

EFFECT OF GAMMA IRRADIATION ON EMBRYOGENIC CALLI OF RICE AND SUBSEQUENT SHOOT AND REGENERATION

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Abstract

This study was carried out during May 2021 to January 2022 at the Tissue Culture Laboratory of Biotechnology Division, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh, Bangladesh for callus induction and regeneration ability of plantlet from low doses irradiated embryogenic calli of Binadhan-13 and BR5 (Dulabhog). The effect of low gamma rays such as 4, 6 and 8 Gy were observed *in vitro* shoot, root and plantlet formation. Two different incubation conditions such as light and dark incubation were maintained. Between two varieties, Binadhan-13 showed the highest ability of callus induction in both conditions. Callus induction was significantly higher under dark condition (77.14%) than light condition (67.14%) at 21 days. Data revealed that gamma rays affect both shoot and root regeneration ability of embryogenic callus. Shoot regeneration ability was the highest at 4 Gy in Binadhan-13 (60%) and gradually decreased with the increased doses of gamma rays. Among the treatments, shoot regeneration ability (60% in Binadhan-13 and 50% in BR5) was higher at 4 Gy dose of gamma ray followed by 6 Gy where shoot regeneration ability was 55% and 45% in Binadhan-13 and BR5 respectively. In root induction, the highest root induction ability (80%) was observed in the cultivar Binadhan-13 at control condition. Like shoot regeneration, 4 Gy gamma ray showed better root induction in both the varieties (70% in Binadhan-13, 60% in BR5). The *in vitro* regenerated plantlets from irradiated embryogenic calli were successfully transferred to soil in pots. The regeneration protocol could be further used for varietal improvement of rice varieties using nuclear technique (⁶⁰Co gamma ray).

Keywords: Embryogenic callus, Irradiation, Regeneration protocol, Rice.

Introduction

Rice (*Oryza sativa* L.) is the most important human food crops and directly supplies food of more than three billion people in the world (Tyagi *et al.*, 2004). It is also the staple food around Asia where half of the world people live and is becoming increasingly important Latin America and Africa (Muthayya *et al.*, 2014). Compare to other crop in the world, rice is grown by more people than any other crops. Bhuiyan *et al.* (2002) reported that rice supplies 92.85% of food requirement, 55% of protein and 75% of daily calorie intake where these have led overall improvement in health, literacy and life

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expectancy and more importantly declined the poverty level in Bangladesh. Rice belongs to Gramineae family and the genus *Oryza* and believed to have originated 130 million years ago (Khush, 1997). Cultivated rice (*Oryza sativa* L.) is divided into 3 subspecies namely *indica*, *japonica*, and *javanica* (Datta *et al.*, 2003). Indica type rice feeds more than two billion people, predominately in developing countries.

In Bangladesh, rice is the most dominant and main food crop, which grows in all the three crop growing seasons of the year. The climate and geographical conditions of Bangladesh are favorable for year-round production during Aus, Amon and Boro seasons. Bangladesh ranks 3rd in area and production (FAO, 2021) and 39th in yield of rice among the rice growing countries (Calpa, 2004). Rice is grown over 11.5 million hectares of land with total production of 35.65 million metric tons in Bangladesh (BBS, 2022). It occupies about 77% of the total cropped area of about 13.9 million hectares. A modest estimate suggests that the demand for rice in Bangladesh will increase by over 80% in the next 20 years to feed the growing population (Zaman, 1996). Furthermore, the agriculture production is challenged by numerous natural disasters due to the impact of climate change. Therefore, at present plant biotechnology (Hoque *et al.*, 2007) or various tissue culture techniques (Zapata *et al.*, 1987), widely recognized breeding tools, are being used for the genetic improvement of rice plant throughout the world (Raina, 1989).

