

Somatic Embryogenesis and *In vitro* Irradiation in the Indigenous Rice Genotype ‘Lombur’

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Key words: Somatic embryogenesis, Lombur, LD₅₀, *In vitro* mutagenesis, Gamma ray

Abstract

Somatic embryogenesis was carried out following *in vitro* irradiation using the mature embryo of an indigenous rice genotype, Lombur in this study. Different doses of γ -radiation i.e., 10, 20, 30, 40, and 50 Gy were applied on mature embryo-derived embryogenic calli to optimize standard dose levels. The highest callus induction of 97.7% was found on MS medium supplemented with 13.65 μ M 2, 4-D under dark conditions. The highest embryogenic calli of 78.42% was observed in MS medium containing 20.47 μ M 2, 4-D. These embryogenic calli were transferred to MS medium fortified with 8.8 μ M BAP in combination with 0.27 μ M NAA and 46.42 % green spots on the calli were found upon transferring into light condition. A maximum of 74.86% of shoots were obtained on the same medium. The highest healthy rooted plantlets were shown on MS medium supplemented with 4.0 μ M NAA. The threshold level was estimated 33 Gy using the survival rate of embryogenic calli for the production of plantlets. Subsequent development of calli i.e. inoculated embryogenic calli, the survival rate of calli, green spots, regeneration frequency, and plantlet survived after callus induction was strongly negatively correlated with gamma irradiation. A successful somatic embryogenesis protocol has been established and irradiation doses for embryogenic calli has been optimized.

Introduction

Sustainable agriculture, of which rice makes up almost half, is considered to be the core heart of food security. Rice is the staple food for more than half of the global population. By 2050, the continuously increasing world's population is projected to have increased to 9.6 billion. Around 165 million hectares are used for rice farming which produces more than 776 million tons of rice per year (FAOSTAT 2023). The rice production needs around a 70% increase from the existing status to meet the demand (fao.org). This is one

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of the main concerns for the scientific community to meet the demand for such a huge population in the upcoming world. As a geographical location of Bangladesh, the Bay of Bengal is adjacent to the southern part of the country. Due to sea-water intrusion, the arable lands are reducing day by day, resulting in constraints to rice production. Though middle-income country with a high population density, Bangladesh achieved the third position in rice production, next to China and India (Wikipedia 2023). Increasing urbanization is reducing the amount of land available for rice farming and the gross production is insufficient for the nation's population, ideas for improving current cultivars are being considered. For the crop improvement, researchers perform the plant breeding program, covering everything from the selection of desired agronomical traits to biotechnological tools. Plant breeding becomes the highest priority program to develop new cultivars that are fitted regarding production to withstand adverse climatic conditions (Saud et al. 2022). Frequently addressed desired agronomical traits of plants are belong to the biotic and abiotic stresses. The conventional plant breeding cycle is associated with being time-consuming and costly compared to modern plant breeding, and it is responsible for reducing diversity, which is susceptible to pests and diseases. Plant mutation breeding, the burning topic of the modern approach, can boast unique and superior features on existing genotypes.

Raising of rice genotypes with improved tolerance towards salinity, various methods such as introduction, hybridization, marker assisted selection (MAS), genomic selection (GS), genetic transformation, mutation breeding and *in vitro* mutagenesis are used all over the world (Phillips 2008, Abe et al. 2012, Desta and Ortiz 2014, Mallick et al. 2015, Moore 2015, Takagi et al. 2015). However, to make these methods competent, different protocols such as effective callus induction and plant regeneration, have to be validated (Nishimura et al. 2006, Sahoo et al. 2011). Somatic embryogenesis is one of the preferred methods for the regeneration of rice. Plants derived from somatic embryos contain uniformity and genetic stability which is also an important trait. Modern plant improvement efforts rely heavily on *in vitro* culture techniques, such as somatic embryogenesis, which facilitate mutagenesis, genetic transformation, and clonal propagation (Hassan et al. 2021). Additionally, combining somatic embryogenesis with mutant breeding and *Agrobacterium*-mediated transformation has created opportunities for precision breeding, which enables the induction or inclusion of desired features in superior cultivars. To increase transformation efficiency and regeneration rates, for example, improved protocols have recently been devised for Chilean temperate Japonica rice cultivars (Barrera et al. 2024). In this event, researchers have a scope to look into its further improvement by generating genetic variability through some processes such as gamma irradiation. The main source of genetic variation is mutation and mutagens are the major factors for causing artificial mutations (Wei et al. 2013, Oladosu et al. 2016). *In vitro* mutagenesis has achieved importance since the 1970s because of its own potential to overcome the constraints of conventional mutagenesis, especially in the production and handling of large mutant populations, the establishment of reliable and easy to apply

screening methods (Maluszynski et al. 1995, Suprasanna and Nakagawa 2012). The effectiveness of mutagenic treatments increases when it is applied in plant tissue culture materials.

