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Optimizing Hormonal Effects and Incubation Periods on In Vitro Regeneration in High-Yielding Indica Rice

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ABSTRACT

The research aimed to evaluate the effects of hormones and callus age on *in vitro* regeneration as well as seeking to establish a reliable and effective plant regeneration protocol for the rice varieties BRRI dhan92 and BRRI dhan96. Mature seeds were used to initiate callus induction using MS media containing 2 mg/L of 2,4-D. For the regeneration process, calli were transferred to MS medium supplemented with different hormone combinations: H1 (2 mg/L BAP + 1 mg/L NAA + 1 mg/L Kinetin), H2 (4 mg/L BAP + 0.5 mg/L NAA + 1.2 mg/L Kinetin), and H3 (1 mg/L BAP + 1 mg/L NAA + 1 mg/L Kinetin). Significant variations were observed in the response to plant hormones between the rice varieties. Additionally, calli of ten, fifteen, and twenty-one day old were tested to observe the effect of callus age on plant regeneration. The highest callus induction frequency was recorded in BRRI dhan92 (88.17%). The H2 hormone combination showed the highest regeneration frequencies, achieving 63.67% in BRRI dhan92 and 45.33% in BRRI dhan96. Furthermore, the highest regeneration frequencies were found in ten-day-old calli, with BRRI dhan92 at 67.00% and BRRI dhan96 at 49.00%. This optimized regeneration protocol for BRRI dhan92 and BRRI dhan96 can be effectively used for *Agrobacterium*-mediated genetic transformation and their subsequent improvement.

Key words: Incubation days, Hormone, *In vitro,* Regeneration, Callus induction.

INTRODUCTION

About one-third of the global population relies on rice (*Oryza sativa* L., 2n = 2x = 24), a member of the Graminae family and subfamily Oryzoidea, as their primary food source. Remarkably, rice also occupies about one-fifth of the world's cereal-cultivated land. Today, a staggering 90.5% of the world's total rice production comes from Asia, with significant contributions from China, Indonesia, Pakistan, India, Vietnam, Thailand, Myanmar, Bangladesh, the Philippines, and Japan (FAO, 2022). In Bangladesh, rice is the cornerstone of agriculture, accounting for half of the country's agricultural GDP and one-sixth of its total income. As the third-largest rice producer globally, Bangladesh dedicates approximately 11.70 million hectares to rice cultivation (FAO, 2022). With the population expected to soar to 215.4 million by 2050, the country will require 44.6 million tons of clean rice (Kabir *et al*., 2015). Although rice production in Bangladesh has quadrupled over the past five decades, the increasing population growth rate (1.22%) and diminishing arable land (-0.69%) (Khalequzzaman *et al.*, 2005) necessitate further enhancements in rice productivity. To meet this growing demand, biotechnology, particularly genetic engineering and tissue culture, can be synergistically applied alongside traditional breeding methods

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(Binte Mostafiz and Wagiran, 2018). Genetic engineering and tissue culture present potent solutions to the limitations of conventional breeding, offering the potential to develop rice varieties with enhanced quality, yield, and resilience to both abiotic and biotic stresses (Tuteja *et al*., 2012).

Successful *in vitro* plant regeneration is paramount for leveraging these technologies to boost rice yields (Alam *et al.*, 2012). An efficient *in vitro* regeneration protocol is crucial for the successful application of genetic engineering in developing transgenic rice varieties. However, plant regeneration through tissue culture is challenging across different rice varieties, with success heavily dependent on reliable callus culture and regeneration procedures. This technique has been pivotal in enhancing the quality and yield of desired plants (Ghorpade *et al.,* 2012).

A significant barrier to genetic modification of many plant species is the lack of an effective *in vitro* regeneration method (Azizi *et al.*, 2015). Many agronomically desirable rice varieties exhibit resistance to *in vitro* regeneration due to inadequate callus development and regeneration capabilities (Khatun *et al*., 2003). Particularly, many indica rice cultivars are less susceptible to genetic alteration owing to their limited regeneration abilities. Factors influencing regeneration efficiency include genotype, type and physiological status of explants, culture medium composition, plant growth regulators, incubation period, and culture conditions, with genotype and nutrient composition being the most impactful (Mohiuddin *et al.*, 2006).

