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Comparison of in vitro regeneration and transformation efficiency among five vietnamese indica rice cultivars

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Abstract. This study optimized the *in vitro* regeneration and transformation processes for five popular indica rice cultivars in Vietnam named as BC15, Bac Thom 7 (BT7), Khang Dan 18 (KD18), OM5451, and TBR225. The highest callus induction rates (83–93.67%) were achieved on a medium containing 2.5 mgL^{-1} 2,4-D, supplemented with 500 mg L^{-1} each of L-proline, L-glutamine, and casein. Higher or lower concentrations of 2,4-D resulted in reduced callus induction. Among tested cultivars, BT7 exhibited the highest callus induction rate of 93.67%. Shoot regeneration was optimized on a medium with a combination of 0.5 mg L^{-1} kinetin, 2.0 mg L^{-1} BAP, and 0.5 mg L^{-1} NAA, resulting in regeneration rates ranging from 80.23% to 90.77% across cultivars, with BT7 exhibiting the highest regeneration rate (90.77%). Hygromycin at 20 mg L^{-1} was optimal for selecting transgenic calli, with BT7 showing the highest transformation efficiency opdetermined to be optimal for selecting transgenic calli, with BT7 achieving the highest transformation efficiency (44% hygromycin-resistant callus and 29% GUS expression). PCR analysis confirmed a high proportion (87.5%) of transgenic plants in the BT7 cultivar. These findings indicate that the BT7 is the most suitable cultivar for genetic transformation and gene editing applications.

Key words: Callus, indica rice, in vitro, regeneration, shoot.

INTRODUCTION

Rice is a staple food for more than half of the world's population, particularly in Asia, where approximately 90% of the world's rice is produced and consumed (Khush, 2005). In Vietnam, rice also plays a critical role and is the most widely cultivated crop. According to the report of the Vietnam General Statistics Office (GSO), the total rice area exceeds 7.0 million hectares and produces more than 40.0 million tonnes annually. This not only meets domestic consumption demands but also supports exports of approximately 6–7 million tonnes per year, making Vietnam one of the world's leading rice exporters in recent years (GSO, 2023, https://www.gso.gov.vn/en/px-web/). However, the area dedicated to rice cultivation appears to be decreasing, for example, it

was recorded at around 7.5 million hectares in 2019 but declined to 7.2 million hectares by 2023, likely due to the impacts of climate change and industrialization (GSO, 2023, https://www.gso.gov.vn/en/px-web/).

Global climate change significantly impacts rice cultivation in Vietnam, particularly in the two most important rice-growing regions as the Mekong River Delta and the Red River Delta. These areas are highly vulnerable to the effects of climate change, which pose serious challenges for rice farmers and threaten Vietnam's position as a leading global rice exporter.

Vietnam has implemented a range of strategic initiatives to enhance its rice sector's resilience to climate change, addressing critical challenges such as rising sea levels, salinity intrusion, erratic weather patterns, and water scarcity. These efforts prioritize the modernization of farming practices and the development of climate-resilient rice varieties through advanced biotechnological approaches to ensure sustainable production. As part of this strategy, the Vietnamese government approved the project on Developing the Vietnamese rice brand, emphasizing the selection and cultivation of high-quality rice varieties tailored to market demand (MARD, 2015). Consequently, greater attention has been directed toward rice breeding programs in recent years. However, the predominant breeding methods still rely on traditional techniques, such as crossing and mutation induction, which are time-consuming and financially demanding for developing rice varieties.

To overcome these challenges, a biotechnology approach must be applied to accelerate the development of rice varieties that are more resilient to environmental stresses such as drought, salinity, and disease, while also enhancing yield and quality. Although conventional breeding has made significant achievments in rice improvements, but its progress is slow and limitation of genetic pool. Combining conventinal breeding with genetic engineering and tissue culture methods could save time and reduce costs in developing new varieties (Gosal & Kang, 2012).

In vitro propagation through somatic embryogenesis is an important method widely used in various crop improvement programs including transformation, somatic selection and genome editing (Mishra et al., 2018). In this process, callus induction and plant regeneration are critical prerequisite steps before applying any genetic improvement techniques (Gosal & Kang, 2012). Various factors affecting clonal propagation have been investigated, including genotypes, used explants, medium composition, … (Khanna et al., 1998; Hoque et al., 2004). Although many efforts to establish an effective tissue culture system have been reported, optimization is still needed for different varieties.

Thus, the most viable and feasible option is the optimization of several parameters in a rice transgenic improvement program. Previous studies have shown that the recalcitrance of indica rice was attributed to its lower callus induction and regeneration abilities compared to japonica rice. Additionally, significant variation has been observed in the *in vitro* culture responses among different genotypes within the indica subspecies (Rahman et al., 2021).

