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Response to Callus Induction and Regeneration of Newly Released BRRI Rice Varieties

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ABSTRACT

A study was carried out for developing an efficient callus induction and regeneration system for three newly developed BRRI varieties namely BRRI dhan86, BRRI dhan87 and BRRI dhan89. Dehusked seeds were plated onto MS and N6 media with two hormone combinations for callus induction. Calli obtained from each callus induction medium were transferred to four different regeneration media. Callus induction frequency and regeneration ability were significantly influenced by rice varieties, and interactions of variety and media. Among the media compositions, the highest callus (59.44%) were obtained from C_1 (MS+2mg/l 2,4-D) followed by C_2 (MS+2 mg/l 2,4-D+0.5 mg/l kinetin) , C_3 (N6+2 mg/l 2,4-D) and C_4 (N6+2 mg/l 2,4-D+0.5 mg/l kinetin) medium. The highest regeneration (45.74%) was obtained from R_2 (MS+4 mg/ml BAP+1.2 mg/ml kinetin+0.5 mg/ml NAA), followed by R_3 (1 mg/ml BAP+1 mg/ml Kinetin+1 mg/ml NAA), R_4 (2 mg/ml kinetin+1 mg/ml NAA+300 mg casein hydrolysate) and R_1 (2 mg/ml BAP+1 mg/ml kinetin+1 mg/ml NAA). BRRI dhan86 showed the highest regeneration ability (53.06%) than the other two varieties. It is observed that all varieties performed better in C_1 medium for callus induction and R_2 medium for regeneration. This study also revealed that BRRI dhan86 was more responsive to callus induction and regeneration of green plants than the other two varieties.

Key words: Rice, callus, regeneration, hormone

INTRODUCTION

Rice is consumed as a staple food by more than half of the world population. Asia produces 90% of rice to meet up the demand (Bandumula *et al*., 2017). But rice production faces a threat of biotic (diseases and insects) and abiotic (salinity, drought, submergence, cold, heat) stresses due to rapid climatic changes (Das *et al*., 2019). Various research approaches like conventional breeding, somaclonal variation and marker assisted selection are being carried out since the last decades to develop tolerant rice varieties to reduce above mentioned stresses. Though some remarkable progresses are achieved, those are not worthy of satisfaction. Rice has become the prime target for genetic manipulations due to much dependence upon it as a staple food. Genetic transformation is an important biotechnological tool for developing stress tolerant rice varieties. However, the success of genetic transformation depends on several factors like genotype, media, light, hormonal effect, etc. *Agrobacterium* mediated transformation of rice requires an efficient regeneration system from a transformed callus and ironically, shoot regeneration represents a major bottleneck in this endeavour (Lim *et al*., 2017). 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 potentiality (Sripriya *et al*., 2017). Although reporting is abundant on callus induction, regeneration and also transformation in japonica rice but 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 the mature embryo in *indica*

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rice is still challenging (Pawar *et al*., 2015). Researchers still struggle to optimize the callus induction procedures and efficiency of plant regeneration for rice mature embryos because it varies with variety to variety. In this context, the evaluation of varieties with different types of media and their manipulation for efficient callusing and green plant regeneration from the dehusked mature embryo in *indica* is still a challenging field (Pazuki and Sohani, 2013). Many studies have been conducted to optimize the techniques and composition of culture medium for callus induction from dehusked rice seed for various purposes (Benlioglu *et al*., 2015). However, it's application is still limited by many factors influencing the culture efficiency such as medium composition explants source (Din *et al*., 2016), genotype and environment (Islam *et al*., 2014). Among them, the genotype and nutrient composition are considered to be the major sources of variation for *in vitro* culture (Kido *et al*., 2015). For carrying out successful genetic transformation in rice, identification and screening of useful cultivars and the establishment of efficient regeneration protocols are very essential (Vennapusa *et al*., 2015). With the concern of the above situations, the present study was carried out to compare the performance of three newly developed high yielding rice varieties to find out the best combination of callus induction media and regeneration media for each variety and to identify the best responsive variety among them for transformation study.