In light of the above discussion, the present study was carried out using an indigenous rice genotype, Lombur to identify the most suitable concentration and combination of plant growth regulators for the induction of re-generable callus, somatic embryogenesis, plant regeneration and to optimization of gamma irradiation dose level for the improvement of agronomic traits.

Materials and Methods

Indigenous rice genotype Lombur was collected from shrimp cultivated areas in Teknaf, Bangladesh. This rice genotype is putatively salt tolerant. Height is around 240 cm. Due to long height, it faces lodging and hence low yield. The dehusked 2000 mature rice seeds were used as explants. Dehusked mature rice seeds were disinfected with distilled water, followed by washing with a few drops of detergent, Trix and kept under running tap water for 30 min. Then seeds were soaked in 70% ethanol for 1-2 min, followed by 0.1% mercuric chloride (w/v) for 9-10 min and finally thoroughly washed 5-6 times with sterile distilled water in the laminar airflow cabinet. After disinfection, the seeds were inoculated on semi-solid media with different concentrations and combinations of plant growth regulators in this study.

The basic medium was used composed of MS salts and organic compounds, 30 g/l sucrose and 8 g/l agar. The pH was adjusted to 5.8 and then autoclaved for 20 min at 121°C and 1-07 kg/cm². For embryogenic callus induction, sterilized dehusked 1-2 rice seeds were inoculated in test tubes containing different concentrations and combinations of 2,4-D, Kn and NAA (Table 1). Some (50%) of these cultures were then kept at 25 ± 2°C in a dark condition for 6-8 weeks and some (50%) were kept at 25 ± 2°C under a 16 h light (60 μmolm⁻²s⁻¹) /8 h dark photoperiod for 8 weeks. The callus was observed every alternate day up to 8 weeks. The percentage of callus induction (%CI) was estimated for different treatments as the following fashion.

$$\%CI = \frac{\text{Total number of callus formed}}{\text{Total number of seeds inoculated}} \times 100$$

To induce somatic embryos, reproducible calli were subcultured after 8 weeks containing different concentrations and combinations of 2,4-D, Kn and NAA. The number of somatic embryos was obtained by counting under a microscope. Embryogenic cells were smaller, whitish, friable, glossy and globular in shape, dense cytoplasm and distinct nucleus whereas non-embryogenic cells were elongated and vacuolated, less cytoplasm and with one or devoid of nucleus. The percentage of somatic embryos formed from callus was calculated. After that, the somatic embryos were then transferred to MS media containing different concentrations and combinations of BAP and NAA like 0, 4.44, 8.88 μM and 0, 0.27 μl/L respectively. The shoots regenerated from the embryogenic calli were counted. The shoots were placed on ½ strength of MS media

alone or with the combination of NAA for root induction. The rooted plantlets were taken out from the culture vessels and washed carefully to remove the adhering media and transferred into small plastic pots for acclimation. Somatic embryo-derived plantlets were then placed in large clay pots filled with a 1 : 1 v/v mixture of soil and compost and maintained in a shed house for hardening.

Embryogenic calli were subjected to gamma irradiation at Gamma Source Division, Institute of Food and Radiation Biology, Atomic Energy Research Establishment, Bangladesh Atomic energy commission. Various dosages of γ - radiation (10, 20, 30, 40, and 50 Gy) were administered to mature embryogenic callus of indigenous rice genotype Lombur, along with control. After irradiation, the cultures were transferred onto fresh medium on that day or the following day. These cultures were then maintained as described in the materials and methods.

The data were analyzed for the estimation of variance, linear correlation, coefficient of variance (CV) at 0.05% level of significance through analysis of variance (ANOVA). The mean differences were adjusted with Duncan's Multiple Range Test (DMRT) using the Statistical Tools for Agricultural Research (STAR). LD50 was determined with Curve Expert 1.4.