In Vietnam, five predominantly indica rice cultivars: Bac thom 7 (BT7), BC15, Khang Dan 18 (KD18), TBR225 and OM5451 are widely grown in the Red River Delta (BT7, BC15, KD18, and TBR225) and Mekong River Delta (OM5451) regions. However, these cultivars are sensitive to abiotic and biotic stresses such as heat,

submerge, and diseases. To improve these cultivars for adapting to adverse environmental conditions through a genetic engineering approach, their *in vitro* culture capabilities and transformation responsiveness must be tested.

In this study, we evaluated the callus induction and *in vitro* regeneration abilities, as well as the genetic transformation potential, of those five rice cultivars to identify the most suitable cultivar for Vietnam's rice improvement program using genetic transformation or gene editing technologies.

MATERIALS AND METHODS

Plant materials and seed sterilization

Mature seeds of five indica rice genotypes (*Orya sativa* L.) namely Bac Thom 7 (BT7), BC15, Khang Dan 18 (KD18), TBR225, and OM5451 were obtained from the ThaiBinh seed company and Cuu Long Delta Rice Research Institute (CLRRI), Vietnam, and used as explants for *in vitro* culture and transformation tested. To sterilize the seeds, dehusked healthy seeds were initially washed twice with distilled water to remove visible floating particales. Subsequently, the seeds were surface sterilizied with 70% (v/v) ethanol (sigma, US) for 30 seconds, followed by a 4% (v/v) sodium hypochlorite solution (Fujifilm Wako, Japan) containing 2 drops of tween 20 for 20 minutes. The seeds were further rinsed 4–5 times with sterile distilled water to remove any residual disinfectant and blotted dried on sterile filter paper for 10 minutes.

Optimization of the medium composition for callus induction and plant regeneration

In order to optimize the medium for callus induction, various concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D, Sigma, US) and either alone or combination of L-proline (Sigma, US), L-glutamine (Sigma, US) and casein hydrolysate (Sigma, US), were evaluated based on Murashige and Skoog (MS, Duchefa) basal medium (Murashige & Skoog, 1962) supplemented with 3% sucrose (Duchefa), 0.4% phytagel (Sigma, US). A total of 12 different types of medium (C_1-C_{12}, Table 1) were tested for callus induction. The pH media were adjusted to 5.8 using 1M NaOH or 1M HCl and autoclaved at 121 °C for 15 minutes. Approximately 22–25 seeds were placed per plate medium at 28 °C under condition of 16/8 h light/dark photoperiod using plant growth chamber (JSPC-300C, JSR, Korea). Twenty plates were observed for each medium treatment. The callus induction frequency was recorded after 3 weeks of innocuation. The best medium for callus induction was evaluated based on frequency of callus (%), fresh weight and morphology.

$$\text{Callus induction (\%)} = \frac{\text{Number of seeds with callus}}{\text{Number of seeds with callus}} \cdot 100$$

Healthy calli were selected based on texture and color and transferred to shoot regeneration medium supplemented with different concentrations of either benzylaminopurine (BAP, Sigma, US), kinetin (Sigma, US), naphthaleneacetic acid (NAA, Sigma, US), L-proline, L-glutamine, and casein hydrolysate alone or in combination. A total of 6 different types of S medium (S1-S6, Table 1) were tested for shoot regeneration. Approximately 22 pieces of calli per plate were maintained under

the same condition as during the callus induction period. Regeneration efficiency was evaluated based on the number of calli forming buds after 30 days on the medium.

$$\text{Regeneration efficiency (\%)} = \frac{\text{Number of calli with buds}}{\text{Total number of calli cultured}} \cdot 100$$

Table 1. Media tested for callus induction and shoot regeneration in rice

	Callus induction					Shoot regeneration					
Mediu	2,4-D (mg L^{-1})	Proline (mg L^{-1})	Glutamine (mg L^{-1})	Casein (mg L^{-1})	Medium	Kinetin (mg L^{-1})	BAP (mg L^{-1})	NAA (mg L^{-1})	Proline (mg L^{-1})	Glutamine (mg L^{-1})	Casein (mg L^{-1})
C_1	1.0	0	0	0	S_1	2.0	0	0.5	0	0	0
C_2	1.5	0	0	0	S_2	0	2	0.5	0	0	0
C_3	2.0	0	0	0	S_3	0.5	2.0	0.5	0	0	0
C_4	2.5	0	0	0	S_4	2.0	0	0.5	500	500	500
C_5	3.0	0	0	0	S_5	0	2	0.5	500	500	500
C_6	2.5	500	0	0	S_6	0.5	2.0	0.5	500	500	500
C_7	2.5	500	500	0							
C_8	2.5	500	500	500							
C_9	2.5	0	500	500							
C_{10}	2.5	0	0	500							
C_{11}	2.5	500	0	500							
C_{12}	2.5	0	500	0							

Agrobacterium-mediated transformation and selection

Preparation of Agrobacterium suspension

Glycerol stocks of transformed EHA105 Agrobacterium harboring pCAMBIA1301 construct (Fig. 1) were streaked on an AB agar plate containing Spectinomycin 50 mg L^{-1}, hygromycin 50 mg L^{-1} and incubated at 28 °C for two days in an incubator (JSGI-30T, JSR, Korea). Subsequently, bacteria were harvested using sterilized spoon and suspended in 20 mL A200 medium, which consisted of N6 salts supplemented with 0.876 g L^{-1} glutamine, 0.5 g L^{-1} casein hydrolysate, 0.26 g L^{-1} L-aspartic, 0.174 g L^{-1} L-arginine, 10 g L^{-1} D-glucose, 20 g L^{-1} sucrose, and acetosyringone 100 µM. The agrobacterium suspension was incubated for approximately 1 hour at 28 °C, 120 rpm and adjusted to OD_{600} = 0.6 before infection with rice calli.

