Bangladesh J. Nuclear Agric, 38(2): 151-159, 2024 *ISSN 0258-7130*
DOI: https://doi.org/10.3329/bjnag.v38i2.79585 ***Short communication***

PROTOCOL DEVELOPMENT OF IRRADIATED RICE CALLI AND SUBSEQUENT *IN VITRO* REGENERATION

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Abstract

Fine and aromatic rice varieties, such as Kataribhog, hold significant commercial value in Bangladesh, particularly in region like Dinajpur where Kataribhog is primarily cultivated. Despite its popularity, Kataribhog rice has a low yield (less than 2.50 t ha^{-1}) and is prone to lodging. To address these issues, a study was conducted using *in vitro* culture techniques combined with gamma irradiation (^{60}Co) to improve the cultivar. Three different media (PT-100G, PT-011G, and PT-100) were used to induce callus, followed by exposure to varying radiation doses (10Gy, 12Gy, and 15Gy). The PT-100G medium found the most effective, inducing an embryogenic callus formation rate of 32.93%, while PT-011G and PT-100 showed lower rates 27.87% and 11.11%, respectively. A radiation dose of 10Gy was found optimal, with an 80% survival rate for calli and the best plant regeneration outcomes.

Keywords: Kataribhog rice, gamma irradiation, embryogenic callus

Rice (*Oryza sativa* L.) is a vital cereal crop and staple food for over half the global population, especially in Asia, where it is critical to food security by providing a significant portion of daily calorie intake (Tyagi *et al*., 2004; Khush, 2005). With population growth, demand for rice is set to increase significantly in Asia, Africa, and Latin America (Wang and Li, 2005). The United Nations underscored rice's importance by declaring 2004 the International Year of Rice, predicting a 70% rise in demand within 30 years (IRRI, 2003).

Rice belongs to the Gramineae family, consisting of 21 wild species and two cultivated species. *Oryza sativa* is globally widespread, while *O. glaberrima* is grown in West Africa (Dai *et al*., 2012). Economically, rice is a key agricultural commodity, sustaining millions of livelihoods (FAO, 2021). It holds cultural significance in Asia and is a vital source of carbohydrates and micronutrients. Efforts like biofortification aim to enhance its nutritional value (Bouis and Welch, 2010).

In Bangladesh, rice occupies 78% of total cropped area, achieving self-sufficiency for its 169.04 million people. Production has tripled since independence, reaching 36.6 million tons in 2019-20 (BBS, 2020). This growth resulted from improved seeds, fertilizers, and advancements in rice varieties. However, climate change poses challenges, including heat stress, erratic rainfall, droughts, flooding, and salinity (Hossain and Majumder, 2018).

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The aromatic Kataribhog rice, grown in Dinajpur, offers a high yield of 2.54 t ha^{-1} and potential for export due to its quality and low production costs. Research in Bangladesh focuses on improving rice traits using tissue culture, genetic engineering, and mutagenesis to enhance yield, pest resistance, and climate resilience, ensuring sustainable rice production.

This study was conducted at the Tissue Culture Laboratory of the Biotechnology Division, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. Mature Kataribhog rice seeds from BINA were used as explants for callus initiation and plant regeneration.