Embryogenesis is a process in which bipolar structures resembling a zygotic embryo develop from a non-zygotic cell without vascular connections with the original tissue (Arnold *et al.*, 2002). It is a promising method for the establishment of protocols reaching rapid multiplication of new and elite genotypes (Kamle *et al.*, 2011). Biotechnology, while making use of both mutagenesis and conventional methods, can assist in overcoming hurdles during the development of new and improved cultivars for sustainable crop production (Jain and Swennen, 2004). The most commonly used mutagens so far are physical mutagens such as gamma rays (Roux *et al.*, 2004). The use of whole plantlet or part of plant organs and tissues as irradiation treatment materials is generally considered to be prone to chimera. In contrast, the use of callus as irradiation treatment material is theoretically a single cell mutation, which is conducive to the acquisition of stable mutants, and shortens the breeding cycle (Bai *et al.*, 2022). The following ranges of doses are recommended: 10–20 Gy for diploid cultivars (AA and BB), 30–40 Gy for triploid cultivars (AAA and AAB), and 40–50 Gy for triploid cultivars (ABB) (Roux *et al.*, 2004).

However, immature embryo culture and matured seed culture are important in rice to create additional variation and novel rice varieties (Sathish *et al.*, 1995). The use of matured seed embryos has distinct advantage over other explants as starting material for *in vitro* regeneration. Callus induction and subsequently plant regeneration is dependent on the type of explants, growth conditions and plant species (Feng *et al.*, 1995). So, the best growth condition, suitable explants and varieties are needed to be identified for large scale utilization in rice improvement program through biotechnology. Nowadays, callus initiation and use of mutagen for induction of mutation in the callus is one of the proven

tools employed by plant breeders for creating variability in crop plants. This technique is known as *in vitro* mutagenesis that is a sudden change in heritable characters of an organism which serves as a source of creating variability for better selection in short time. Tissue culture as well as callus production followed by irradiation is an important tool for creating variants and could play an important role in crop improvement (Bansal *et al.*, 1990). The application of ionizing radiation in optimal dose to plant tissue culture is to investigate the culture response and *in vitro* culture, in combination with induced mutation could open new ways in which exploit somaclonal variations for plant improvement. The present piece of research work was undertaken to study the callus induction and regeneration ability of Binadhan-13 and BR5 and to study the effect of gamma irradiation on embryogenic calli of Binadhan-13 and BR5, and their subsequent regeneration potential.

Materials and Methods

The experiment was conducted during the period from May 2021 to January 2022 at the Tissue Culture Laboratory of Biotechnology Division, Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh, Bangladesh to establish efficient shoot and root regeneration protocol from mature grains of selected two rice varieties i.e. Binadhan13 and BR5 (*Oryza sativa* L.). MS medium was used for callus induction, shoot differentiation and half strength for root initiation (Murashige and Skoog, 1962, Ducefha, Netherlands). When the regenerated plants were established, plantlets were transferred to the pot containing 50% soil and 50% cowdung.

Preparation of the MS media

To prepare one liter of MS media about 500ml of distilled water was added in the flask to dissolve all the ingredients. After that, MS powder (4.4g) and Sucrose (30g) was added to this solution and gently agitated to dissolve completely. pH of the medium was adjusted to 5.8 with a digital pH meter with the help of 0.1N NaOH or 0.1N HCl as necessary. The whole mixture was then made up to 1000 ml with further addition of distilled water and mixed well. After adjustment of the pH, 6g^l⁻¹ gelrite (Ducefha, Netherlands) was added to obtain semi-solid medium. The mixture then gently stirred to complete dissolution of gelrite. After MS media preparation, all the instruments, glassware were sterilized to ensure aseptic condition.

There are several methods that were applied during the course of culturing different explants. For the explant/embryo culture method, sterilized dehusked seeds were the main source of explants in the experiment. The plant growth regulators and their respective number of combinations of explant/embryo culture were used as callus initiation, where MS medium (Murashige and Skoog Medium), 2.5 g^l⁻¹ 2,4-D growth regulators and sucrose (3%) were required as pretreatment. The shoot differentiation was done using MS medium, i.e., MS powder, sucrose (3%) and Gelrite (6%) supplemented with 1.0 g^l⁻¹ NAA + 2.0 g^l⁻¹ BAP + 2.0 g^l⁻¹ Kinetin. Half-strength MS medium was used

for root initiation.