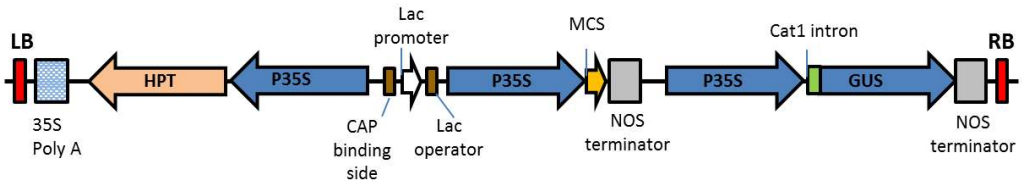

Figure 1. Schematic of the pCAMBIA1301 plant expression vector T-DNA region containing GUS reporter and hygromycin resistance genes (HPT).

Infection of callus with Agrobacterium

Five-day old regenerated callus on C media (Table 2) were used as explants for Agrobacterium-mediated transformation. Approximately 50 calli were submerged in 30 mL *A. tumefaciens* suspension (OD_{600} = 0.6) for 20 minutes inoculation. Subsequently, the inoculated calli were blotted dry on sterilized filter papers (Whatman) under sterile conditions to remove excess bacteria, then immediately transferred to A201 medium, which consisted of N6 salts supplemented with 2.0 mg L^{-1} 2,4-D, 0.5 g L^{-1} casein hydrolysate, 0.5 g L^{-1} L-proline, 10 g L^{-1} D-glucose, 20 g L^{-1} sucrose, 10 g L^{-1} agar, and acetosyringone 100 μM. Inoculated calli were incubated for 3 days at 28 °C in the dark.

Table 2. Effect of medium culture on percentage of callus induction from rice mature seed explants

Callus induction medium	BC15	BT7	KD18	OM5451	TBR225	Average (Medium)
C_1	45.00 ± 1.00^{a}	61.00 ± 1.00^{d}	60.33 ± 0.58^{d}	63.00 ± 1.00^{e}	59.33 ± 0.58^{d}	57.73
C_2	51.00 ± 1.00^{b}	69.33 ± 0.58^{g}	68.00 ± 1.00^{f}	67.33 ± 0.58^{f}	70.33 ± 0.58^{g}	65.20
C_3	55.33 ± 0.58^{c}	78.33 ± 0.58^{k}	73.67 ± 0.58^{i}	74.33 ± 0.58^{i}	74.33 ± 0.58^{i}	71.20
C_4	73.67 ± 0.58^{i}	84.67 ± 0.58^{n}	81.00 ± 1.00^{l}	80.67 ± 0.58^{l}	79.33 ± 0.58^{l}	79.87
C_5	67.33 ± 0.58^{f}	79.33 ± 0.58^{l}	75.67 ± 1.53^{j}	75.33 ± 0.58^{i}	75.33 ± 0.58^{j}	74.60
C_6	77.00 ± 1.00^{k}	88.33 ± 0.58^{k}	83.33 ± 0.58^{n}	83.67 ± 0.58^{n}	82.33 ± 0.58^{n}	82.93
C_7	79.67 ± 0.58^{l}	89.67 ± 0.58^{q}	84.33 ± 0.58^{n}	84.67 ± 0.58^{n}	83.33 ± 1.15^{n}	84.73
C_8	83.00 ± 1.00^{n}	93.67 ± 0.58^{r}	87.00 ± 1.00^{o}	86.67 ± 0.58^{p}	84.33 ± 1.15^{n}	87.33
C_9	79.33 ± 0.58^{l}	89.33 ± 0.58^{q}	84.00 ± 1.00^{n}	84.67 ± 0.58^{n}	83.00 ± 1.00^{n}	84.73
C_{10}	77.67 ± 0.58^{k}	88.00 ± 1.00^{p}	83.67 ± 1.15^{n}	83.33 ± 0.58^{n}	81.67 ± 1.15^{m}	82.87
C_{11}	80.00 ± 1.00^{l}	90.33 ± 0.58^{q}	84.00 ± 1.00^{n}	87.33 ± 1.53^{o}	86.67 ± 0.58^{o}	85.67
C_{12}	78.67 ± 0.58^{k}	87.00 ± 1.00^{o}	83.67 ± 0.58^{n}	82.67 ± 0.58^{m}	81.33 ± 1.15^{m}	84.73
Average (Genotype)	70.63	83.25	79.06	79.81	78.44	
Significance of two-away ANOVA						
Medium (M)	$P < 0.001$					
Genotype (G)	$P < 0.001$					
M × G	$P < 0.001$					

Mean ± standard deviation. Difference letters 'a', 'b', 'c', … indicate significant difference.