Since 1994, BRRI dhan28 and BRRI dhan29 have been mega rice varieties for Boro season. Recently, they have been replaced by BRRI dhan96 and BRRI dhan92, respectively. These high-yielding BRRI varieties, were selected for this experiment due to their outstanding significance on yield performance. The objective was to identify the most effective hormonal combination and optimal incubation period for *in vitro* regeneration. Since there is no available tissue culture protocol of these newly developed rice varieties for transformation work, development of an efficient *in vitro* regeneration protocol is a top priority for the genetic transformation/genome editing and subsequent improvement.

Therefore, this experiment was conducted to evaluate the effects of various hormone combinations on the regeneration frequency of BRRI dhan92 and BRRI dhan96 to identify the most effective combinations. Subsequently, the selected hormone combination was used to assess the impact of callus age on regeneration capability. This research aims to develop a successful regeneration protocol for these rice varieties while minimizing experimental time in tissue culture.

MATERIALS AND METHODS

Seed collection Seeds of BRRI dhan92 and BRRI dhan96 were collected from the Biotechnology Division, Bangladesh Rice Research Institute in Gazipur.

Seed sterilization Eight hundred mature seeds of each variety were manually dehulled and subjected to a sterilization process in a laminar air flow cabinet. First, the dehulled seeds were sterilized with 70% ethanol for one minute and then rinsed with sterile water. Next, they were treated with 50% Clorox (v/v) containing one drop of Tween 20 for 20 minutes, with gentle agitation. To remove the sodium hypochlorite, the seeds were washed five to six times with sterile distilled water. Subsequently, the seeds were sterilized again with 50% Clorox (v/v) without Tween 20, followed by another five washes with sterile water to ensure complete removal of sodium hypochlorite. Finally, the sterilized seeds

were placed on sterile filter papers to remove excess water.

Media Preparation, Observations, Collection and Scoring of data

The sterilized seeds were then placed into a callus induction medium (CIM) consisting of MS media (Murashige and Skoog, 1962) with 2.0 mg/L of 2, 4-dichlorophenoxyacetic acid (2, 4-D). Sucrose at 30 g/L used as the carbohydrate source, and phytagel at 4 g/L was the solidifying agent. The pH of the media was adjusted to 5.8, and it was autoclaved at 15 PSI and 121°C for 20 minutes. Six hundred sterilized seeds of each variety were placed on the callus induction media, and all culture plates were incubated at 25±1°C in the dark to induce callusing. Data on callus induction percentage were recorded 21 days after seed plating.

Three-week-old calli were then transferred into magenta boxes containing regeneration media supplemented with different hormone combinations: H1 (MS + 2 mg/L BAP + 1 mg/L NAA + 1 mg/L Kinetin), H2 (MS + 4 mg/L BAP + 0.5 mg/L NAA + 1.2 mg/L Kinetin), and H3 (MS + 1 mg/L BAP + 1 mg/L NAA + 1 mg/L Kinetin). These calli were maintained under a 16/8-hour light/dark conditions. Data on regeneration percentage and the number of green plants produced were collected.

Callus induction frequency (%) was calculated as follows:

$$\frac{\text{Number of embryogenic calli}}{\text{Total number of calli}} \text{X } 100$$

Regeneration frequency (%) was calculated as follows:

$$\frac{\text{Number of regenerated calli}}{\text{Number of calli incubated}} \text{X } 100$$

Another experiment was conducted to observe the effect of incubation period on regeneration frequency. The same seed sterilization and media preparation procedures mentioned above were followed. Calli aged ten, fifteen, and twenty-one days were transferred to regeneration media using the H2 hormone combination, as it showed the highest regeneration rates. The regenerated plantlets were successfully rooted on half-strength MS medium and later transferred into pots containing soil for acclimatization