Irradiation effect on calli for regeneration of plants from mature seeds of Binadhan- 13 and BR5 when the calli attained at convenient size they were divided into four parts. Each part contains 40 calli. Four parts were irradiated with three different doses of gamma irradiation (4, 6, 8 Gy) from ^{60}Co source at the Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh. Thus, the calli were prepared for subsequent regeneration. After that calli were removed aseptically from the test tube and placed on a sterilized petri-dish and were cut into small pieces, so that no contact of parental tissue remained and these were inoculated to a freshly prepared medium for the maintenance of the calli without root shoot differentiation. After 21 days, these were sub-cultured to freshly prepared medium containing different hormonal supplements for the maintenance of callus or for root-shoot differentiation. After 7 and 14 days, all cultures were examined, and whitish, compact and nodular embryogenic calli were separated and again sub-cultured for further growth. The dishes showing signs of contamination were discarded. Repeated sub-culturing was done at an interval of 15 days for maintenance of calli and organogenesis. Some of the sub-cultured calli continued to proliferate and differentiate into shoots. When these shoots grew upto 3-4 cm in height, they were separated aseptically from each other and transferred into freshly prepared rooting medium to induce roots. The conical flasks containing plantlets were incubated in same environment. Two types of incubation period were maintained in this study for callus initiation; i.e; dark incubation and light incubation. The culture vessels with inoculated explants were incubated in dark condition for 21 days in a temperature controlled growth chamber ($25\pm 1^\circ\text{C}$) less than 16-hour photoperiod with a light intensity of 2000-3000 lux. Day to day observations was made to note the response. Potting mixture containing garden soil and cow dung in the ratio of 1:1 was mixed properly and autoclaved for 1 hr. in 121°C at 1.16 kg/cm^2 . After cooling the soil mixture was taken into 10cm plastic pots for growing the plantlets at *in vivo* condition.

The regenerated plantlets with sufficient root system were ready for transfer in soil. The plantlets were brought out from the controlled environment of the incubation room and were kept in the room temperature for 2-3 days to be acclimatized in the normal environment. The plantlets with sufficient roots was then taken out from the culture vessels and thoroughly washed in running tap water to remove all adherent culture medium. The plantlets were then transplanted to small plastic pots containing sterilized ground soil mixed with cow-dung. After transplantation, the pots with the plantlets were covered immediately with polythene bag to prevent excessive evapotranspiration. The pots were kept in the controlled environment of glass house to reduce shock. Then the plants covered by the polythene bags in the pot were sprayed with Hogland's solution (Hoagland and Arnon, 1938) . After 4 days the polythene bags were gradually perforated to expose the plantlets into natural environment and after 7 days it was removed completely. When the plantlets grew well, these were transferred to earthen pots where they developed into

mature plants.

Recording of data

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

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

$$\text{Percent (\%)} \text{ survival rate} = \frac{\text{Number of irradiated calli survived}}{\text{Number of explants inoculated}} \times 100$$

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

Statistical analysis

The experiment was conducted in growth room and arranged in Completely Randomized Design (CRD) with three replications. The analysis of variance for different characters were performed, and mean values were compared by the Duncan's Multiple Range Test (DMRT) by using statistical package MSTAT-C software (Russel, 1986).

Results and Discussion

A callus is largely unorganized, continued proliferation of undifferentiated parenchyma cells from parent tissue on clearly defined semi-solid media. Various numbers of explants were inoculated on semi-solid media containing 2.0 mg l⁻¹ 2, 4-D concentration for callus formation (Figure 1). To achieve the ultimate goal of plantlet regeneration via embryogenic calli, two rice varieties (Binadhan-13 and BR5) were cultured on MS medium supplemented with different combinations of plant growth regulators. Mature embryos of these varieties were used as explants. Callus initiation performance results of these varieties are presented on (Table 1). Significant difference was observed in callus initiation at light conditions between two varieties, Binadhan-13 and BR5. Among the varieties, in dark condition, the highest average callus initiation percentage was observed in Binadhan-13 (83.27%), while it was 73.65% for BR5. On the other hand, highest average callus initiation (%) in light condition was observed in Binadhan-13 (74.14%) followed by BR5 (61.78%) (Figure 2).

Different doses of gamma radiation (0, 4, 6, and 8 Gy) were applied in calli of two rice varieties Binadhan-13 and BR5 to know the effect of gamma rays on plant regeneration (Hossain and Alam, 2001). After irradiation, callus was transferred on MS sub culture medium supplement with 2,4-D 2.0 gl⁻¹, NAA 0.5gl⁻¹ for better shoot growth.