MATERIALS AND METHODS

This research work was conducted at the Biotechnology Division of BRRI following Complete randomized design with three replications. A total of 1200 dry seeds of each three rice cultivars viz BRRI dhan86, BRRI dhan87, BRRI dhan89 were used for this study. Sterilization was carried out in a laminar air flow cabinet. Dehusked seeds were first sterilized with 70% ethanol for one minute followed by washing with sterile water. Then it was sterilized with 50% sodium hypochlorite (v/v) containing one drop of 20 for 40 minutes with gentle agitation. Seeds were thoroughly washed five to six times with sterile distilled water to remove sodium hypochlorite. The sterilized seeds were finally placed on sterile filter papers also to remove excess water.

Both MS (Murashige and Skoog, 1962) and N6 (Nitsch and Nitsch, 1969) basal salts were used to supplement with two different hormone combinations for calli induction (Table 1). Sucrose 30 g/l and phytagel 4 g/l was used as a source of carbohydrate and solidifying agent, respectively. Four types of media (C_1, C_2, C_3 and C_4) were used. The pH of the media was adjusted to 5.8. The media was autoclaved at 15 psi at 121°C for 20 minutes. Four hundred sterilized seeds of each variety were placed on four callus induction media.

Then all culture plates were placed at 25±1°C under the dark condition for callusing. Data on % callus induction and size of callus were collected after 21 days and 30 days after plating the seeds. Three week's old calli were transferred onto magenta boxes containing regeneration media. Callus obtained from each combination of callus induction media (C_1, C_2, C_3 and C_4 were transferred onto four types of regeneration media (R_1, R_2, R_3 and R_4) (Table 2). Regeneration efficiency was observed on MS media supplemented with four different combinations of naphthalene acetic acid (NAA), Kinetin (Kn) and 6-benzylaminopurine (BAP). The pH of the regeneration media was adjusted to 5.8. Cultural conditions of the study was maintained at 25±1°C, light and dark cycle of 16:8 hours. After 30 days, % regenerated calli and number of regenerated green plants were recorded.

The data were subjected to ANOVA (Analysis of Variance) testing and the mean values were separated by the least significant difference (LSD) using MS Excel and R software (Table 3).

Table 1. Composition of callus induction media are as follows.

Callus induction media	Basal media	Media combination
C_1	MS	MS+2 mg/l 2,4-D
C_2	MS	MS+2 mg/l 2,4-D+0.5 mg/l kinetin
C_3	N6	N_6+2 mg/l 2,4-D
C_4	N6	N_6+2 mg/l 2,4-D+0.5 mg/l kinetin

Table 2. Four hormonal combinations in regeneration media are as follows.

Regeneration media	Basal media	Hormone combination
R_1	MS	2 mg/ml BAP+1mg/ml kinetin+1 mg/ml NAA
R_2	MS	4 mg/ml BAP+1.2 mg/ml kinetin+0.5 mg/ml NAA
R_3	MS	1 mg/ml BAP+1mg/ml Kinetin+1mg/ml NAA
R_4	MS	2 mg/ml kinetin+1mg/ml NAA+300 mg casein hydrolysate

Table 3. Analysis of variance (ANOVA) for callus induction and size of the calli.

Source of variation (SV)	DF	Mean sum of square (MS)	
		Calli induction	Size of calli
Variety (V)	2	1281.60***	0.05**
Calli induction media (C)	3	3041.60***	0.02
C:V	6	561.03***	0.01
Residuals	24	56.86	0.01

** and *** indicates significant at the 5% and the 1% level of significance respectively.

RESULTS AND DISCUSSION

Callus initiation in varieties

The highest callus induction (52.17%) was found in BRRI dhan86 followed by (43.50%) BRRI dhan87 and the lowest (31.58%) was in BRRI dhan89 (Table 4). Thus, BRRI dhan86 showed comparatively better potential in callus induction compared to other varieties. At the beginning, scutellum callus was compact almost in all varieties but turned into friable after two weeks of culture. Calli of BRRI dhan86 were bright yellowish, look most healthy compared to other varieties (Fig. 1a-f).