Media Composition and Preparation

The tissue culture process for this study employed Murashige and Skoog (MS) medium (1962), customized in different formulations to optimize the stages of callus induction and plantlet regeneration in Kataribhog rice. The medium was composed of macronutrients such as KNO_3, $NH4NO_3$, KH_2PO_4, $CaCl_2 \cdot 2H_2O$, and $MgSO_4 \cdot 7H_2O$, prepared as a 10X stock solution, and micronutrients like $MnSO_4 \cdot 4H_2O$, H_3BO_3, $ZnSO_4 \cdot 4H_2O$, KI, $Na_2MoO_4 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, and $CoCl_2 \cdot 6H_2O$, prepared as a 100X stock solution. Additionally, $FeSO_4 \cdot 7H_2O$ and Na_2-EDTA were used as iron sources in a 10x solution, while organic nutrients and vitamins, such as glycine, nicotinic acid, pyridoxine-HCl, thiamine-HCl, myo-inositol, agar, and sucrose, were added as required. For callus induction, MS medium was supplemented with 2, 4-D and additives like sucrose, maltose, proline, and glutamine, creating an environment conducive to callus formation. Shoot differentiation was achieved by enriching the medium with 0.5 mg/L NAA and 10 mg/L kinetin, promoting shoot regeneration from callus tissue, while rooting was induced using half-strength MS medium supplemented with IBA. PT-100G, PT-011G, and PT-100 different three types of media, widely utilized in rice tissue culture, were specifically selected for this research to optimize callus induction and plant regeneration. These media differ in their precise concentrations of macronutrients, micronutrients, vitamins, and plant growth regulators, providing tailored environments for each stage of tissue culture. Their balanced compositions ensured effective nutrient availability, enhancing the developmental processes of Kataribhog rice tissue culture.

Irradiation

Seeds of the local rice cultivar Kataribhog were germinated under aseptic conditions to produce seedlings. Calli were induced from the germinated seedlings by culturing them on callus induction media supplemented with plant growth regulators. The resulting calli were then exposed to gamma irradiation at doses of 10, 12, and 15 Gy using a ^{60}Co gamma source at the Bangladesh Institute of Nuclear Agriculture, Mymensingh. Following irradiation, the treated calli were cultured on regeneration media to evaluate the effects of different gamma irradiation doses on plant regeneration efficiency and morphological responses during the in vitro regeneration process.

Sterilization and Culture Procedures

Rice seeds were dehusked, washed, and surface sterilized with 50% NaOCl combined with 2 µl Tween 20. All media were autoclaved at 121°C and 1.6 kg/cm² for 20 minutes. Glassware and instruments were sterilized at the same conditions. The culture room and transfer area were regularly cleaned and sterilized using 70% ethyl alcohol and UV light.

Recording of Data

To investigate the effect of different treatments of the experiment, data were collected on the following parameters:

$$\text{Percent (\%) callus induction} = \frac{\text{Number of explants induced calli}}{\text{Number of explants inoculated}} \text{ X } 100$$

$$\text{Percent (\%) embryogenic callus induction} = \frac{\text{Number of explants induced embryogenic calli}}{\text{Number of explants inoculated}} \text{ X } 100$$

$$\text{Percent (\%) shoot regeneration} = \frac{\text{Number of calli showing shoot}}{\text{Number of inoculated calli}} \text{ X } 100$$

Percent root induction

The percentage of root induction was calculated on the basis of the number of regenerated shoots transferred to the rooting medium and the number of regenerated shoots produced root.

$$\text{Percent (\%) establishment} = \frac{\text{Number of established plantlets}}{\text{Total Number of plantlets}} \text{ X } 100$$

Statistical Analysis

The data for the characters were statistically analyzed wherever applicable. The experiments were conducted in a growth chamber and arranged in CRD. Different characters underwent analysis of variance, and Duncan's Multiple Comparison method was used to compare the mean values utilizing the MSTAT-C statistical package (Russel, 1986).

This study investigated three types media–PT-100G, PT-011G, and PT-100–for embryogenic callus formation using mature embryos of the local rice cultivar Kataribhog. The experiment aimed to achieve embryogenic callus formation by culturing mature embryos on MS medium with various hormone concentrations and combinations.