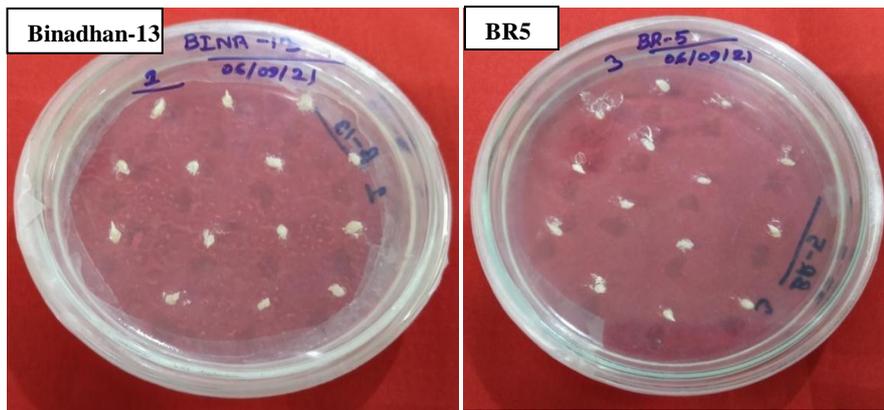


Figure 1. Inoculation of mature explant in MS medium.

Influence of light and dark condition on callus initiation from mature embryos of two rice varieties

Mature embryos of two rice varieties (Binadhan-13, BR5) were cultured in two different photoperiod's condition to observe callus response (24-hour under dark condition and 16/8-hour light using 3000 lux intensity). Calli grown under dark condition had higher cell mass than that under light condition, because the enhanced peroxidase activity and absence of oxidative stress might favor accumulation of polymers (Kevers *et al.*, 1995). Light is very important factor for callus induction, cell growth and production of plant secondary metabolites (Summart *et al.*, 2008). Among the conditions, average callus initiation efficiency was observed higher in dark condition followed by light condition (Table 1). From the present study, it was clear that calli induced and grew slightly better in dark than light condition.

Table 1. Effect of dark and light condition on callus initiation from mature embryos

Varieties	Replication	Average callus initiation in dark (%)	Average callus initiation in light (%)
Binadhan-13	R ₁ -R ₆	83.277	74.120
BR5	R ₁ -R ₆	73.650	61.785
Level of significance		*	*
LSD (0.05)		1.24	3.95
CV(%)		1.07	3.93

* = Significant at 5% level of probability

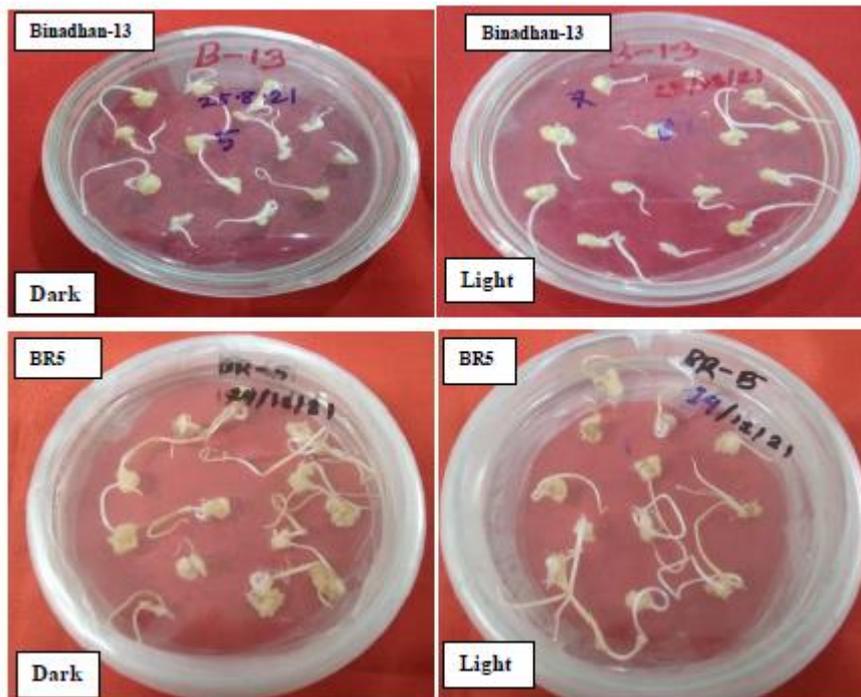


Figure 2. Callus initiation of two rice varieties cultured in MS medium supplemented with 2,4-D (2.0 mg/L) under dark and light condition at (25±3). Photo was taken after 15 days of inoculation.