Results and Discussion

A plant regeneration through embryogenesis has been found from different types of explants such as root, mature embryo, leaf, immature embryo and inflorescence of rice. Among these, mature embryo is arguably one of the best explants because of its have more potential for callogenesis as compared to node or tip (Rashid et al. 2000). In this case, different concentrations and combinations of plant growth regulators (PGRs) were used to induce callus from the dehusked seeds of indigenous rice genotype, Lombur. From the Table 1, it was observed that the highest 97.7% of explants induced callus induction on MS media supplemented with 13.65 μ M 2, 4-D under a dark condition, whereas 32% was found in light condition. This result is similar with the results of Choi et al. (1997) and Islam et al. (2013) where MS medium was shown to be more effective in terms of callus induction in rice cultivars (Choi et al. 1997, Islam et al. 2013).

After 3 to 4 weeks, only healthy calli were subcultured on MS media in combination with 13.65 μ M 2, 4-D and kept in a dark condition. It was observed that calli were formed after 3-4 weeks later. Different types of calli (Fig. 2A.) were found in this stage like compact white and creamy, loose friable and some calli responded with small roots (Table 1). The high degree of dedifferentiation and maximum somatic embryogenesis were shown in friable callus on MS media fortified with 20.47 μ M 2, 4-D (Table 1). This is also supported in this investigation that 2,4-D was responsible for producing the maximum meristematic system consistent with the result of Souza et al. (Souza et al. 2014). This finding is similar with the result of Lindsey and Jones demonstrated that friable callus was loosely aggregated cells with lower density whereas compact aggregated cells were thicker, with higher density (Lindsey and Jones 2000). The degree

of callusing was found with better manner among the treatments and the highest percentage of somatic embryogenesis was observed 78.42 upon culturing on MS medium supplemented with 20.47 μM 2,4-D (Fig. 2A).

Table 1. Effects of 2, 4-D alone or in combination with Kn/NAA on callus induction as well as somatic embryogenesis of *Lombur* in light and dark conditions.

Treatments (μM)			Response explant (%)		Colour and types of callus	Degree of callusing	Somatic embryogenesis (%)
2,4-D	Kn	NAA	Light	Dark			
4.55	-	-	25 ^{cd}	58.33 ^e	Creamy & Compact	+	51.33 ^{de}
9.1	-	-	26.67 ^{bc}	82 ^c	Whitish & Friable	+++	61.6 ^c
13.65	-	-	32^a	97.7^a	Whitish & Compact	+++	71 ^{ab}
18.2	-	-	28 ^b	87.3 ^{bc}	Yellowish & Compact	+++	63.7 ^{bc}
4.55	2.32	-	27 ^{bc}	69 ^d	Yellowish & Hardy	++	39.9 ^f
4.55	-	50	25.5 ^{bcd}	57.67 ^e	Brown & Compact	++	43.26 ^{ef}
4.55	4.65	-	23 ^d	65.33 ^d	Creamy & Hardy	++	51.86 ^d
20.47	-	-	26 ^{bc}	89.67 ^b	Creamy & Friable	++++	78.42^a
CV (%)			5.53	5.03			8.09

In a column, the figures with similar letter (s) do not differ significantly by DMRT (Duncan's multiple range test) at $p < 0.05$; CV: Coefficient of variation. Degree of callusing (+, ++, +++) means semi-quantitative scoring to denote the amount of callus formation.

In the case of observing, two types of cells were found under the microscope based on the stages of embryo formation. Globular cells with glossy apex, abundant in cytoplasm and distinct nuclei are embryogenic (Fig. 2A). On the other hand, elongated cells with uneven in shape and vacuolated with less cytoplasm are non-embryogenic is in evidence in this study (Fig. 2A) (Moghaddam and Taha 2005).

