Studies on *In Vitro* Response to Callus Induction and Regeneration of Five High Yielding *Indica* Rice Varieties

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Abstract. Due to growing population, there is an increasing demand of rice production but the productivity of rice is lessened day by day. To overcome this problem various biotechnological tools can be used for developing various rice varieties. However, the lack of a simple and efficient protocol for callus induction, embryogenic callus formation and quick plant regeneration in this cereal crop. In this study embryogenic calli from mature seeds of five indica rice varieties viz. Binadhan-5, Binadhan-6, BRRI dhan-48, BRRI dhan-58 and IR-64 were observed that is done in four different types of media composition. The highest callus induction were observed in media containing Sucrose as a carbon source. Among those varieties Binadhan-6 and BRRI dhan-48 showed highest rate of callus induction respectively. This study will be useful for selecting suitable callus induction medium for callus induction in future that will be useful for not only national but also international plant breeders for producing new variety and so on.

Introduction

Rice (*Oryza sativa* L.) is a monocot plant that belongs to the genus *Oryza*, family Gramineae (Poaceae) and tribe Oryzeae. It also comprised two subspecies, *Indica* and *Japonica* [16]. It is being cultivated for more than 10,000 years [14]. It is cultivated in 10.1 million ha and produces 95 % of the total food grain of Bangladesh. Out of the all crops that are using as feed in all over the world rice provide 23 % of the calories consumed by the whole population [9]. In Asia, two main subspecies named *indica* and *japonica* are grown among the three species. *Indica* rice encompasses 80% of the world rice cultivation [15] and this types of rice is mainly grown in South and South-East Asian countries of the world [18]. Larger than half of the population of the world using rice as a staple food [7] that serves more than 90% of the Asian population and is occupy the second position among all cereal crops cultivated in the world, after wheat [12]. For coping with the high rate of increasing population of the world the total rice production has to be intensified 50% within 2025 [8]. This goal cannot be accomplished by increasing the cultivation area under rice cultivation because of limitation of proper lands. On the other hands the farm areas are being lessen by residential areas in the developing countries. Previous experiments showed that the production rate of rice is unpleasantly affected by abiotic stress and that is highly defenseless with the change of weather [5, 6, 19, 3]. Various Modern biotechnological tools requires the optimization of an efficient regeneration protocol for specific cultivar(s) is an essential for the composition of the nutrient media is a major factor that influencing the regeneration frequency of rice [2]. Various researchers used different types of media for callus induction and regeneration [20],[21]. This experiment considering the objectives of identifying a suitable medium for callus induction from mature dehusked seeds. We hope that this study will be useful for local as well as for international plant breeders for improving the callus induction and regeneration efficiency in Rice in future.

Materials and Methods

This study was conducted in the Laboratory of Biotechnology division, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh.

Plant material

Five different healthy and dehusked seeds of indica rice (*Oryza sativa* L.) named Binadhan-5, Binadhan-6, BRRI dhan 48, BRRI dhan 58, and IR 64 were used as experimental material to study different parameters associated with callus induction of plant [4].

Surface sterilization of seeds

Seeds were surface sterilized by dipped in 70% ethyl alcohol for 1 minute with vigorous shaking after washing 5 to 6 times with distilled water. After that it were washed with double distilled water for 3 to 4 times and Surface disinfection was done by using of 50% NaOCl and 1 drop Tween-20 for 15 minutes at 300 rpm in a shaker. Then the seeds were subsequently washed 7 to 10 times with sterile distilled water to remove traces of sterilant that would be toxic to the seed materials if kept for longer duration. The surface sterilized seeds were then dried for 5-6 min on a sterilized filter paper.

Callus induction

Sterilized seeds were cultured directly in MS medium supplemented with different carbon source and media composition required as per treatment. In this experiment we used four types of media composition. MS medium containing 30g/L sucrose (T1) [23]; 30g/L Maltose (T2) [5]; 30 g/L Sucrose, 0.5 g/L Proline and 0.5 g/L Glutamine (T3) [24] and 30 g/L Maltose, 0.3 g/L Casein hydrolysate, 0.6 g/L L- proline and 0.25 mg/L BAP (T4). The culture media were autoclaved at 1.16 kgcm⁻² pressure and 121°C temperature for 30 minutes. Sterilized Seeds were cultured horizontally on to the media with different treatments under aseptic conditions and then incubated at 27⁰ C in dark conditions. Every treatment contained three replications. The callus induction frequency was recorded after 2 weeks of culturing. The following Equation was used for determining the callus induction frequency as given below.

$$\text{percent (\%) callus induction} = \frac{\text{Number of callus induction}}{\text{Number of seeds incubated}} \times 100$$

Percent of embryogenic callus were calculated on the basis of the number of induced callus and the total number of embryogenic callus.

$$\text{Percent (\%) of embryogenic callus} = \frac{\text{Number of embryogenic callus}}{\text{Number of callus induction}} \times 100$$

Subculture

Two weeks after the inoculation of explants, the embryogenic callus attained convenient size. Then they were removed aseptically and the root and shoot are discarded from the callus on a sterilized glass plate inside the laminar airflow cabinet and were placed again on a freshly prepared sterilized medium containing appropriate hormonal supplements. Then the subcultured callus were incubated in growth chamber at controlled temperature (27±1⁰ C) in dark.