Optimization of hygromycin for calli selection

After three days on co-cultivation medium, calli were transferred to a 50-mL sterile tube and washed 3–5 times with sterile distilled water. Then the calli were blotted dry on sterile filter paper, the coleptile and seed were removed before placing them on selection medium (N6 salts containing 2.0 mg L^{-1} 2,4-D, 0.5 g L^{-1} casein, 0.5 g L^{-1} L-proline, 0.5 gL^{-1} glutamine, 36 g L^{-1} mannitol, 20 g L^{-1} maltose, 5 g L^{-1} phytagel, 200 mg L^{-1} cefotaxime, and 200 mg L^{-1} vancomycin) containing hygromycin (Sigma, US) antibiotic at different concentrations of 10 mg L^{-1}, 20 mg L^{-1}, 30 mg L^{-1}, 40 mg L^{-1} and 50 mg L^{-1}. Approximately 20–25 calli were cultured on plate medium and maintained in a growth chamber under the condition of 28 °C, 16/8 hours light/dark for 14 days.

Hygromycin resistant calli were subcultured every 14 days for proliferating calli. The frequency of resistant calli was recorded based on calli morphology with healthy creamish while colour.

$$\text{Callus resistant efficiency (\%)} = \frac{\text{Number of hygromycin resistant calli}}{\text{Total number of selected calli}} \cdot 100$$

GUS staining

The GUS-staining assay was conducted according to the previous method (Jefferson, 1987). The transformed callus was immersed in a GUS-staining solution containing 10 mg^{-mL} X-Gluc (Thermo Scientific, US), 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 10 mM Na_2EDTA, 0.2 M $NaH_2PO_4 \cdot 2H_2O$, 0.2 M $Na_2HPO_4 \cdot 12H_2O$, 0.1% Triton X-100, and 4% methanol and incubated at 37 °C for 12 hours. The calli were decolorized with 70% ethanol until completely decolorized. The calli selected with hygromycin for 45 days were stained with the GUS staining solution.

$$\text{GUS transformation efficiency (\%)} = \frac{\text{Number of GUS positive calli}}{\text{Total number of GUS stained calli}} \cdot 100$$

Plantlet regeneration and validation

Proliferating calli were transferred to RN regeneration medium (MS salts containing 0.5 g L^{-1} casein, 0.5 g L^{-1} L-proline, 0.5 g L^{-1} glutamine, 2 mg L^{-1} BAP, 0.5 mg L^{-1} NAA, 0.5 mg L^{-1} kinetin, 30 g L^{-1} sucrose, 5 g L^{-1} phytagel, 30 mg L^{-1} hygromycin) and maintained at 28 °C, 16/8 h light/dark. Calli were subcultured until the appearance of shoots. Calli with shoots were transferred to MS hormone- free medium for plantlet development. Plants with a root system longer than 3 cm in length were transferred to soil and grown in greenhouse conditions. DNA was extracted from young leaves of individual plants using the CTAB method (Doye & Doye, 1998) and checked using a NanodropOne spectrophotometer (Thermo Scientific, USA) for DNA quality and concentration. Transgenic plants were validated by PCR analysis using Hygromycin phosphotransferase gene (*HPT*) primers (HPT-For: 5'-CGCATAACAGCGGTCATTGACTGGAGC-3' and HPT-Rev: 5'-GCTGGGGCGTCGGTTTCCACTATCCG-3'). PCR reaction was conduct in 20 µL for 35 cycles of denaturaion at 94 °C for 30s, annealing at 55 °C for 40s and extension at 72 °C for 15s. PCR products were run on 1% agarose gel.

Statistical analysis

The effects of genotype and hormone treatment on callus induction were tested using two-way analysis of variance (ANOVA) in the INDOSTAT program. The further effects of genotype, callus formation and shoot induction medium on *in vitro* regeneration were tested by using three-way ANOVA.

RESULTS AND DISCUSSION

Callus induction of five rice cultivars

Five indica rice cultivars named BC15, BT7, KD18, OM5451, TBR225 were investigated for their ability to induce callus induction on C media containing various concentrations of 2,4-D either alone or combined with L-proline, L-glutamine, and casein (Table 1). After 3 weeks of cultivation, all five cultivars showed an increase in

the rate of callus formation and weight that corresponded with a rising concentration of 2,4-D from 1.0 mg L^{-1} to 2.5 mg L^{-1} across the cultivars. In detail, the callus formation frequency and callus weight of BC15 cultivar increased from 45.0% (2.7 g) to 73.67% (4.48 g), BT7 from 61.0% (3.67 g) to 84.67% (5.2 g), KD18 from 60.33% (3.65 g) to 81.0% (4.89 g), OM5451 from 63.0% (3.78 g) to 80.67% (4.88 g), TBR225 from 59.33% (3.57 g) to 79.337% (4.82 g) at 1.0 mg L^{-1} and 2.5 mg L^{-1}, respectively (C_1-C_4, Table 2 and Table 3). In constrast, the callus formation rate and weight of all tested rice cultivars decreased in the C_5 medium supplemented with 3.0 mg L^{-1} 2,4-D (Table 2 and Table 3).