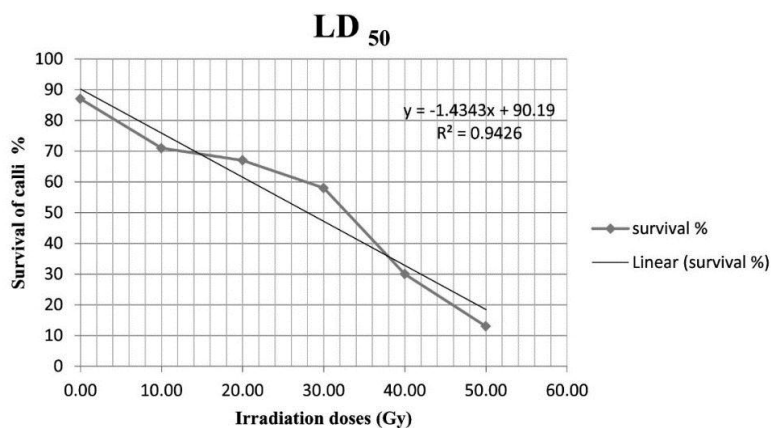


Fig. 1. Determination of LD₅₀ of the embryogenic calli of *Lombur* upon gamma irradiation with different doses.

Different dose levels of acute γ - irradiation i.e. 10, 20, 30, 40 and 50 Gy were applied to mature embryo derived embryogenic calli. The highest percentage of survived calli was found 87 in the control. Among the irradiated treatments, the highest (71) percentage

of survived calli was found in 10 Gy and the lowest (13) was obtained in 50 Gy. LD₅₀ was determined using Curve Expert 1.4 after 2 weeks on MS media with 8.88 μM BAP and 0.27 μM NAA and 50% survival rate was found at 33 Gy of gamma irradiation (Fig. 1). It was reported that in mutation breeding, the dose should be chosen in the result of the highest survival of irradiated explants but low inhibition rate of the production of new shoots which would give the highest efficiency to overcome useful mutants (Laneri et al. 1990).

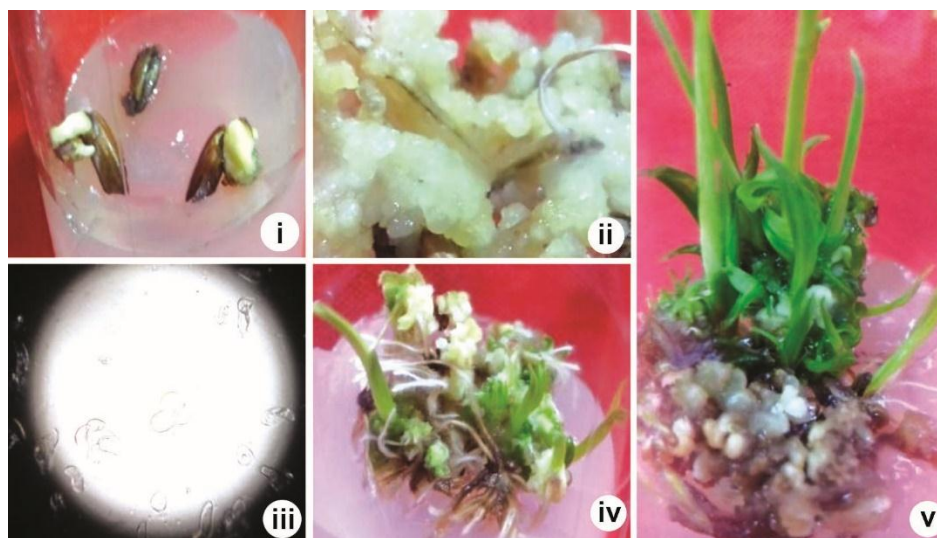


Fig. 2(A). Somatic embryogenesis: (i) initiation of calli on MS medium supplemented with 20.47 μM 2,4-D after 5-7 days of cultures, (ii) proliferation of calli after 4 weeks, (iii) microscopic view ($\times 10$) of callus cells of both embryogenic and non-embryogenic (iv) initiation of green plant parts on MS medium containing 8.88 μM BAP and 0.27 μM NAA, and (v) elongation of shoots.

The calli were observed for the greening percentage and average number of regenerated plantlets from different irradiation doses shown in Table 2. For greening of callus, five treatments were given in which we found that a radiation level of 10 Gy resulted in the highest greening percentage of 46.42 % followed by 20 Gy (40.62), 30 Gy (33.33), 40 Gy (20.83) and 50 Gy (03.84) (Fig. 2B). Among these treatments, 10 Gy and 20 Gy showed equally good performance to initiate greening. But the greening percentage decreased sharply followed with the increase in dose level suggesting they are negatively correlated with each other. In a study on sugarcane callus cultures, gamma irradiation at doses of 20, 40, 60, and 80 Gy caused a progressive drop in shoot regeneration percentages. In particular, regeneration rates decreased from more than 90% 20 Gy to 0% at 80 Gy, suggesting a significant inverse relationship between irradiation dose and the development of greening or shoots (Kaur and Gosal 2009). Studies on gamma-irradiated rice calli revealed that as radiation dosages increased, callus growth and plant regeneration abilities declined (Snigdhya et al. 2024).

