RESEARCH ARTICLE

Callus induction and plant regeneration in five Bangladeshi rice landraces

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ABSTRACT

A successful callus induction and *in vitro* plant regeneration method has tremendous potential to regenerate rice landraces, which could be used for further genetic improvement. Research on rice landraces is scarce, and these landraces are becoming extinct in nature. Therefore, the study aimed to create an optimized plant regeneration protocol using plant growth regulators (PGRs) on N6 media for five Bangladeshi rice landraces: Hingairmanik, Moynashail, Haloi, Noyaraz, and Prabini. N6 media were enhanced with various concentrations and combinations of PGRs to find out the greatest PGR composition for callusing and regeneration. Hingairmanik, Moynashail, and Haloi showed maximum calli formation on N6 medium supplemented with 2.5 mg/l 2,4-D (2,4-dichlorophenoxy acetic acid), achieving 80%, 90%, and 76.67% callus induction, respectively. In contrast, the largest callus induction was found in Noyaraz (76.67%) and Prabini (66.67%) on N6 medium having 3.0 mg/l 2,4-D. For complete plant regeneration from embryogenic calli, N6 medium supplemented with three different combinations of NAA (1-naphthalene acetic acid) and BA (6-Benzylaminopurine) concentrations was employed. In the case of Moynashail, Prabini, and Haloi, the highest rates of regeneration were obtained on N6 medium amended with 1.5 mg/l NAA and 3.0 mg/l BA, resulting 70%, 55%, and 60% of regeneration, respectively. In addition, both Hingairmanik and Noyaraz showed maximum regeneration frequency (65%) at medium having 1.5 mg/l NAA and 3.5 mg/l BA. This study has the potential to contribute significantly to future genetic research on these Bangladeshi rice landraces.

Key words: Rice landraces, Callus induction, Plant regeneration, N6 media

Introduction

Rice (*Oryza sativa* L.), the staple food crop of Bangladeshi people, is crucial for ensuring food security not only in Bangladesh but also in Asia, where the largest part of the world's rice (> 90%) is consumed (Khush, 2005). It is also known as a high-calorie food commodity with high biological protein content (Shiferaw et al., 2013). Bangladesh produced 38.15 million tonnes of rice in 2021/2022, ranking it as the third largest rice producer in the world (BBS, 2022), while demand for rice continues to increase every year worldwide. To mitigate our global demands, the production of rice needs to increase by 40% by 2030 (Khush, 2005). Thus, it is imperative to develop rice varieties having high production abilities, superior qualities, and resilience to both biotic and environmental challenges. To achieve considerable improvement of rice varieties by using natural hybridization, various conventional breeding techniques are already in practice. But traditional techniques for varietal improvement are a tedious and lengthy process (Wang et al., 2011). Although these techniques brought success in the last century, these efforts may be unable to mitigate the rising demand for rice consumers in the future. To address this situation, improved techniques like molecular breeding or biotechnological approaches such as genetic transformation can be used along with conventional techniques (Gosal & Kang, 2012). Yet, enhancement of rice genetics through *Agrobacterium-mediated* gene transfer relies on the availability of a robust protocol for callus induction and *in vitro* regeneration (Hiei & Komari, 2008). So, tissue culture continues to be a crucial method for advancing the genetic characteristics of rice plants (Komari et al., 2007).

Both callus formation and later regeneration of plantlets require all the necessary nutrients supplied as a growth medium along with plant growth regulators (PGRs). These factors should be considered to develop an optimized *in vitro*

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culture and regeneration process of a specific genotype. Additionally, the response to plant growth regulators (PGRs) supplemented media is limited for some genotypes like *indica*, widely grown rice types (Hiei & Komari, 2008; Sahoo et al., 2011). This limits the possibility of the genetic improvements of that type of genotype by genetic engineering. However, the low regeneration potential of some *indica* genotypes is a bottleneck in genetic transformation, but efforts need to be made to develop efficient regeneration methods for rice landraces. Rice landraces are ecologically special populations with various desirable characters (Ram et al., 2007), and their important trait need to be exploited (Islam et al., 2013). Moreover, genetic modification using biotechnological approaches may turn existing landraces into high-yielding varieties as well as preserve valuable genetic resources. Considering these, the present research was done to prove an efficient callus induction and *in vitro* plant regeneration method for five Bangladeshi rice landraces, namely Hingairmanik, Moynashail, Haloi, Noyaraz, and Prabini. Here, we found their genotypic response on N6 medium (Chu et al., 1975) having various concentrations of PGRs. The findings of this research could be used for further improvements of these genotypes through biotechnological approaches.