RESULTS AND DISCUSSION

Callus induction The aim of the present study was to develop an effective *in vitro* regeneration protocol for the rice varieties BRRI dhan92 and BRRI dhan96. In this experiment, 2 mg/L of 2,4-D was used for callus induction. As a potent synthetic auxin, 2,4-D is often sufficient to initiate and sustain embryogenic callus growth in rice, hence its exclusive use at a concentration of 2 mg/L. Previous studies, including those by Shahsavari *et al*. (2010) and Hoque and Mansfield (2004), have shown that 2 mg/L is optimal for callus induction. This was further corroborated by Htwe *et al.* (2011). Figure 1 illustrated the callus induction frequency from dehisced seeds of both rice varieties. Callus induction in rice is highly variable and genotype-specific. Similar result was achieved by Joya *et al* (2019). Among the two varieties, BRRI dhan96 exhibited poorer callus induction compared to BRRI dhan92. The highest callus induction frequency was observed in BRRI dhan92 (88.17%), followed by BRRI dhan96 (71.61%). This difference highlights the genotype-dependent nature of callusing efficiency, as confirmed by Rashid *et al*. (2003), who reported significant variations in callusing among rice varieties. A prior study also indicated that tissue culture generates a wide range of variation correlated with incubation time and cultivar-specific factors (Rasheed *et al.,* 2005).

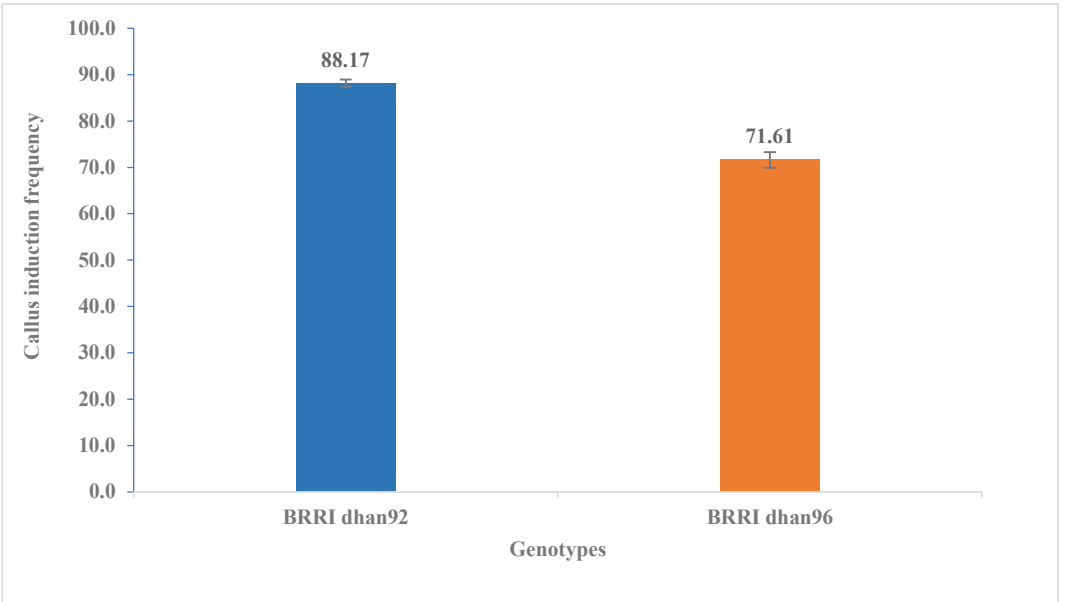

Fig. 1. Effect of genotypes to callus induction frequency.

Effect of hormone on regeneration frequency (%): Calli produced on MS media supplemented with 2.0 mg/L 2,4-D were evaluated for their plant regeneration ability under three regeneration media with different hormone combinations: H1 (2 mg/L BAP + 1 mg/L NAA + 1 mg/L Kinetin), H2 (4 mg/L BAP + 0.5 mg/L NAA + 1.2 mg/L Kinetin), and H3 (1 mg/L BAP + 1 mg/L NAA + 1 mg/L Kinetin). BRRI dhan92 exhibited the highest regeneration ability in H2 (63.67%) and the lowest in H3 (30.00%) (Fig. 2). Conversely, BRRI dhan96 also showed the highest regeneration ability in H2 (45.33%) and the lowest in H3 (23.67%) (Fig. 2). BRRI dhan92 generally responded better to all tested regeneration media compared to BRRI dhan96. This disparity may be attributed to the genotype and culture environment, as noted by Hoque and Mansfield (2004). Among the three media, the highest regeneration was observed in H2 and the lowest in H3. The study found that regeneration efficiency is influenced by varying concentrations of BAP, NAA, and Kinetin. The highest regeneration frequency was achieved with a high concentration of BAP (4 mg/L) and a low concentration of NAA (0.5 mg/L), similar to the findings of Tariq *et al*. (2008). A low concentration of Kinetin has been reported to enhance embryogenic calli and shoot formation efficiency in indica rice (Nhut *et al*., 2000; Humera and Jafar, 2011). Another study confirmed that successful plant regeneration is largely dependent on auxins and cytokinins combinations (Lee *et al*., 2002).