Table 2. Effects of gamma irradiation on calli and their subsequent developments towards plantlet formation in rice genotype on MS+ 8.88 μ M BAP and 0.27 μ M NAA.

Dose levels (Gy)	Clump of embryogenic calli inoculated (no.)	Survival of calli (%)	Green spots (%)
0	18	87 ^a	52.63 ^a
10	28	71 ^{ab}	46.42 ^{ab}
20	32	67 ^b	40.62 ^b
30	27	58 ^{bc}	33.33 ^{bc}
40	24	30 ^c	20.83 ^c
50	26	13 ^d	03.84 ^d

In a column, the figures with similar letter (s) do not differ significantly by DMRT (Duncan's multiple range test) at $p < 0.05$.

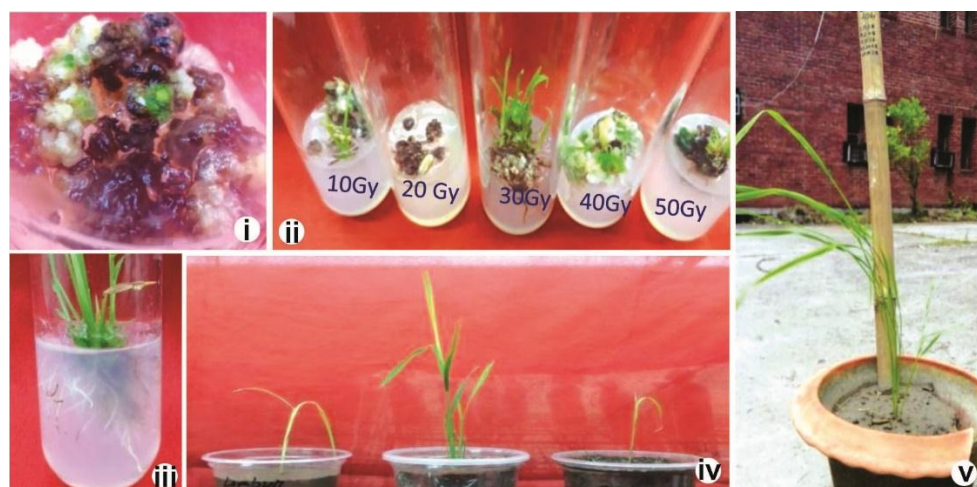


Fig. 2(B). *In vitro* mutagenesis and acclimatization: (i) effect of irradiation on embryogenic callus, (ii) different developmental stages of survived embryogenic cell upon gamma irradiation doses of 10, 20, 30, 40 and 50 Gy and (iii) root initiation of shoots on half MS medium containing 4.0 μ M NAA, and (iv & v) acclimatization of survived plantlets of Lombur in small plastic pots for 7 days, then transferred to large clay pots.

It was found that a very low percentage (3.84 %) of greening observed in the case of a 50 Gy gamma irradiation dose compared to other dose levels (Fig. 2B). This study indicated that gamma irradiation has detrimental effects on callus to initiate greening and towards plantlet formation. Significant changes in callus color and shape were observed in *Artemisia annua* at higher doses, demonstrating the effect of irradiation on callus development (Azeez et al. 2017). After greening of callus, calli were transferred to regeneration media containing MS with 8.88 μ M BAP and 0.27 μ M NAA. Plant regeneration was observed in different doses of gamma rays. The highest 74.86 % plant regeneration was found in 10 Gy on MS medium supplemented with 8.88 μ M BAP in combination with 0.27 μ M NAA followed by 20, 30, 40 and 50 Gy (Table 3).

Table 3. Effects of PGRs on plant regeneration from somatic embryos of Lombur.

Treatments (μ M)		Clump of embryogenic calli (no.)	Regeneration (%)
BAP	NAA		
4.44	-	20	65.10c
4.44	0.27	23	72.97a
8.88	-	21	66.32bc
8.88	0.27	24	74.86a
13.32	-	23	60.42d
13.32	0.27	22	67.86b
CV%			1.03

In a column, the figures with similar letter (s) do not differ significantly by DMRT (Duncan's multiple range test) at $p < 0.05$; CV: Coefficient of variance.