BRRI dhan87 performed better in media C_2 followed by C_4 and C_3. The results found of this study are in agreement with the findings of other researchers largely depending on the use of harmonious combinations of nutritional constituents and growth regulators. There are many factors influence callus induction in rice like genotype potentiality, P^H of the media, plant growth regulators (PGRs) supplement, solidification of culture medium, light intensity etc. Joyia *et al*., (2012). Yaqoob *et al*., (2016) reported that mature dehusked rice seeds

Table 4. Interaction between the genotypes and media for callus induction of three rice genotypes.

Calli induction media → Varieties↓	MS		N_6		Mean of varieties
	C_1	C_2	C_3	C_4	
BRRI dhan86	83.00d	70.67cd	26.00a	29.00ab	52.17A
BRRI dhan87	49.67bc	71.00cd	24.33a	29.00ab	43.50B
BRRI dhan89	45.67ab	29.67ab	24.67a	26.33a	31.58C
Calli induction media mean	59.44A	57.11A	28.11B	25.00B	-

*In interaction means in both row and column indicate a common small letter(s) are not different significantly. (P≤0.05)
*In a row, calli induction media mean indicates a common capital letter(s) are not different significantly. (P≤0.05)
*In a column mean of varieties indicate a common capital letter(s) are not different significantly. (P≤0.05)

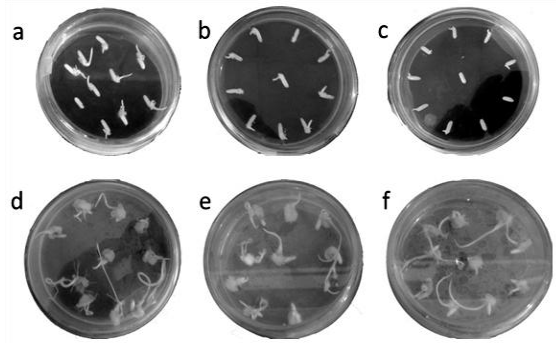

Fig. 1. Seed placement of BRRI dhan86 (a), BRRI dhan87 (b) and BRRI dhan89 (c) and callus initiation of BRRI dhan86 (d), BRRI dhan87 (e) and BRRI dhan89 (f) into media.

were good for callus induction because of callus initiated from scutellum of mature rice seeds have high embryogenic potentiality. Amer *et al*., (2016) and Ahmad *et al*., (2015) also reported that embryogenic calli have high regeneration capacity and excellent material for the transformation of rice by using *Agrobacterium*.

Effect of medium on callusing

From this study, it was found that the varieties, media compositions and their interactions significantly affected on callus induction at the 5% level of significance. Among the media composition, C_1 and C_2 were significantly higher than C_3 and C_4 media for effective callus induction. The rate of callus induction was similar in C_3 and C_4 media (Table 4). The rate of callus induction was also similar in C_1 and C_2 media, but calli obtained from C_1 medium were quite good in texture and friable in nature than that of C_2 medium. MS and N_6 are the most commonly used basal media for calli induction and regeneration (Azizi *et al*., 2015). BRRI dhan86 in MS medium gave better callus induction as compared to N_6 media, these findings were similar to the report by Islam *et al*., (2014) who indicated that variety was one of the major determinants in embryogenic callus induction. Kido *et al*. (2015), Narciso *et al*. (2010) and Roy *et al*. (2015) also reported that embryogenic callus formation and plantlet regeneration were influenced by culture medium and variety.

The mean frequency of calli size over media (Table 5) showed that the variety BRRI dhan87 produced larger calli (0.47%). Among the media composition we found, calli size on C_3 media were higher than C_1, C_2 and C_4 media. BRRI dhan87 produced larger size calli from C_3 (0.54%) and C_2 (0.51%) media and BRRI dhan89 produced lower size calli in C_2 (0.27%) than the other two varieties. Variations were observed among the three varieties but there was no significant differences.