Materials and Methods

Collection of seeds

Five rice landraces were collected from farmers in the Sylhet and Mymensingh divisions of Bangladesh. Among them, two rice landraces (Hingairmanik and Moynashail) were collected from the Sylhet division, while the remaining three rice landraces (Haloi, Noyaraz, and Prabini) were collected from the Jamalpur district of the Mymensingh division.

Callus induction media preparation and seed inoculation

Fresh N6 media having sucrose (30 mg/l) and different concentrations of 2,4-D were prepared and employed to determine the best concentration for callogenesis. Eight concentrations of 2,4-D (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg/l) were tested, with N6 media without 2,4-D serving as a control. Mature and healthy seeds were properly dehusked, washed enough with autoclaved water, and sterilized in 70% ethanol for 2 minutes, followed by treatments (10 minutes) with 0.1% $HgCl_2$ (50 ml) and Tween-20 (two drops). After drying on sterile filter paper, seeds were aseptically inoculated into the prepared N6 media and kept in darkness at 25 ± 2°C to induce callus formation. Once callus developed, they were moved to light conditions under white, fluorescent light (2500 lux) with a 16-hour light /8-hour dark photoperiod. After two weeks, the rate of callus induction was assessed employing the following formula:

$$\text{Callus induction frequency (\%)} = \frac{\text{Number of explants produced calli}}{\text{Total number of explants inoculated}} \times 100$$

Plantlet regeneration from the callus

The best callus-inducing media was selected and used to subculture the calli in that media to promote continued growth. From the proliferated callus, a small portion of healthy, embryonic callus was transferred to shoot-inducing media. N6 media supplemented with three different combinations of NAA and BA concentrations (1.0 mg/l NAA + 2.5 mg/l BA; 1.5 mg/l NAA + 3.0 mg/l BA; and 1.5 mg/l NAA + 3.5 mg/l BA) was used for shoot regeneration. N6 media without PGR served as the control. The frequency of shoot regeneration was figured out employing the following formula:

$$\text{Shoot regeneration frequency (\%)} = \frac{\text{Number of calli producing shoots}}{\text{Number of inoculated calli}} \times 100$$

After sub-culturing of regenerated shoots into the shooting media, they were transferred to N6 medium devoid of PGRs to assess their rooting ability. This was calculated by using the following formula:

$$\text{Root initiation frequency (\%)} = \frac{\text{Number of shoot producing roots}}{\text{Number of inoculated shoot}} \times 100$$

After sufficient root formation, the plantlets were planted in a pot having sterilized soil and acclimatized for 2 weeks.

Statistical analysis of data

The experiments were done with three replications of ten seeds per treatment for callus induction, and two replications of ten embryonic calli per shooting hormonal treatment for plant regeneration. A two-way ANOVA followed by Fisher's LSD test was conducted using GraphPad Prism version 10.0.0 to assess the significance ($P \leq 0.05$) of variation among the treatments.

Results and Discussion

Callus Induction

Efficient embryogenic callus production and high regeneration efficiency are fundamental prerequisites for the progress of genetic engineering of rice landraces. In the present study, we used mature seeds for callogenesis due to their year-round availability. Many other studies have developed rice regeneration systems using embryogenic calli derived from mature seeds (Jiang et al., 2000; Oh et al., 2005; Kant et al., 2007; Karthikeyan et al., 2009). Embryonic calli originating from the scutellum of mature seeds are considered the most effective for developing *in vitro* regeneration systems and to produce transgenic rice (Hiei et al., 1994; Rashid et al., 1996). In this study, we assessed the efficiency of N6 medium having various concentrations of 2,4-D to induce callus formation in five rice landraces, viz., Hingairmanik, Moynashail, Haloi, Noyaraz, and Prabini. Typically, 2,4-D is used alone or in combination with cytokinin to promote the initiation and maintenance of callus (Castillo et al., 1998). Our results showed that callus was successfully developed from

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seeds of these landraces using N6 medium, consistent with the observations of Rashid et al. (2001, 2004), who previously documented callus growth on N6 media. Callus induction from isolated seeds after about two weeks was clearly visible on N6 medium containing various concentrations of 2,4-D (Figure 1).