Table 3. Effect of medium culture on fresh callus weight from mature rice seed explants rice

Callus induction medium	BC15	BT7	KD18	OM5451	TBR225	Average (%)
C_1	2.70 ± 0.06^{a}	3.67 ± 0.07^{c}	3.65 ± 0.05^{c}	3.78 ± 0.06^{d}	3.57 ± 0.03^{c}	3.47
C_2	3.08 ± 0.08^{b}	4.27 ± 0.12^{e}	4.09 ± 0.08^{e}	4.14 ± 0.06^{f}	4.24 ± 0.06^{e}	3.96
C_3	3.52 ± 0.07^{c}	4.79 ± 0.06^{h}	4.40 ± 0.03^{f}	4.51 ± 0.10^{g}	4.58 ± 0.07^{g}	4.36
C_4	4.48 ± 0.03^{f}	5.20 ± 0.07^{j}	4.89 ± 0.06^{h}	4.88 ± 0.08^{h}	4.82 ± 0.07^{h}	4.86
C_5	4.05 ± 0.03^{e}	4.78 ± 0.03^{h}	4.54 ± 0.09^{g}	4.53 ± 0.03^{g}	4.53 ± 0.03^{g}	4.49
C_6	5.39 ± 0.07^{k}	6.20 ± 0.04^{n}	5.67 ± 0.04^{l}	5.78 ± 0.07^{m}	5.61 ± 0.04^{l}	5.73
C_7	6.21 ± 0.03^{n}	6.78 ± 0.07^{p}	6.67 ± 0.04^{p}	6.59 ± 0.05^{p}	6.32 ± 0.04^{o}	6.51
C_8	7.55 ± 0.05^{r}	8.50 ± 0.04^{t}	7.96 ± 0.05^{s}	7.98 ± 0.06^{s}	7.56 ± 0.09^{r}	7.91
C_9	6.25 ± 0.10^{n}	6.78 ± 0.09^{p}	6.64 ± 0.06^{p}	6.59 ± 0.04^{l}	6.57 ± 0.07^{p}	6.57
C_{10}	5.37 ± 0.06^{k}	6.15 ± 0.06^{n}	5.67 ± 0.07^{l}	5.68 ± 0.04^{l}	5.58 ± 0.07^{l}	5.69
C_{11}	6.32 ± 0.08^{o}	7.23 ± 0.05^{q}	6.73 ± 0.09^{p}	6.86 ± 0.07^{p}	6.80 ± 0.03^{p}	6.79
C_{12}	5.64 ± 0.09^{p}	6.14 ± 0.07^{n}	5.77 ± 0.08^{n}	5.60 ± 0.04^{l}	5.59 ± 0.07^{l}	5.75
Average (Genotype)	5.85	6.05	5.56	5.58	5.48	
Significance of two-away ANOVA						
Medium (M)	$P < 0.001$					
Genotype (G)	$P < 0.001$					
M × G	$P < 0.001$					

Mean ± standard deviation. Difference letters 'a', 'b', 'c', ... indicate significant difference.

Interestingly, the frequency of callus induction and callus weight increased significantly with the addition of 2.5 mg L^{-1} 2,4-D, either alone or in combination with L-proline, L-glutamine, and casein. The highest calli frequency of the BC15, BT7, and KD18 cultivars were obtained at 83.0%, 93.67%, 87.0%, respectively, in the C_8 medium supplemented with 2.5 mg L^{-1} 2,4-D along with 500 mg L^{-1} L-proline, 500 mg L^{-1} L-glutamine, 500 mg L^{-1} casein. Meanwhile, the OM5451 and TBR225 cultivars showed the highest callus induction rate of 87.33% and 86.67%, respectively, in the C_{11} medium containing 2.5 mg L^{-1} 2,4-D along with 500 mg L^{-1} L-proline and 500 mg L^{-1} casein (Table 2).