The concentrations of the plant growth regulators also affect the callusing of the genotypes. In this study, 2, 4-D was used at the rate of 2 mg/l. Previous studies also showed that callus could be induced better in 2 mg/l (Shahsavari *et al*., 2010). These results are confirmatory to the findings of the other researchers (Htwe *et al*., 2011). Thus present investigation revealed that both genotypes and media compositions and their interaction largely affected the callus.

Table 5. Interaction between the varieties and media for calli size of three rice varieties.

Media→ Varieties↓	MS		N_6		Mean of varieties
	C_1	C_2	C_3	C_4	
BRRI dhan86	0.45a	0.39a	0.41a	0.33a	0.39AB
BRRI dhan87	0.47a	0.51a	0.54a	0.37a	0.47A
BRRI dhan89	0.37a	0.27a	0.41a	0.31a	0.34B
Calli induction media mean	0.43A	0.39A	0.45A	0.34A	-

*In interaction means in both row and column indicate a common small letter (s) are not different significantly. (P≤0.05)

*In a row, calli induction media mean indicates a common capital letter (s) are not different significantly. (P≤0.05)

*In a column mean of varieties indicate a common capital letter(s) are not different significantly. (P≤0.05)

Regeneration of calli and green plant

In vitro plant regeneration was investigated on MS medium supplemented with different combinations of BAP, Kinetin, and NAA (Kaswan *et al*., 2012). Several factors, such as variety, developmental stage of cells in the explants, plant growth hormone composition in the medium, carbohydrates source, have been reported to improve the frequency of plantlet regeneration in rice (Muhammad *et al*., 2014). Plant regeneration was observed as early as three weeks and continued to occur up to four weeks after calluses were placed on regeneration media (Fig. 2a-e). Likewise callus proliferation, regenerative capacities varied considerably among rice genotypes. Table 4 shows the ANOVA results for the regeneration of calli and green plants. It was found that varieties were highly significant for regenerated calli induction and variety and regenerated media interaction showed highly significant differences for regenerated green plants.

The addition of day to the regeneration medium appeared to exert the least influence of all factors studied for plant regeneration frequency (Table 6). Nevertheless; day differentially affected subsequent plant regeneration of three varieties.

Mean frequency of regenerated calli induction over varieties (Table 7) showed that the variety BRRI dhan86 produced maximum callus (53.06%) which was not significantly higher than other varieties BRRI dhan87 (26.43%) and BRRI dhan89 (26.32%). Among the media composition, media R_2 (45.74%) was found to be most effective for regeneration. Although other varieties performed better in media R_3, R_4 and R_1, the quality of regenerated calli were not as good as those induced in R_2.

Mean frequency of green plants over varieties (Table 8) showed that the variety BRRI dhan86 produced maximum green plants (41.37%) which was not significantly different than the rest of the varieties BRRI dhan87 (32.71%) and BRRI dhan89 (31.01%). Among the media composition for producing green plant, R_2 was the found highest (47.34%) followed by R_3 (42.23%) which did not significantly differ from the other media R_1 and R_4. Although some varieties performed better in media R_3, R_4 and R_1, the quality of regenerated green plants was not as good as those induced in R_2. Given the highly significant genotype effect, the statistical analysis is shown separately for each of them (Table 8).

Table 6. Analysis of variance (ANOVA) for regeneration of calli induction and green plants.

Sources of variation	DF	Mean sum of square (MS)	
		Regenerated calli (%)	Green plants (%)
Variety (V)	2	15174.00***	1513.50
Calli induction media (C)	3	128.00	3962.67
Regeneration media (R)	3	1860.33	5258.33**
V:R	6	1480.17	9364.50***
V: D	2	2265.00	338.50
C:R	9	859.22	2184.33
V:C:R	12	1479.50**	1856.67
V: R: D	9	742.78	1592.44
Residuals	144	767.58	-