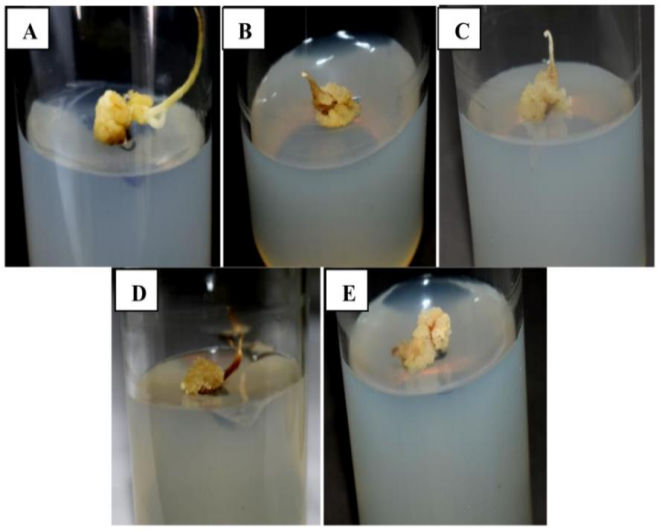

Figure 1. *Callus of rice landraces. A) Hingairmanik, B) Moynashail, C) Haloi, D) Noyaraz, and E) Prabini.*

The callus induction frequency varied significantly across the different concentrations of 2,4-D tested compared to the control (Figure 2). It was observed that all the landraces exhibited the highest frequency of callus induction in the presence of 2.5 to 3.0 mg/l 2,4-D, whereas both higher and lower concentrations of 2,4-D resulted in reduced callus initiation. Among the five landraces, Hingairmanik (80%), Moynashail (90%), and Haloi (76.67%) showed the highest frequency of callus initiation at 2.5 mg/l 2,4-D. In contrast, Noyaraz and Prabini displayed 76.67% and 66.67% callus induction at 3.0 mg/l 2,4-D, respectively, with optimal size and color (Figure 2). These results align closely with those reported by Tariq et al. (2008), who observed that three Basmati rice varieties exhibited the highest frequency of callus induction (60-75%) when treated with 2.5 to 3.0 mg/l 2,4-D. Several studies have also revealed that 2,4-D at concentrations of 2.5-3.0 mg/l is the optimal growth regulator for inducing callus in rice (Visarada & Sarma, 2002; Saharan et al., 2004; Liu et al., 2009; Panjaitan et al., 2009; Wani & Gosal, 2011). Additionally, Jan (2001) reported that N6 media having 2.0 mg/l of 2,4-D was optimal to induce calli of four rice genotypes (i.e., Swat I, Swat II, Dilrosh 97, and Pakhal), commonly cultivated in Pakistan's North-West Frontier Province (NWFP). However, Prabini rice showed a lower frequency of callus induction compared to the other landraces tested. The variation in these results may be due to differences in the *in vitro* culture systems, such as different nutrients, hormone compositions, and explant types (Torbert et al., 1998; Ramesh et al., 2009). Additionally, genetic variability among rice genotypes may also be a significant contributing factor (Khanna & Raina, 1998).

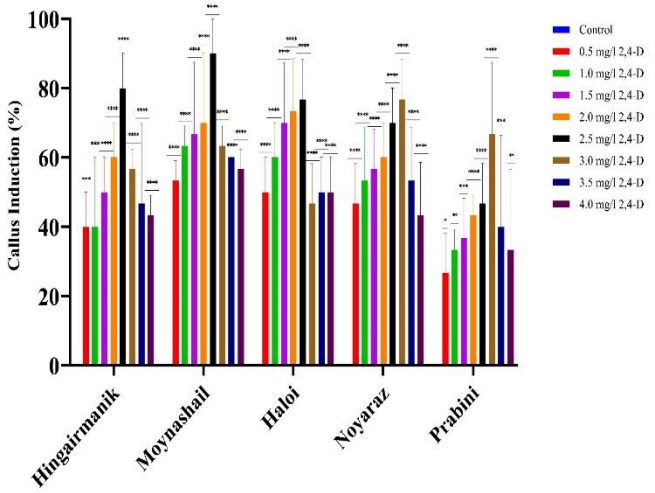

Figure 2. *Callus induction frequency of landraces on different 2,4-D concentrations. Significant differences were indicated by labeling the means with different numbers of asterisks (*).*