Shoot regeneration from rice calli

All induced calli from the C_6 medium were selected for evaluation of shoot regeneration. The calli were transferred to 6 different types of shot regeneration media (S_1-S_6, Table 1). The results showed that combination of kinetin, BAP, and NAA influenced regeneration ability. A combination of 2.0 mg L^{-1} kinetin and 0.5 mg L^{-1} NAA

(S_1) or 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} (S_2) resulted in a lower percentage of calli regenerating shoots compared to the S_3 medium, which contained a triple combination of 0.5 mg L^{-1} kinetin, 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA. The average of shoot regeneration rates were 40.95%, 67.9% and 75.24% in the S_1, S_2 and S_3, respectively (Table 4). Interestingly, the shoot regeneration rate increased significantly when these media were supplemented with a triple combination of 500 mg L^{-1} L-proline, 500 mg L^{-1} L-glutamine, 500 mg L^{-1} casein, resulting in rate of 47.22%, 74.26% and 84.47% on the S_4, S_5 and S_6 media, respectively (Table 4). A comparison of regeneration among the five cultivars revealed that the BT7 cultivar exhibited the highest shoot regeneration rate on the same media as other rice cultivars. Among these, the S_6 medium provided the highest shoot regeneration rates for all five tested rice cultivars compared to the other media (S_1-S_5, Table 4), ranging from 80.23% to 90.77%. The BT7 cultivar achieved a regeneration rate of 90.77%, surpassing BC15 (82.73%), KD18 (87.03%), OM5451 (81.58%) and TBR225 (80.23%) after 30 days of regeneration (Table 4).

Table 4. Effect of culture medium on shoot regeneration efficiency from callus

Medium	BC15	BT7	KD18	OM5451	TBR225	Average (%)
S1	45.80 ± 1.75^{h}	56.52 ± 2.04^{l}	34.10 ± 0.77^{d}	39.10 ± 0.77^{f}	29.25 ± 0.28^{c}	40.95
S2	68.67 ± 0.38^{p}	83.98 ± 0.28^{t}	62.46 ± 1.34^{n}	63.16 ± 0.41^{n}	61.23 ± 1.18^{n}	67.9
S3	75.52 ± 0.46^{r}	85.77 ± 0.57^{u}	75.85 ± 1.08^{r}	71.06 ± 1.55^{q}	67.99 ± 0.83^{p}	75.24
S4	48.60 ± 1.24^{i}	56.94 ± 1.43^{l}	39.08 ± 0.45^{f}	50.00 ± 0.56^{i}	41.50 ± 1.30^{f}	47.22
S5	73.92 ± 2.21^{q}	83.98 ± 1.10^{t}	70.12 ± 1.96^{q}	73.31 ± 1.56^{q}	69.97 ± 0.96^{p}	74.26
S6	82.73 ± 0.72^{t}	90.77 ± 0.65^{v}	87.03 ± 0.58^{u}	81.58 ± 0.59^{s}	80.23 ± 0.85^{s}	84.47
Significance of three-way ANOVA						
Callusing medium (C)			$P < 0.001$			
Shooting medium (S)			$P < 0.001$			
Genotype (G			$P < 0.001$			
C x S x G			$P < 0.001$			

Mean ± standard deviation. Difference letters indicate significant difference.

Effect of hygromycin for calli selection

Seed explants pre-cultured for 5 days on the C_8 medium were innoculated with *Agrobacterium tumefacien* carrying pCAMBIA1301 harboring the hygromycin phosphotransferase resistant gene (Fig. 1 and Fig. 2, b). After three days on co-cultivation medium, resistant calli were selected on a medium supplemented with hygromycin at various concentrations, range from 10 to 50 mg L^{-1}.

After 30 days of selection, the number of surviving calli reduced rapidly with increasing concentrations of hygromycin from 10 to 50 mg L^{-1} for the five rice cultivars (Table 5). At 10 mg L^{-1} hygromycin, the frequency of surviving calli ranged from 54.33% to 67.67%, depending on the cultivars and continuously decreased in media containing higher concentrations of hygromycin at 20 mg L^{-1} (34.67–47.33%), 30 mg L^{-1} (13.67–32.33%), 40 mg L^{-1} (5.33–17.0%). At 50 mg L^{-1} hygromycin, most of callus had died. While a low frequency of surviving calli was observed for BT7 (3.6%), KD18 (3.0%) and OM5451 (0.33%), no resistant calli were obtained from BC15 and TBR225 (Table 5 and Fig. 2, c–e). These results indicate that 20 mg L^{-1} hygromycin was suitable for selection of trangenic calli from these cultivars.