** and *** indicates significant at the 5% and the 1% level of significance respectively.

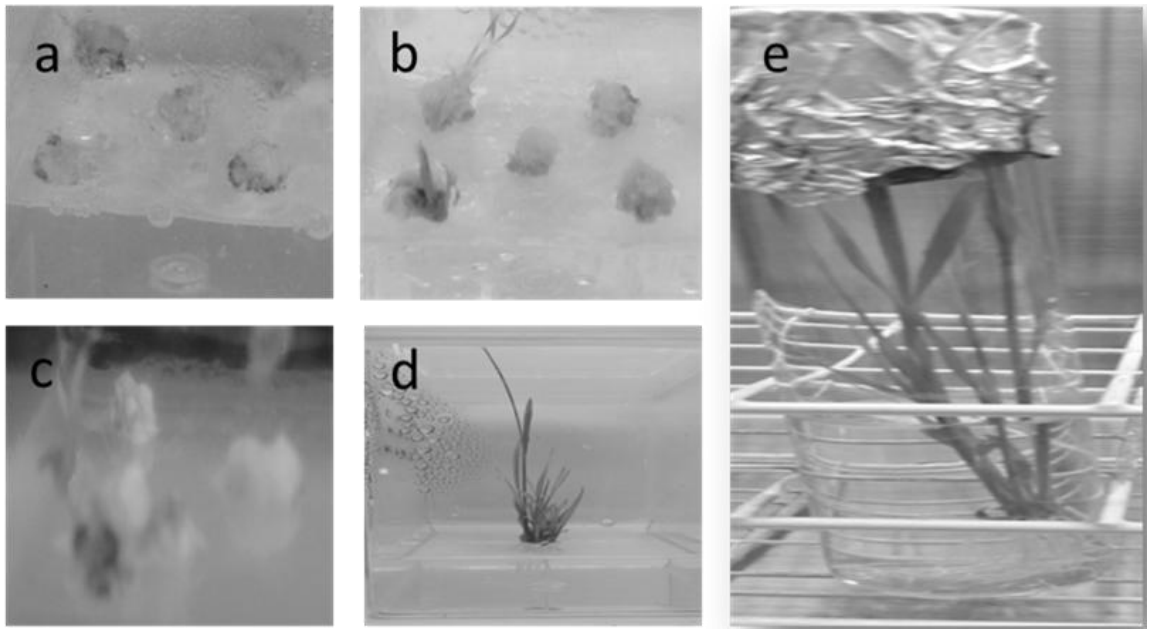

Fig. 2. Green bud initiation and induction of shoot (a-d), and established regenerated green plantlets of BRRI dhan86 (e).

Table 7. Interaction between the varieties and regeneration media for regenerated calli of three rice varieties.

Regeneration media (R) → Varieties (V)↓	R_1	R_2	R_3	R_4	Mean of varieties
BRRI dhan86	50.10a	76.10a	69.90a	67.00a	53.06A
BRRI dhan87	40.90a	38.10a	40.40a	37.30a	26.43B
BRRI dhan89	31.70a	72.80a	39.90a	41.40a	26.32B
Regeneration mean	29.26A	45.74A	38.64A	36.37A	-

*In interaction means in both row and column indicate a common small letter (s) are not different significantly. (P≤0.05)
*In a row, calli induction media mean indicates a common capital letter (s) are not different significantly. (P≤0.05)
*In a column mean of varieties indicate a common capital letter (s) are not different significantly. (P≤0.05)

Table 8. Interaction between the varieties and regenerated media for green plants of three rice varieties three rice varieties.

Plant regeneration media (R) → Varieties (V)↓	R_1	R_2	R_3	R_4	Mean of varieties
BRRI dhan86	31.20a	69.00a	82.70a	58.60a	41.37A
BRRI dhan87	69.40a	53.00a	40.20a	44.30a	32.71A
BRRI dhan89	38.80a	104.20a	71.00a	30.10a	31.01A
Regeneration media mean	27.55A	47.34A	42.23A	27.03A	-

*In interaction means in both row and column indicate a common small letter (s) are not different significantly. (P≤0.05)
*In a row, calli induction media mean indicates a common capital letter (s) are not different significantly. (P≤0.05)
*In a column mean of varieties indicate a common capital letter (s) are not different significantly. (P≤0.05)