Embryogenic callus initiation from mature embryos of rice varieties

The embryogenic callus is the cell with strong division ability and the potential of differentiation into somatic embryos. The mature embryos of two rice varieties were inoculated on the same media for callus initiation. Embryogenic callus formation invariably developed within 21 days. Results on embryogenic callus initiation performance of these varieties are presented in (Table 2). Among the varieties, high average embryogenic callus initiation (%) was observed in Binadhan-13 (77.14%) and BR5 (67.14%) in dark condition (Figure 3). There CV (%) value is (1.34) and LSD value (1.37) and there has significant difference in embryogenic callus initiation at dark condition between two varieties Binadhan-13 and BR5.

CV (%) value (5.39) found greater than LSD value (4.66); so, there has significant difference in embryogenic callus initiation at light condition between two varieties Binadhan-13 and BR5.

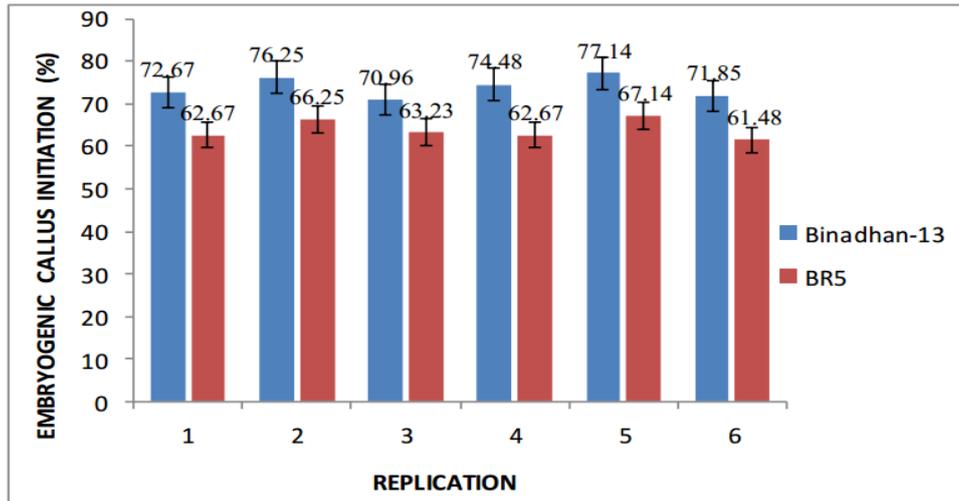


Figure 3. Embryogenic callus initiation response of mature embryos of two rice varieties in dark condition.

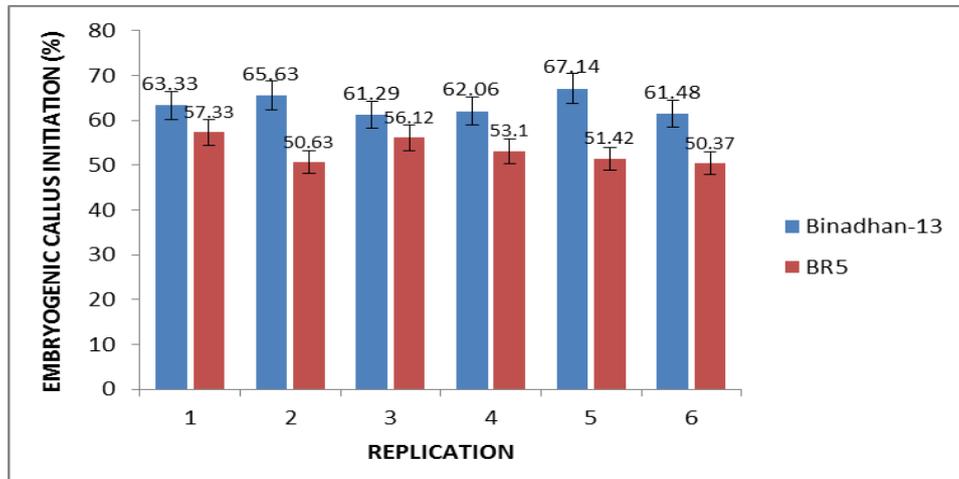


Figure 4. Embryogenic callus initiation response of mature embryos of two rice varieties in light condition.