Regeneration from embryogenic calli

After 2 weeks the subcultured calli were transferred onto regeneration media containing either two or three growth regulators comprised of MS salts, 30 g/l maltose, 2 mg/l kinetin, 0.2 mg/l naphthalene acetic acid (NAA), P^H 5.8. The media was supplemented with 8 g/l agar during the first phase of regeneration and 10 g/l agar during the second phase of regeneration. These microcalli were incubated at 27±1⁰ C in dark for 7 days for the first phase of regeneration. During the second phase of regeneration these microcalli were transferred to fresh regeneration medium with 10 g/l agar concentration and incubated in light at 27±1⁰ C temperature [13].

Results

Plant regeneration is a system that is essential for establishing an efficient application of biotechnology that can be utilized for crop improvement in future. Therefore, in vitro regeneration from induced callus from mature embryos of five *indica* rice (*Oryza sativa* L.) varieties was investigated in this study.

The experiment was accompanied using five varieties of *indica* rice named Binadhan-5, Binadhan-6, BRRI dhan-48, BRRI dhan-58 and IR-64. Exploration on in vitro regeneration of these varieties was accomplished with callus induction, desiccation of callus, embryogenic callus, and finally plantlet regeneration. Results of different steps of the experiments are described under the following heads.

Callus induction

The first step of this experiment was callus induction from mature embryo. To meet this purpose, callus induction potentiality of mature embryo on different MS media composition was investigated.

Embryo of mature seeds of five varieties of rice used as explants were cultured on four types of MS media (MS + Sucrose, MS + Maltose, MS + Sucrose + Proline + Glutamine, and MS + Maltose + Casein hydrolase + L- proline + BAP) for callus induction. Callus induction performances of all the varieties in each treatment were evaluated and presented in Table-1.

Table 1: effects of different ms media on callus induction

Treatment	Varieties	No. of seed plating	Percent of callus induction	Percent of embryogenic callus
MS + Sucrose +2,4-D	Binadhan-5	100	92	48
	Binadhan-6	100	93	62
	BRRI dhan-48	100	89	39
	BRRI dhan-58	100	38	21
	IR-64	100	59	23
MS + Maltose+ 2,4-D	Binadhan-5	100	94	21
	Binadhan-6	100	93	55
	BRRI dhan-48	100	83	54
	BRRI dhan-58	100	89	37
	IR-64	100	79	63
MS + Sucrose + Proline + Glutamine +2,4-D	Binadhan-5	100	42	15
	Binadhan-6	100	90	52
	BRRI dhan-48	100	84	57
	BRRI dhan-58	100	79	22
	IR-64	100	91	40
MS + Maltose + Casein hydrolase + L- proline + BAP + 2,4- D	Binadhan-5	100	52	17
	Binadhan-6	100	49	31
	BRRI dhan-48	100	57	37
	BRRI dhan-58	100	39	21
	IR-64	100	49	29

Figure 1: Callus induction from mature embryos of five indica rice varieties: a) Binadhan-5, b) Binadhan-6, BRRI dhan-48, d) BRRI dhan-58 and e) IR 64

Callus initiation started from six days from incubation and took about fourteen to sixteen days for the completion followed by Binadhan-5, Binadhan-6, BRRI dhan-48, BRRI dhan-58 and IR-64 respectively (Figure-1). The percentage of callus induction was highest in Binadhan-6 (69.17%), followed by BRRI dhan-48 (66.17%), Binadhan-5 (52.50%), IR-64 (52.50) and lowest in BRRI dhan-58 (43.17%). Highest (72.87%) percentage of callus induction was observed in T1 (MS + Sucrose + 2 mg/L 2,4-D), T2 (MS + Maltose+ 2 mg/L 2,4-D) is near the T1 (72.73%) and lowest (24.20%) percentage of callus induction was observed in T4 (MS + Maltose + Casein hydrolase + L- proline + BAP + 2 mg/L 2,4- D) (Table 2). .

Effects of different treatments on callus induction

Mean square values of four different combinations of treatments were found statistically significant for the callus such as percent of callus induction and percent of embryogenic callus. Among those treatments MS + Sucrose + 2 mg/L 2,4-D was found to be the best of all variety (Table 2).

T1 (MS + Sucrose + 2 mg/L 2,4-D) showed the highest percentage (72.87%) of callus induction closely followed by T2 (MS + Maltose+ 2 mg/L 2,4-D) 72.73%, T3 (MS + Sucrose + Proline + Glutamine +2,4-D) 57% and lowest (24.20%) percentage of callus induction was observed in T4 (MS + Maltose + Casein hydrolase + L- proline + BAP + 2 mg/L 2,4- D). But T2 (MS + Maltose+ 2 mg/L 2,4-D) showed the highest percentage (38.60%) of embryogenic callus production, closely followed T1 (MS + Sucrose + 2 mg/L 2,4-D) 35.73%, T3 (MS + Sucrose + Proline + Glutamine +2,4-D) 28.33%, and lowest 7% of embryogenic callus was produced in T4 (MS + Maltose + Casein hydrolase + L- proline + BAP + 2 mg/L 2,4- D).