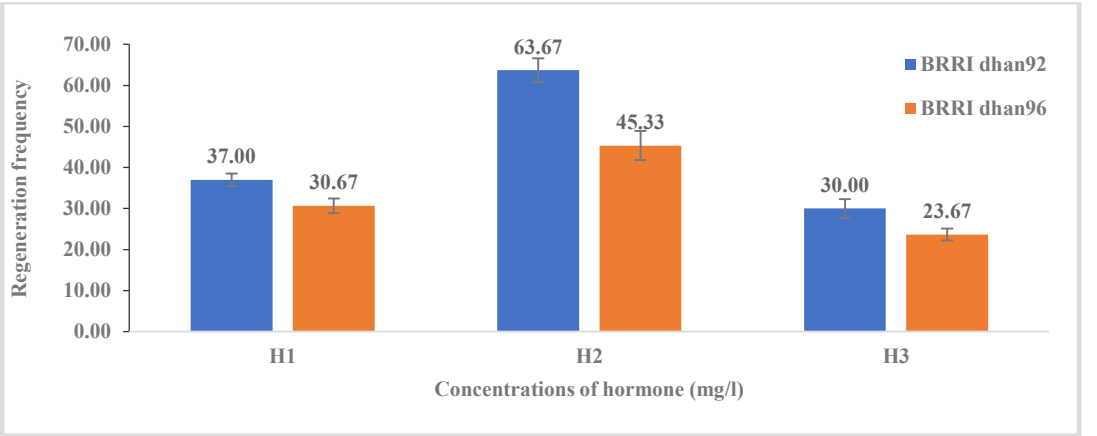

Fig. 2. Effect of different concentrations of hormone to regeneration frequency of BRRI dhan92 and BRRI dhan96.

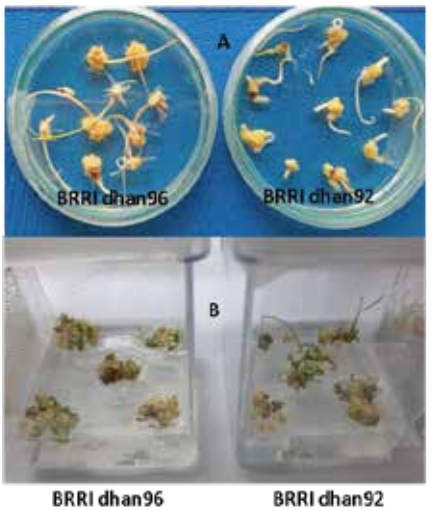

Fig. 3. Comparison of callus induction (A) and plant regeneration (B) between BRRI dhan92 and BRRI dhan96.

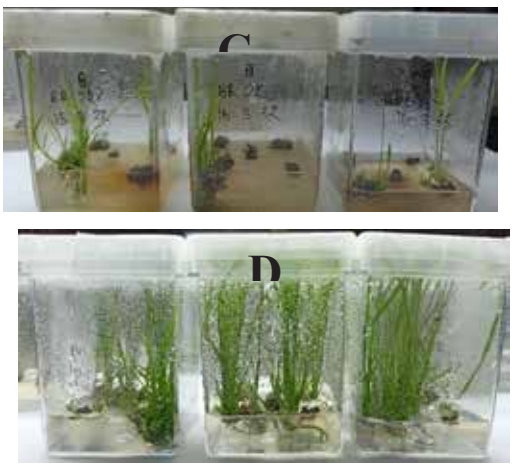

Fig. 4. Hormonal response to plant regeneration of BRRI dhan92; C= H1 and D= H2.