Callus initiation response of Kataribhog seeds on MS medium

In the study, seeds of the local rice cultivar Kataribhog were cultured on MS medium supplemented with 2, 4-D (2.0 mg/L). Callus developed from the scutellum region within 14 days, with the highest callus initiation observed on PT-100G (T1), followed by PT-011G (T2) and PT-100 (T3), resulting in an average callus initiation rate of 58.72%. The study confirms previous research of callus initiation in rice (Katiyar *et al*., 1999; Zhenyu *et al*.,

1999; Briside *et al*., 1990; Azria and Bhalla, 2000). Similar findings were reported by Gao and Huang,1999; and Aditya *et al*. 2004, who also identified 2.0 mg/L as optimal. Higher concentrations of 2, 4-D were found to inhibit callus induction, likely due to cell damage (Seraj *et al*., 1997) (Fig. 1).

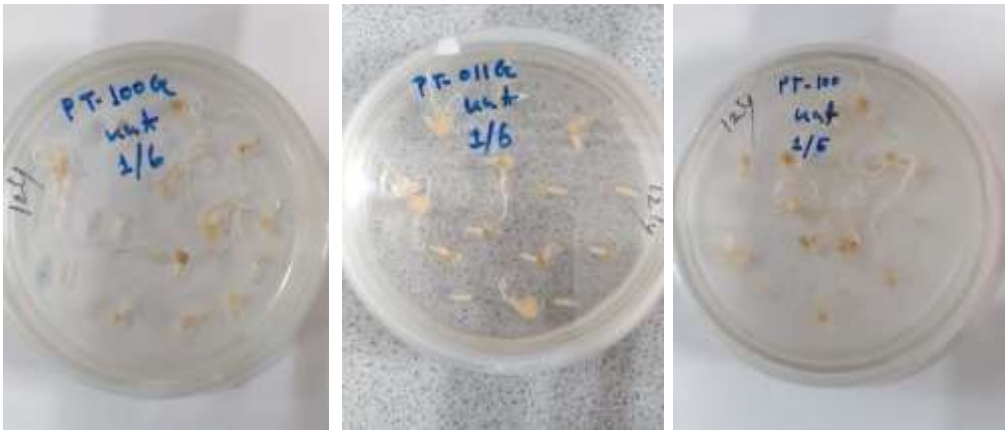

Fig. 1. Callus initiation efficiency from mature embryos of Kataribhog on MS medium.

Table 1. Callus initiation efficiency from mature embryos of local rice cultivar (Kataribhog) on MS medium

Treatment	No. of explants inoculated	No. of explants showing callus initiation	Callus initiation (%)
PT-100G	82	65	79.27
PT-011G	61	32	52.46
PT-100	63	28	44.44

Embryogenic callus initiation response of mature embryos of Kataribhog rice on various treatments

Mature embryos of the local rice cultivar Kataribhog were subjected to various treatments to evaluate embryogenic callus formation. Embryogenic calli developed within 21 days. callus initiation performance of the local rice cultivar (Kataribhog) is presented in Table2. Among the treatments, PT-100G (T1) showed the highest embryogenic callus initiation rate at 32.93% followed by PT-011G (T2) at 27.87% and PT-100 (T3) at 11.11% with an overall average of 23.97%. The initiation of embryogenic calli is recognized as a crucial step in rice tissue culture. Embryogenic calli are compact, nodular, cream to yellowish-white structures with a smooth, firm texture. They are identified by their organized appearance, regeneration potential on media, and distinct color compared to non-embryogenic calli. Histological examination reveals densely packed cells with small vacuoles and prominent nuclei, confirming embryogenic competence. (Fig. 2).

