 mg/L Kinetin). The lowest regeneration ability was observed (7.3%) for T1 (10 µg/L NAA + 2 mg/L Kinetin) in the genotype Zenshan97.

Table 1. Effect of different concentration of 2,4-D on callus induction for the genotype RM-AC-2, BRR1 dhan89, BRR1 dhan88, Nipponbare, Koshihikari and Zenshan97

Treatment	Concentration of 2,4-D (mg/L)	Genotype					
		RM-AC-2	BRR1 dhan89	BRR1 dhan88	Nipponbare	Koshihikari	Zenshan97
Control	0	0e	0e	0e	0e	0e	0c
T ₁	1	64.2d	58.5d	55.1d	69.7d	60.7d	52.7b
T ₂	2	92.7a	87.8a	84.6a	82.9s	86.2a	62.9a
T ₃	3	84.4b	74.7b	71.4b	75.7b	71.5b	52.7b
T ₄	4	79.5c	70.2c	64.7c	70.7c	65.7c	50.1b
CV%		1.63	1.47	0.73	0.68	1.77	5.91

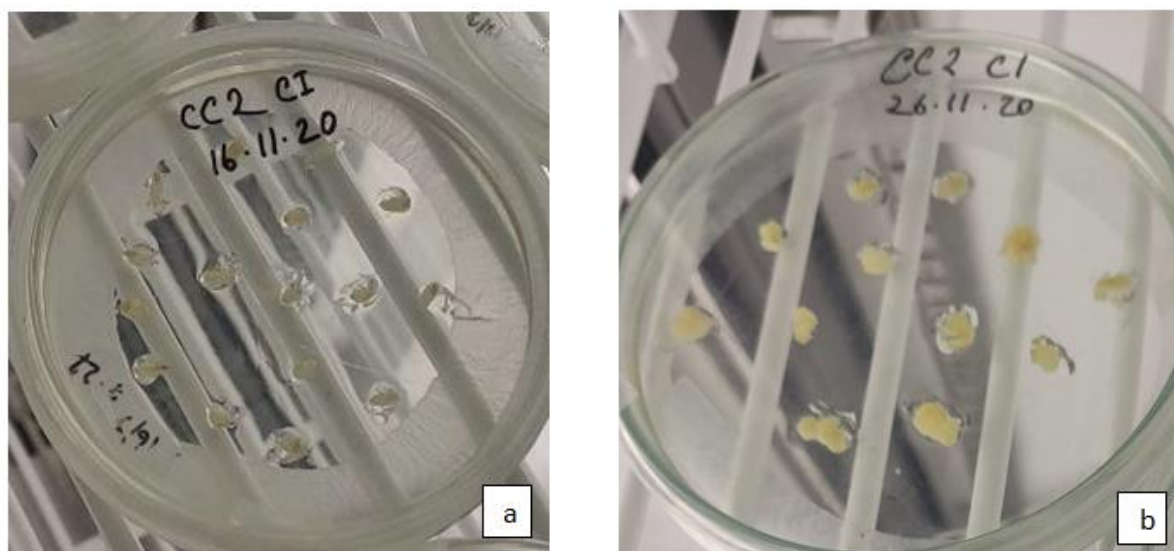


Fig. 1. a. Mature endosperm plating in callus induction media, b. Callus induction in callus induction media

Table 2. Effect of different concentration of kinetin and NAA on callus regeneration for the genotype RM-AC-2, BRR1 dhan89, BRR1 dhan88, Nipponbare, Koshihikari and Zenshan97

Treatment	Kinetin (ml/L) + NAA (µg/L)	Genotype					
		RM-AC-2	BRR1 dhan89	BRR1 dhan88	Nipponbare	Koshihikari	Zenshan97
Control	00 + 00	2.8h	5.6f	3e	5.667g	1f	0e
T ₁	01 + 05	19.2g	23.3e	15.3d	12.3f	8.7e	7.3d
T ₂	01 + 10	25.7f	25.3e	23.7c	16e	8.7e	10d
T ₃	01 + 15	25.1f	26e	28c	18.3e	10.7e	11.3d
T ₄	02+ 05	65.1b	61.3b	48.3b	59.3b	32cd	31b
T ₅	02 + 10	72.4a	66.9a	62.5a	63.3a	48a	39.6a
T ₆	02+ 15	63.7bc	60.7b	60.7a	59.3b	43.3b	32.3b
T ₇	03+ 05	61.1cd	58bc	51b	51.3c	36c	19.6c
T ₈	03+ 10	58.5d	54.7c	50b	51.3c	33.3cd	23.3c
T ₉	03+ 15	53.3e	47.7d	47b	47.7d	30d	21c
CV%		4.68	6.25	8.38	5.30	10.72	10.91