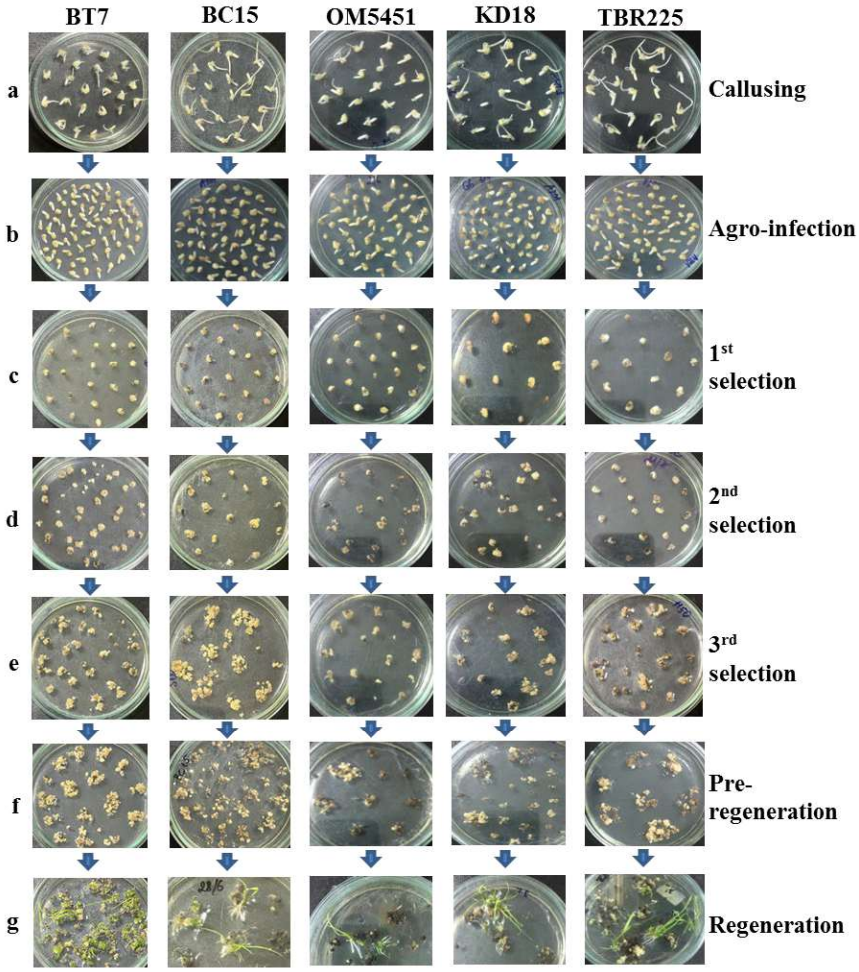

Figure 2. A summary of steps for Agrobacterium mediated transformation of rice calli. Five indica rice cultivars: BT7, BC15, KD18, OM5451 and TBR225 were used as explant.

(a) Callus intiation on callus induction meedium; (b) Agro-infection and co-cultivatiom of calli with *Agrobacterium tumefaciens*; (c–e) First, second and third hygromycin selection cycles calli, respectively; (f) Hygromycin resistant calli on pre-regeneration medium; (g) Shoot regeneration from hygromycin resistant calli.

Table 5. Effect of Hygromycin on callus selection

Hygromycin (mgL^{-1})	Hygromycin resistant calli (%)				
	BC15	BT7	KD18	OM5451	TBR225
0	100.00 ± 0	100.00 ± 0	100.00 ± 0	100.00 ± 0	100.00 ± 0
10	54.33 ± 1.52^{h}	67.67 ± 2.51^{i}	66.33 ± 3.05^{i}	55.00 ± 2.00^{h}	55.67 ± 3.05^{h}
20	34.67 ± 1.53^{f}	47.33 ± 2.08^{g}	46.33 ± 1.25^{g}	35.00 ± 3.00^{f}	36.33 ± 1.53^{f}
30	13.67 ± 1.53^{c}	32.33 ± 2.00^{e}	32.00 ± 2.00^{e}	16.00 ± 2.00^{d}	16.67 ± 1.53^{d}
40	5.33 ± 1.53^{b}	17.00 ± 1.00^{d}	15.67 ± 2.08^{c}	4.33 ± 1.53^{b}	5.00 ± 1.00^{b}
50	0	$3.67 \pm 1,15^{a}$	3.00 ± 1.00^{a}	0.33 ± 0.58^{a}	0

Mean ± standard deviation. Difference letters 'a', 'b', 'c', ... indicate significant difference $p > 0.05$.

Response of rice cultivar for gene transformation

Based on optimized medium, transformation efficiency of five rice cultivars with pCAMBIA3310 was investigated, with results shown in Table 6. The rate of hygromycin- resistant (HyR) calli ranged from 28.0% to 44.0%. The BT7 cultivar exhibited the highest percentage of HyR calli (44.0%), followed by KD18 (35.0%), BC15 (32.0%), OM5451 (31.0%) and TBR225 (28.0%). Consistantly, GUS staining assay was also revealed that highest rate of calli expressing the GUS signal was observed in BT7 (29.0%), followed by KD18 (19.67%), OM5451 (15.0%), TBR225 (14.33%) and BC15 (13.67%). HyR calli were transferred to shoot regeneration medium (Fig. 2, f) and the resulting plantlets were confirmed positive by PCR analysis (Fig 2, g). In total, 58 transgenic plants were transferred to soil pots in a greenhouse and analyzed for the *HPT* gene by PCR, with 48 plants (82.7%) confirmed positive for the *HPT* gene. Among these, BT7 had the highes number of positve plants (10/12, 87.5%), followed by TBR225 (6/7, 85.7%), OM5451 (5/6, 83.3%), KD18 (10/12, 83.3%), and BC15 (6/9, 66.67%). These results indicate that the BT7 cultivar is the most suitable for genetic applications.