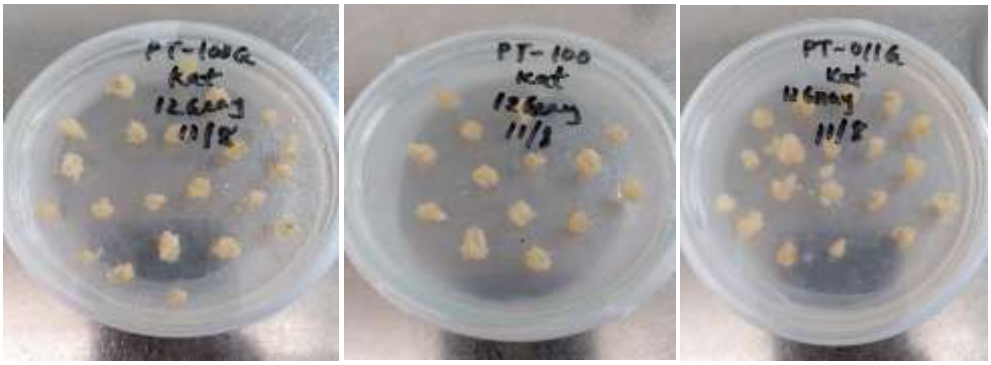

Fig. 2. Embrogenic callus initiation from mature embryos of Kataribhog rice cultivar under various treatment 3 types of media (PT-100 G, PT-011G and PT-100).

Table 2. Embryogenic callus initiation response of mature embryos of Kataribhog rice on various media treatments

Treatment	No. of explants inoculated	No. of explants showing embryogenic callus initiation	Embryogenic callus initiation (%)	Average of embryogenic callus initiation (%)
PT-100G	82	27	32.93	23.97
PT-011G	61	17	27.87	
PT-100	63	7	11.11	

Effect of Gamma Irradiation of Callus on Shoot Regeneration

In this study, different doses of gamma irradiation (10, 12, and 15 Gy) were applied to calli of the local rice cultivar Kataribhog to assess the impact on plant regeneration. The irradiated calli were transferred to MS medium supplemented with 1.5 mgL^{-1} NAA and + 2.5 mgL^{-1} BAP for plantlet initiation. After 21 days, it was clearly observed (Table 3) that the regeneration rates varied based on the irradiation dose with 10 Gy showing the highest regeneration percentage (80%) followed by 15 Gy (71.43%) and 12 Gy (66.67%) (Fig. 3). This aligns with previous findings, as gamma irradiation at optimal doses has been reported to stimulate cellular activity, leading to enhanced regeneration (Majeed *et al*., 2018; Rahman *et al*., 2020). However, excessive doses may cause cellular damage, explaining the reduced regeneration at 12 and 15 Gy compared to 10 Gy. These results suggest that irradiation can be a valuable tool for inducing beneficial mutations and improving regeneration in rice tissue culture.

Table 3. Effect of irradiation on callus for shoot regeneration of Kataribhog rice

Dose (Gy)	No. of irradiated calli sub-culture	No. of calli survived	No. of survived calli showing shoot induction	Shoot regeneration (%)
10	18	15	12	80.00
12	23	3	2	66.67
15	41	7	5	71.43

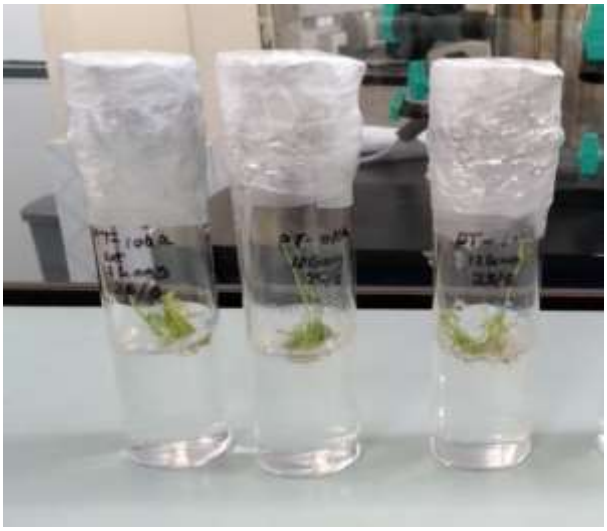

Fig. 3. Shoot regeneration from callus of Kataribhog rice cultivars with gamma irradiation (10Gy) treatment on 3 types of media (PT-100 G, PT-011G and PT-100).