Table 2: Effects of different treatments on callus induction

Treatments combination	Percent of callus induction
MS + Sucrose +2,4-D (T ₁)	72.86667 a
MS + Maltose +2,4-D (T ₂)	72.73333 a
MS + Sucrose + Proline + Glutamine +2,4-D (T ₃)	57.00000 b
MS + Maltose + Casein hydrolase + L- proline + BAP + 2,4- D (T ₄)	24.20000 c
CV(%)	32.34381
LSD(0.05)	13.534
In the column figures followed by same same letter (s) in a do not statistically significant	

Table 3: Varietal differences in callus induction

Variety	Percent of callus induction
Binadhan-5	52.50000 bc
Binadhan-6	69.16667 a
BRRI dhan-48	66.16667 ab
BRRI dhan-58	43.16667 c
IR-64	52.0000 bc
CV (%)	32.34381
LSD (0.05)	15.13147
In the column figures followed by same same letter (s) in a do not statistically significant	

Effects of different varieties on callus characters

The genotypic mean square values of rice were found statistically significant for all the characters of callus induction such as percent of callus induction and percent of embryogenic callus under those treatment (Table 3).

Highest (69.17%) percentage of callus induction was observed in Binadhan-6 closely followed by BRRI dhan-48 (66.17%), Binadhan-5 and IR-64 showed the same percentage (52.50%)

and lowest (43.17%) in BRRI dhan-58. Which were statistically significant. Percentage of embryogenic callus was higher (37.75%) in Binadhan-6 closely followed by BRRI dhan-48 (36.17%), IR-64 (27.83%), Binadhan-5 (18.83%) and lowest (16.50%) in BRRI dhan-58 which were statistically significant. We can say that rate of callus induction depends on genotype. It has been showed that callus induction frequency is related with genotypic variability [4],[10]. In this experiment the percentage of callus induction varied variety to variety.

Regeneration from embryogenic calli

Two different media containing either two or three growth regulators are used. One (R1) comprised of MS salts, 30 g/l maltose, 2 mg/l kinetin, 0.2 mg/l naphthalene acetic acid (NAA), pH 5.8. The media was supplemented with 8 g/l agar during the first phase of regeneration and 10 g/l agar during the second phase of regeneration. While other media (R2) consisted of MS salts, 30 g/l maltose, 2.7 mg/l BAP, 1.2 mg/l kinetin, 0.5 mg/l NAA, pH 5.8 the regeneration occur within 25 days of inoculation (Figure 3) [13].

a) After 7 days of inoculation

b) After 14 days of inoculation

c) After 21 days of inoculation

d) After 25 days of inoculation

Figure 3: Regeneration from transformed calli

Discussion

In previous study it were showed that the callus induction frequency were between 60% and 90% [22], in the present study the callus induction rate reached up to 72% that support the previous study. However, high callus induction and regeneration may differ in different media composition and varieties [4],[5]. In this study, whitish and globular calli were found in media containing maltose and sucrose treated media formed brownish calli. In parallel to the above observations, Visarada et al. and Abiri et al. [1],[17] reported that nodular, compact and whitish calli were highly

embryogenic while watery, yellowish, rhizogenic and necrotic calli were stated to be non-embryogenic. Non-embryogenic calli are not competent for regeneration [12]. Based on color and structural morphology, the calli of the present study were mostly embryogenic in maltose treated media where they reached up to 38 %. Previous studies illustrated that maltose gave high embryo formation in *indica* rice [11]. It has been reported that Callus induction is highly genotype specific [4]. In this experiment there is a huge difference of callus induction among the five rice varieties.

Conclusions

In this experiment a thorough investigation was carried out for knowing the callus induction ability of five *indica* Rice varieties named Binadhan-5, Binadhan-6, BRRI dhan-48, BRRI dhan-58, IR-64. Mature embryos of five *indica* rice varieties were cultured on four different media composition for inducing callus and embryogenic callus. A wide range of variation was observed in the ability of callus induction and embryogenic callus formation. The maintenance of callus was essential for getting efficient regeneration for this purpose, embryogenic callus were desiccated and cultured on callus induction media. MS + Sucrose (T1) media performed better compared to other types of media for inducing callus. The percent of callus induction was highest (69.17%) in Binadhan-6 and lowest (43.17%) in BRRI dhan-58. Callus induction was highest (72.87%) in T1 (MS + Sucrose +2,4-D) and lowest (24.20%) in media T4 (MS + Maltose + Casein hydrolase + L-proline + BAP + 2,4-D). highest (38.60%) percentage of embryogenic callus was formed in T2 (MS + Maltose +2,4-D) and lowest (7%) embryogenic callus was formed in T4 (MS + Maltose + Casein hydrolase + L-proline + BAP + 2,4-D). Binadhan-6 produced highest (37.75%) rate of embryogenic callus and BRRI dhan-58 showed lowest (16.50%) embryogenic callus production.

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