Effect of irradiation on shoot regeneration of Binadhan-13 and BR5

Embryogenic calli of two rice varieties were cultured in MS medium with different plant growth regulators (NAA g l^{-1} + BAP g l^{-1} + Kn g l^{-1}). After 21 days of irradiation (0, 4, 6, and 8Gy), it was clearly observed (Table 2) that the extent of regeneration ability varied from different radiation doses. In both genotypes, regeneration percentage were found to be higher in the control and decreased with the increase of gamma ray dose. The high percentage of shoot induction from calli were in Binadhan-13 and BR5 in lower doses. After 4 weeks, these calli were turned into shoots and the shoot formation efficiency was measured on the basis of their vigor (Figure 5). This experiment was also conducted to check out root induction ability for first 3 replications for control and another 3 replications within different doses of gamma radiation. The reason was may be the tissues of these varieties were more sensitive to inhibition by gamma radiation because increase of high doses created low survivability of callus. Both growth and regeneration capacity decreased with increasing levels of gamma rays; however, plant regeneration capacity was more sensitive to gamma rays than growth (Hossain and Alam, 2001).

Table 2. Effect of irradiation on callus for shoot induction of Binadhan-13 and BR5 using MS media with growth regulators

Varieties	Dose (Gy)	No. of calli inoculated	No. of calli showing shoot induction	Shoot regeneration %
Binadhan-13	0	20	14	70
	4	20	12	60
	6	20	11	55
	8	20	9	45
BR5	0	20	12	60
	4	20	10	50
	6	20	9	45
	8	20	8	40

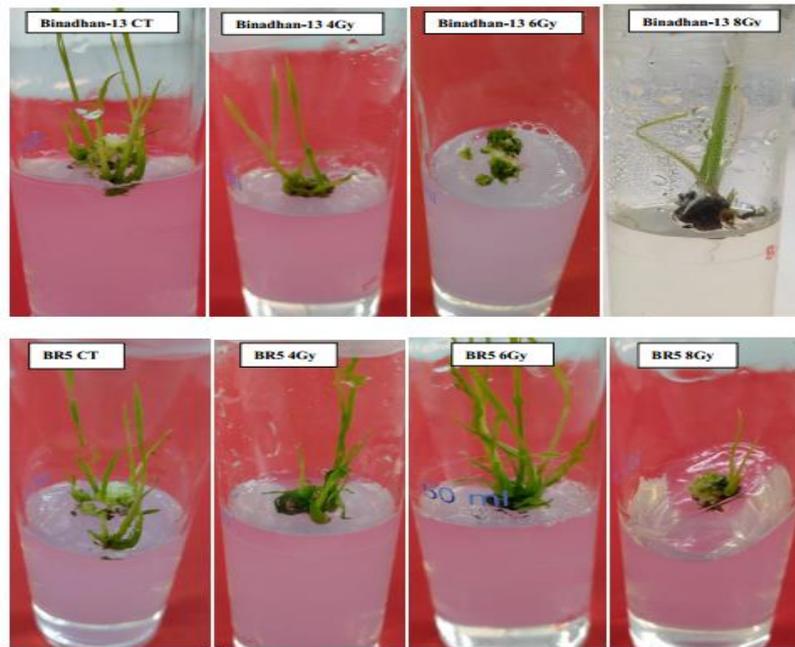


Figure 5. Effect of radiation on embryogenic calli of Binadhan-13 and BR5 at different doses of gamma ray (CT=Control). Photos were taken after 21 days of inoculation.

Effect of gamma irradiation on root induction from shoot

Half strength of MS media was used to see the rooting response of the regenerated shoot. Shoots were collected from irradiated calli exposed to the different doses (0, 4, 6 and 8Gy) of gamma rays. After 15 days, it was clearly observed that the extent of root induction ability varied from shoots that were differentiated due to different doses of irradiated calli. In both varieties, root induction percentage were found to be higher in the control and gradually decreased with the increasing doses of gamma rays (Figure 6). But in some cases, root induction prolificacy followed irregular trend in the higher doses of gamma rays. The root induction ability from shoot was higher in control treatment (0 Gy). Root induction frequency of two varieties under the various doses was observed and the results are presented in (Table 3). When the roots sprouted, these were turned into complete plant after 2-3 weeks later. This experiment was performed to check out root induction ability in control condition and within different doses of gamma irradiation. This finding is similar to Sarwar, (2003) who reported that root induction ability was decreased gradually with increase in the radiation dose.