Effect of incubation days to callus induction frequency (%): BRRI dhan92 showed callus induction frequencies of 85.83%, 84.16%, and 83.33% at ten, fifteen, and 21 days of incubation, respectively (Table 1). In contrast, BRRI dhan96 exhibited callus induction frequencies of 72.33%, 68.50%, and 66.67% at the same respective intervals (Table 1). These results may be influenced by genotype and different incubation periods. Previous research has confirmed that tissue culture produces a wide range of variance due to the incubation period and cultivar specificity (Rasheed *et al.,* 2005). The experiment suggested that ten-day-old calli could be transferred to regeneration media, thereby reducing the time frame of the experiment.

Table 1. Effect of incubation days to callus induction frequency.

Days of transfer	Variety	Callus induction frequency (%)
Ten days	BRRI dhan92	85.83
	BRRI dhan96	72.33
Fifteen days	BRRI dhan92	84.16
	BRRI dhan96	68.50
Twenty-one days	BRRI dhan92	83.33
	BRRI dhan96	66.67

Effect of calli age on regeneration: In this experiment, calli aged ten, 15 and 21 days were transferred to the H2 hormone combination (H2 = 4 mg/L BAP + 0.5 mg/L NAA + 1.2 mg/L Kinetin) to observe the effect of callus age on plantlets regeneration. This experiment aimed to not only establish a regeneration protocol but also to minimize the experimental duration. The highest regeneration frequency (67.00%) for BRRI dhan92 was found in ten-day-old calli, with the lowest (62.00%) observed in 21 day-old calli (Table 2). The maximum number of regenerated plants (150) was also found in ten-day-old calli. For BRRI dhan96, the maximum regeneration frequency was 49.00% from ten-day-old calli, and the lowest was 44.00% from 21 day-old calli. The highest number of regenerated plants (60) was from ten-day-old calli, while the lowest number (47) was from 21 day-old calli (Table 2). A study highlighted that prolonged incubation of rice calli led to decreased regeneration efficiency due to increased oxidative stress and hormonal imbalances (Ali *et al.,* 2013). Another study also found that calli incubated for prolonged periods showed reduced regeneration efficiency due to decreased cellular viability and increased ROS levels (Mishra *et al*., 2020). From this phase of the experiment, it became clear that greater numbers of plantlets can be derived from ten-day-old calli. The insight of this study can significantly reduce the experimental time required in tissue culture.

Table 2. Effect of incubation days to plantlet regeneration and number of regenerated plants.

Days of transfer	Variety	% Plantlet regeneration	Number of regenerated plants
Ten days	BRRI dhan92	67	150
	BRRI dhan96	49	60
15 days	BRRI dhan92	65	140
	BRRI dhan96	46	52
21 days	BRRI dhan92	62	120
	BRRI dhan96	44	47

CONCLUSION

An efficient and reproducible regeneration system is crucial for controlling molecular mechanisms and achieving stable genetic transformation. Key factors for successful regeneration include genotypes, tissue source of explants, combination and concentration of growth regulators, and culture conditions. Considering this, our experiment evaluated the effect of different hormonal combinations on the regeneration rate of the newly developed rice varieties BRRI dhan92 and BRRI dhan96. The highest regeneration response was observed in H2 media, followed by H1 and H3. Based on these results, we conducted a further experiment to identify the shortest time required for optimal regeneration. Calli aged ten, 15, and 21 days were transferred to H2 media, which had demonstrated the highest regeneration frequency for both varieties. Remarkably, the ten-day-old calli showed the highest regeneration rate in the H2 hormone combination. This optimized regeneration protocol will significantly contribute to the enhancement of nutritional quality, resistance to biotic stresses (e.g., disease and pest resistance), tolerance to abiotic stresses (e.g., drought and salinity), and the improvement of specific traits in BRRI dhan92 and BRRI dhan96. Furthermore, gene editing technologies like CRISPR-Cas9 can be integrated with these regeneration protocols to precisely modify specific genes through *Agrobacterium*-mediated genetic transformation, enabling the production of genome-edited plants within a shorter timeframe. We hope our study will contribute to food security and agricultural sustainability.

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