Regeneration of Plantlet

The calli (soft friable, compact) of five rice landraces were proliferated by sub-culturing in N6 medium enriched with 2.5-3.0 mg/l 2,4-D, as these concentrations were identified as the optimal for inducing callus. Embryogenic calli of convenient size were excised and transferred to N6 media fortified with different concentrations and combinations of phytohormones, specifically lower concentrations of auxin (NAA) and higher concentrations of cytokinin (BA), to develop shoot generation. Many studies have already reported the stimulatory function of BA in combination with NAA, with or without the addition of other hormones such as KIN, in the regeneration of rice callus cultures (Boissot et al., 1990; Ramesh & Gupta, 2005; Radziah et al., 2017; Binte Mostafiz & Wagira, 2018). In this research, N6 medium containing three different combinations of NAA and BA was used to identify the shoot regeneration efficiency. The cultures were incubated at room temperature under a 16-hour light /8-hour dark photoperiod. Within 2-3 weeks, multiple shoot apices appeared from the calli, which grew into long and robust multiple shoots after another 2 weeks of culturing. The results of the shooting response are shown in Figure 3.

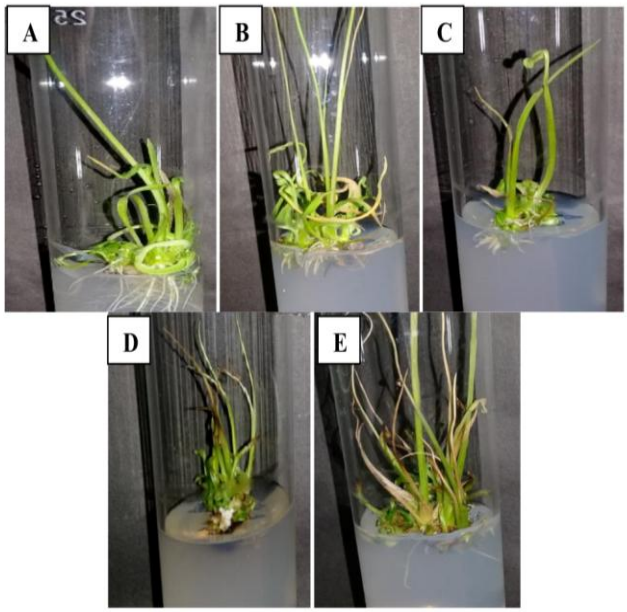

Figure 3. *Shoot regeneration from embryogenic calli. A) Hingairmanik, B) Moynashail, C) Haloi, D) Noyaraz, and E) Prabini.*

The frequency of shoot regeneration significantly varied across the treatments compared to the control. This study revealed that Moynashail, Haloi, and Prabini had the best shoot regeneration response at 1.5 mg/l NAA and 3.0 mg/l BA, with rates of 70%, 60%, and 55%, respectively, which is consistent with a previous study (Hiei & Komari, 2006). On the other hand, both Hingairmanik and Noyaraz showed satisfactory results at 1.5 mg/l NAA and 3.5 mg/l BA, with a percentage of 65% (Figure 4).

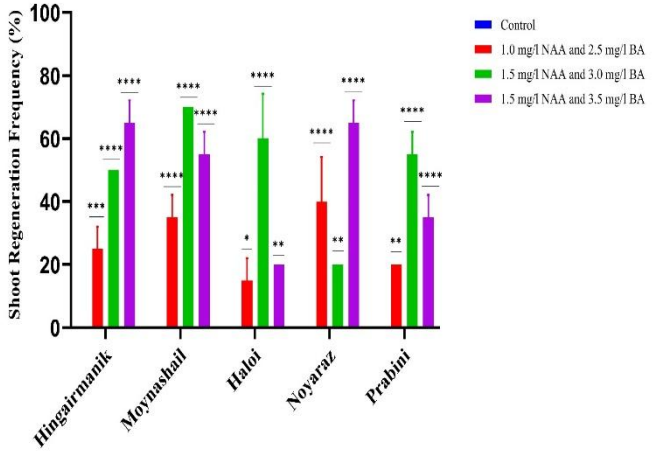

Figure 4. *Shoot regeneration frequency of landraces on different shoot regeneration medium. Significant differences were indicated by labeling the means with different numbers of asterisks (*).*