Table 4. Study of correlation on subsequent development of calli with gamma irradiation.

	Dose levels	IEC (no.)	Survival of calli (%)	Green spots (%)	RF (%)	Plantlet survived (no.)
Dose levels	1					
IEC (no.)	0.22	1				
Survival of calli (%)	-0.97	-0.11	1			
Green spots (%)	-0.97	-0.10	0.99	1		
RF (%)	-0.96	-0.10	0.98	1.0	1	
Plantlet survived (no.)	-0.99	-0.18	0.98	0.99	0.98	1

IEC: Inoculated Embryogenic Calli; RF: Regeneration Frequency; Correlation is an effect size and so we can verbally describe the strength of the Correlation using the guide that Evans (1996) suggests for the absolute value of r : (.00-.19 very weak; .20-.39 weak; .40-.59 moderate; .60-.79 strong; .80-1.0 very strong).

In Table 4 the study of correlation between subsequent developments of calli i.e. inoculated embryogenic calli, survival rate of calli, green spots, regeneration frequency, and plantlet survived with gamma irradiation was done. Gamma irradiation dose had a very strong negatively linear correlation with callus survival ($r = -0.97$), green spot formation ($r = -0.97$), regeneration frequency (RF) ($r = -0.96$), and plantlet survival ($r = -0.99$), according to the correlation analysis. This represents that the regenerative capacity of embryogenic calli significantly decreased with the increase in gamma irradiation doses. Higher gamma-ray doses have been shown to have similar detrimental effects on tissue culture response and plant regeneration in rice and other crop species, where callus growth and morphogenesis were hampered by radiation-induced cellular damage, oxidative stress, and chromosomal abnormalities (Mba et al. 2010, Hossain et al. 2021). These results are also similar with the study reported by Sakr et al. where they observed that number of shoot culture were increasing at low level gamma irradiation (Sakr et al. 2013). On the other hand, green spot development ($r = 0.99$), regeneration frequency ($r = 0.98$), and plantlet survival ($r = 0.98$) all showed very substantial positive relationships with callus survival. Additionally, green spot production was substantially

connected with plantlet survival ($r = 0.99$) and perfectly correlated with regeneration frequency ($r = 1.00$), indicating that efficient maintenance of viable calli is a precondition for shoot regeneration and subsequent plant establishment. These results are consistent with earlier findings that callus viability and the capacity of irradiated tissues to maintain cellular competence for organogenesis are directly related to regeneration efficiency (Ahloowalia et al. 2004, Jain 2010).

The survived plantlets were transferred to small plastic pots containing soil and compost ratio (1 : 1) and were kept for 7 days in growth room for acclimatization. Then they were transplanted in large clay pot under shed house for hardening with 10 % survival rate (Fig. 2B iv & v).

Two weeks, after inoculation, was needed for callus formation where somatic embryos were formed after 7 weeks. The time of shoot initiation and root initiation were required 2-3 weeks where only 1 week was needed for acclimatization. These results revealed that 13 to 17 weeks was needed for establishing plantlets under *ex vitro* conditions.

Effective *in vitro* mutagenesis is firmly established by the successful development of a somatic embryogenesis process employing mature embryos of the native rice genotype Lombur. Hormonal combinations that were tuned for callus induction, embryogenic callus development, and shoot regeneration showed great efficiency; 4.0 μM NAA was the most effective for subsequent rooting. Gamma irradiation therapy demonstrated a 33 Gy threshold dose, above which regeneration potential precipitously decreased, suggesting its crucial function in striking a balance between tissue viability and mutagenesis efficacy. Increasing γ -ray doses are negatively correlated with regeneration parameters, confirming embryogenic tissues' susceptibility to ionizing radiation. This study supports the promise of integrated somatic embryogenesis and *in vitro* mutagenesis techniques in expediting rice breeding efforts for sustainable agriculture in Bangladesh and abroad, in addition to aiding in the genetic development of locally adapted rice varieties.

Acknowledgement

Authors are grateful to the Gamma Source Division, IFRB, BAEC for helping in irradiation of the embryogenic calli during this study.

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