Effect of Gamma Irradiation on Root Induction from Shoot

The regenerated shoots of the local rice cultivar (Kataribhog) required sufficient roots to establish them in the soil. MS media supplemented with 0.5 mgL^{-1} IBA was used to see the rooting response of the regenerated shoots. Root induction (%), number of roots per plant were studied in this experiment. After 15 days, it was clearly observed (Fig. 4) that the extent of root induction ability varied from shoots that were differentiated due to different doses of irradiated calli. In the genotype (Kataribhog), root induction percentages were found to be higher in the 15 Gy (60%) followed by 10 Gy (58.33%) and 12 Gy (50%). These results suggest that gamma irradiation at certain doses can positively influence rooting ability in regenerated plants, likely by enhancing auxin sensitivity or stimulating cellular activity in the shoot base. Similar findings have been reported by Alam *et al*., 2021 and Khan *et al*., 2019, who demonstrated improved rooting responses in irradiated tissues of rice and other crops when cultured with IBA. The slight decrease in root induction at 12 Gy may be attributed to sub-lethal damage caused by irradiation, which affects cellular differentiation and root initiation. These observations highlight the role of gamma irradiation in improving rooting potential for successful acclimatization in tissue culture-derived plants.

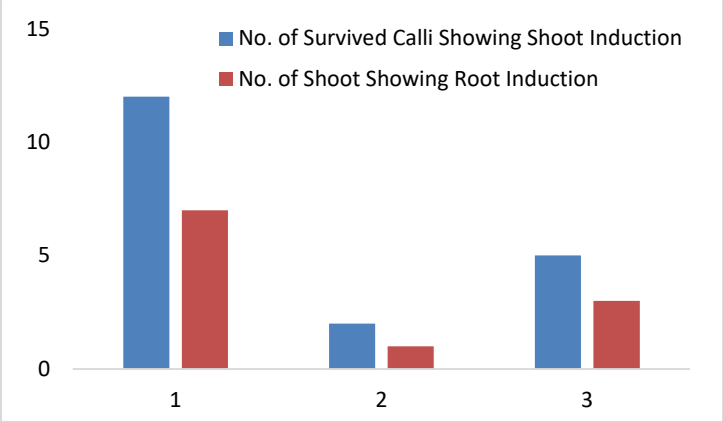

Fig 4. Effect of gamma irradiation on root induction in Kataribhog rice calli (Y axis = % of shoot or root induction, X axis = replication).

Fig. 5. Root induced from shoot of Kataribhog rice cultivars on MS + 0.5 mgL^{-1} IBA+3 types of media (PT-100G, PT-011G and PT-100).

Fig. 6. Established plants of Kataribhog rice cultivars in earthen plot on 3 types of media (PT-100G, PT-011G and PT-100).

After sufficient development of the root system, the small plantlets were taken from the culture vessels without damaging the roots. Excess agar around the roots was washed off by tap water to prevent microbial infection. Then the plantlets were transplanted in small plots. When the plantlets grew to a height of above 10 cm and sufficient roots were proliferated, those were transferred to earthen pots following the procedure described in materials and methods. The growth condition, the tillering capacity of plantlets, and the survival rate of the plantlets in the plot (Fig. 6) were satisfactory. Variation due to irradiation was observed in plantlet growth, tillering capacity, and survival rates, with 10 Gy showing the highest performance compared to 12 and 15 Gy.

This research successfully optimized the callus initiation and regeneration protocols for the local rice cultivar Kataribhog. Gamma irradiation emerged as an effective tool for enhancing in vitro regeneration, with 10 Gy producing the highest shoot regeneration rate (80%) and improved root induction. These results provide a reliable method for regenerating plants from mature embryos and demonstrate the potential of gamma irradiation in boosting regeneration efficiency. This study not only establishes a practical protocol for Kataribhog but also offers a framework for improving other rice cultivars, contributing significantly to the advancement of rice improvement programs and crop development strategies.

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