These results contradict the findings of another study, which found that plant regeneration of Basmati varieties is higher in N6 medium having 1.0 mg/l NAA and 2.5 mg/l BA (Tariq et al., 2008). Many recent reports have also mentioned that MS medium having different combinations of NAA and BAP is the best for the regeneration of plants from embryogenic calli (Ramesh et al., 2009; Upadhyaya et al., 2015; Radziah et al., 2017; Binte Mostafiz & Wagira, 2018). The successful creation of transgenic rice plants using tissue culture methods depends on the high regeneration efficiency of embryogenic calli through sub-culturing. A frequent problem in tissue culture is the production of Albino plants, especially when using anther- or pollen-derived callus (Torrizo et al., 1986; Raina et al., 1987). However, our study observed green regenerated shoots without any albinos, as we utilized mature seed-derived embryogenic calli for plant regeneration. These results are consistent with those of Hoque et al. (2007), who also found no albino plants when regenerating from mature seed-derived embryogenic rice callus.

Root Induction

The regenerated shoots were transferred to N6 media without PGRs for root development, which resulted in a 100% rooting efficacy across all landraces. Plantlets with well-formed shoots and roots were gently removed from the regeneration media and thoroughly washed to eliminate any remaining medium and avoid contamination. These plantlets were then hardened in the laboratory before being subjected to acclimatization. Eventually, the hardened plantlets were transplanted into plastic pots containing sterile soil and kept under regular supervision, where they showed normal growth and development (Figure 5).

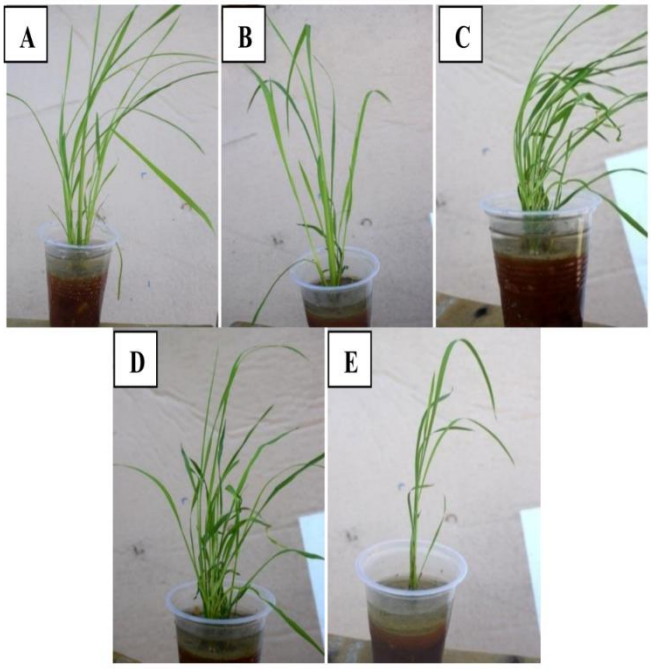

Figure 5. *Plantlets of five landraces. A) Hingairmanik, B) Moynashail, C) Haloi, D) Noyaraz, and E) Prabini.*

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In this study, the landraces showed high regeneration with significant variation. In callus induction, the histogram represents the callus induction frequency of five landraces at nine different concentrations, each in a different color. The callus induction output is highly significant, as indicated by an asterisk. In shoot regeneration, the regeneration frequency of four different combinations is represented in different colors, with the level of significance indicated by an asterisk. The outcome of the study indicates that these landraces are suitable for genetic transformation. A significant level of variance was observed in callus induction and regeneration frequency, which suggests the need for further molecular studies to explore the observed diversity at the allelic or genotypic level. Genetic variation motivates plant breeders to cross these landraces using different methods to achieve novel morphological traits (e.g., resistance varieties, grain production, etc.). Molecular study predicts the necessity of conservation based on allele frequency, which is vital for these local landraces to become extinct.

Conclusion

In conclusion, we have optimized a high-frequency callus induction and regeneration protocol for five rice landraces collected from different regions of the country, demonstrating a simple and highly reproducible approach. To fulfill the ever-increasing global demand for rice, production must be increased. Efficient callus induction and regeneration protocol development is a primary step for various genetic transformations. Rice landraces harbor important traits and are now becoming extinct in Bangladesh. Thus, improving these indigenous rice landraces may address this issue. This *in vitro* regeneration system provides a pathway to developing transgenic plants with improved productivity. The method presented here will also be helpful for further research activities and can be incorporated into a genetic engineering program to introduce desirable agronomic traits in rice landraces.

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