In a subsequent study, in comparison to the results presented here, higher regeneration frequency was observed in scutellar calli of all the three genotypes tested when dehusked seeds used as explants. Dehusked seeds proved to be better explants than mature-embryos, possibly due to the involvement of endosperm factor(s) and some possible damage to the embryos during the process of embryo isolation from mature seeds. However, in the present study isolated embryos rather than dehusked seeds were used to eliminate any variation due to the involvement of endosperm. The results have demonstrated that the composition of basal media used for callus induction is not always optimum for plant regeneration as the nutritional requirements of the two phases of development may vary. Furthermore, this differential requirement was found to be variety dependent. Transfer of embryo-calli from callus induction medium to plant regeneration medium involved either a change of only the growth regulators or a change of both, growth regulators as well as the basal medium. Within a variety, significant differences affecting the regeneration parameters were observed between the two types of regeneration media (MS based regeneration medium and callus-induction basal medium based regeneration medium). In cultivar BRRI dhan86, variation was mainly due to regeneration media effect whereas in BRRI dhan87 and BRRI dhan89, callus induction medium affected the most. After transferring the calli into the regeneration medium, green spots became visible on the surface of the calli within 5-7 days were developed. MS medium supplemented with 4 mg/ml BAP+1.2 mg/ml kinetin+0.5 mg/ml NAA was found the best in respect to percent regenerated planlet (47.34%) as well as for the growth of plantlets *in vitro*. The addition of a small amount of Kinetin has been reported to improve embryogenic calli and shoot formation efficiency (Barbosa *et al*., 2014) in *indica* rice. Present finding agrees with the result of (Azizi *et al.,* 2016 and Barman *et al*., 2016) where it was reported that kinetin was found to be more effective for plantlets regeneration compared with BAP (6-benzylaminopurine). Combinations of Auxin and cytokinin along with the effect of basal salts played an important role in plant regeneration (Kumar *et al*., 2013).

The interaction effect of variety and medium on callus induction was not varied significantly. The highest callus induction was recorded in BRRI dhan86 (90.00%) in C_3 for callus induction and R_2 (Table 9). The lowest callus induction was observed in BRRI dhan87 (07.14%) in C_3 and R_1.

However, higher frequencies of regeneration were associated with certain calli induction media (Table 9). Interestingly, for BRRI dhan86 was appeared to be an equal relationship between callus induction and highest plant regeneration capacity.

CONCLUSIONS

In summary, rice varieties showed significant divergence for their *in vitro* response to callus induction. The quality and frequency of callus induction and subsequent plant regeneration, however, ultimately depend on the composition of initial callus induction treatment. Therefore, selection of better responsive rice variety like BRRI dhan86 and medium designated as C_1 for callus induction as like as R_2 for regeneration would offer great promise for the induction of higher level of desired somaclones and quality of callus for various means of genetic transformation as well as in the selection of stress tolerant cultivar development program and other relevant studies for improving this world's staple food crop.

Table 9. Interaction between the varieties and calli induction media on percent regenerated calli induction.

Variety	Callus induction media	Regeneration media			
		R_1	R_2	R_3	R_4
BRRI dhan86	C_1	47.08bc	61.07bc	50.00bc	75.00bc
	C_2	45.00bc	52.92bc	70.00bc	47.14bc
	C_3	39.58bc	90.00c	45.83bc	60.00bc
	C_4	8.33b	60.00bc	74.29bc	44.58bc
BRRI dhan87	C_1	22.5b	23.75bc	21.67bc	25.00bc
	C_2	25.00bc	23.75bc	46.67bc	20.00bc
	C_3	07.14b	23.33bc	20.00b	16.03bc
	C_4	63.75bc	35.00bc	30.00bc	30.83bc
BRRI dhan89	C_1	11.58b	0a	0a	0a
	C_2	0a	46.75bc	0a	0a
	C_3	0a	0a	19.72b	0a
	C_4	0a	0a	0a	20.30b

Zero (0) represented by non-regenerated plants.
*In interaction means in both row and column indicate a common small letter(s) are not different significantly. ($P \leq 0.05$)

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