Fig. 2. a. Regenerated calli in regeneration media, b. Plantlet in regeneration media, c. Plantlet obtained from tissue in rooting media

For plant regeneration, the ideal concentration of NAA in combination with 2.0 mg/L kinetin was 10 µg/L, which resulted in the maximum plant regeneration frequency for all genotypes, producing both shoots and roots. The findings of the present study also revealed that as NAA concentrations increased, the frequency of callus-producing shoots and roots reduced. This could be owing to the high concentration of NAA (15 µg/L), which could restrict growth and morphogenesis by inhibiting cytokinin accumulation [23]. The effect of varying NAA concentrations in conjunction with kinetin on root production was the polar opposite of the effect on shoot production. With an increment in NAA concentration, the average number of roots generated per callus was increased. As the concentration of NAA in the medium rises, the ratio of auxin to cytokinin rises as well, resulting in improved root regeneration. Thadavong *et al.* 2002, described a similar phenomenon. The number of roots generated was reduced when the NAA concentration exceeded 10 µg/L. This NAA severely inhibited growth and morphogenesis at this dose.

3.3 Acclimatization of Rice Plantlets

The *in vitro* grown shoots were green and healthy, and they were moved for acclimation (100 percent vermiculate) and kept at a growth chamber at 25°C, 16 h photoperiod, and 30–40 mol/m²s cool white fluorescent light (Fig. 3a & 3b), with about 75 percent survival. The roots sprouted and grew nicely within 3 weeks. After being transferred to large pots and grown in a shaded area for two weeks (Fig. 3c) and then in normal conditions (Fig. 3d). During acclimatization, rooted shoots grew actively and showed no stress symptoms or morphological abnormalities (Fig. 3e).

We had continued with RM-AC-2 because the regeneration of the genotype of RM-AC-2 was successful. Effective tiller per hill, Plant height, Panicle length, grain per panicle, filled grain per panicle, grain length/breadth and hundred seed weight was recorded 32, 85.7 cm, 21.3 cm, 144.37, 118.3, 3.32 and 2.45 respectively (Table 3).



Fig. 3. a. Plantlets cover with polybags, b. Plantlet grown in vermiculite, c. Plantlets in soil at vegetative stage, d. Plants at reproductive stage, e. Mature plants obtained from tissue culture

Table 3. Yield and yield contributing characters of the rice plant derived from the RM-AC-2 genotype

Genotype	No of effective tiller per hill	Plant Height (cm)	Panicle length (cm)	Grain per panicle	Filled Grain	Grain length/breadth	100 seed weight (g)	Yield per plant (g)
RM-AC-2	32	85.7	21.3	144.37	118.3	3.32	2.45	89

4. CONCLUSION

The MS medium supplemented with 2.0 mg/L 2, 4-D produced the maximum frequency of callus induction from matured embryo of the genotypes RM-AC-2, BRRI dhan89, BRRI dhan88, Nipponbare, Koshihikari, and Zenshan97, according to the current study. The maximum effective concentration of NAA was 10 µg/L in combination with 2.0 mg/L kinetin for plant regeneration from rice genotype RM-AC-2, BRRI dhan89, BRRI dhan88, Nipponbare, Koshihikari, and Zenshan97. Plants with the RM-AC-2 genotype produced 32 panicles and yielded 89 g per plant. This result might be helpful for future rice genetic transformation. The plant obtained from this study produced significant number of effective tillers as well as yield. Therefore, this plant could be used as a high yielding genotype for developing a rice variety for cultivation in Bangladesh.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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