Table 3. Effect of irradiation on root induction of Binadhan-13 and BR5

Varieties	Dose(Gy)	No. of shoot inoculated	No. of shoot showing root induction	Root induction %
Binadhan-13	0	10	8	80
	4	10	7	70
	6	10	6	60
	8	10	4	40
BR5	0	10	7	70
	4	10	6	60
	6	10	5	50
	8	10	3	30

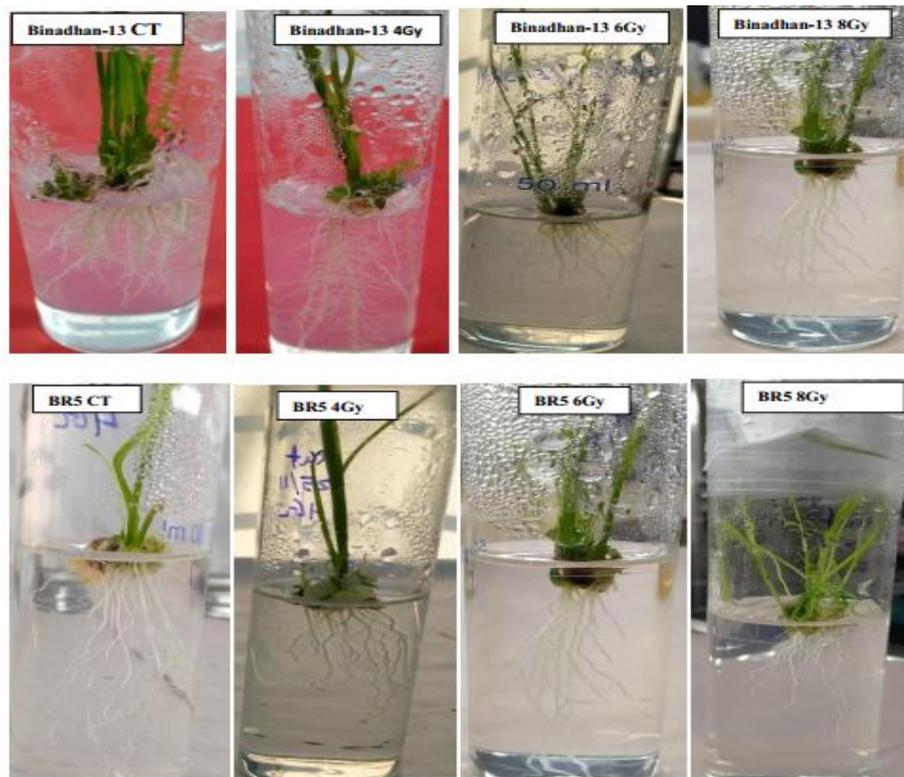


Figure 6. Root formation from regenerated shoots of Binadhan-13 and BR5 (CT= Control). Photos were taken after 28 days of inoculation.

Establishment of Plantlets

After sufficient development of root system, the small plantlets were taken out from the culture vessels without damaging roots. Excess agar around the roots was washed off by tap water to prevent microbial infection. Then the plantlets were transplanted in small pots. 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, tillering capacity of plantlets and survival rate of the plantlets in the pots were satisfactory.

Conclusion

This comparative study investigated optimal conditions for callus initiation and the impact of gamma rays on regeneration in Binadhan-13 and BR5 rice varieties. Callus initiation efficiency was influenced by genotypes, growth regulators, and light conditions, with 2.0 mg/L 2,4-D in dark conditions proving most effective. Gamma irradiation, particularly at 8 Gy, negatively affected *in vitro* regeneration, while 4 Gy demonstrated superior shoot and root regeneration. The study highlights the potential of gamma irradiation for inducing useful variability in rice varieties. The developed *in vitro* regeneration protocol, which successfully produced healthy plantlets, shows potential for improving different rice varieties in future breeding programs.

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