Table 6. Result of transformations in five rice cultivars

Cultivar	#seeds	#callus inoculation	Callus formation	HyR callus	GUS^+ callus	No. plants	PCR^+ with hpt gene
BC15	410	396	83.00 ± 1.00^a	32.00 ± 1.00^b	13.67 ± 0.57^b	9	6
BT7	405	400	93.67 ± 0.58^c	44.00 ± 1.00^d	29.00 ± 2.64^d	24	21
KD18	403	391	87.00 ± 1.00^b	35.00 ± 1.00^c	$19.,67 \pm 1.53^c$	12	10
OM5451	400	319	86.67 ± 0.58^b	31.00 ± 1.00^b	15.00 ± 1.00^b	6	5
TBR225	400	322	84.33 ± 1.15^a	28.00 ± 1.00^a	14.33 ± 2.08^b	7	6
Genotype (G)			$P < 0.001$	$P < 0.01$		$P < 0.01$	$P < 0.01$

Mean ± standard deviation. Difference letters 'a', 'b', 'c', ... indicate significant difference.

Callus induction and regeneration are critical steps in rice *in vitro* regeneration process, particularly for genetic transformation approaches (Aldemita & Hodges, 1996). Many studies have concluated that rice callus induction and *in vitro* regeneration capacity strickly depend on genotypes (Abe & Futsuhara, 1986; Hartke & Lörz, 1989; Khalequzzaman et al., 2005; Karthikeyan et al., 2009; Mostafiz & Wagiran, 2018; Yaqoob et al., 2021; Noor et al., 2022; Gao et al., 2024), medium composition (Meneses et al., 2005; Geng et al., 2008; Carsono et al., 2021), explants (Ali et al., 2021, Gao et al., 2024), culture condition and duration (Meesook et al., 2020).

Khalequzzaman et al. (2005) investigated 15 indica rice genotypes for callus induction and *in vitro* regeneration ability, concluding that the percentage of callus induction and regeneration was influenced by genotypes and media, with no significant interaction observed between genotype and media. Callus induction ability varied widely among genotypes, ranging from 0.69% to 83.8%. Similarly, Zaidi et al. (2006) studied 74 indica rice genotypes for callus induction and regeneration, revealting that the number of calli obtained varied significantly, with some genotypes showing no callus formation on the same media. Mostafiz & Wagiran (2018) reported that upland and wetland rices varieties exhibited different frequencies of callus induction. While wetland rice achieved

a high percentage of callus formation (ranging from 76% to 94%), upland rice had a lower callus rate (42%). The variation in callus induction was also compared among Japonica, indica and hybrid types in a study by Yan et al. (2010). Under identical culture conditions, nine japonica varieties showed callus induction rate ranging from 61.7% to 89.2%, while nine indica varieties displayed greater variation from 90.1% to 100%.

Duad et al. (2023) compared the cold resistance of a japonica rice variety and a tropical savanna grass, concluding that high callus induction rates, ranging from 88.2% to 97.7%, were recorded in *Oryza sativa* cv. Fujisaka 5. In a similar study by Chen et al. (2023), six rice cultivars were evaluated for callus induction, resulting in 65% of seeds producing embryogenic calli. Among them, the japonica cultivar ZH11 exhibited the highest callus induction rate at 87%, with most indica cultivars generating calli earlier than japonica cultivars but at lower rates than japonica cultivar. Consistent with previous studies, in this study, under the similar callus induction conditions, five indica rice cultivars also exhibited different callus frequencies. Among them, the BT7 showed the highest frequency (93.67%) of callus induction, followed by KD18 (87%), OM5451 (87.33%), TBR225 (86.67%) and BC15 (83%).

Together with genotype, medium composition also influenced callus induction. Numerous studies have been conducted to optimize the concentration of auxin (2,4-D, NAA) for callus formation (Karthikeyan et al., 2009; Duad et al., 2023, Gao et al., 2024). While NAA was reported to stimulate the frequency of embryogenesis in the initial culture stage, 2,4-D was investegated for its role in promoting DNA hypermethylation during the pre-embryonic phase (Endress et al., 1994*).* Although 2,4-D is a primary auxin used for successful callus induction, high concentration of 2,4-D can reduce callus formation or result in poor quality due to its effects on redifferentiation during the mitotic stage (Rueb et al*.,* 1994*;* Mohd Din et al., 2015).

Numerous previous studies have investigated that a combination of 2,4-D with other hormones results in a higher number of calli, for example, NAA (Bano et al., 2005, Duad et al., 2023), BAP (Sahoo et al., 2011; Yadav et al., 2023). Mohd Din et al. (2015) obtained the highest callus frequencies of 90% with a combination of 3.0 mg L^{-1} 2,4-D and 2.0 mg L^{-1}, consistent with reports by Ali et al. (2004), who found that using BAP along with BAP or NAA enhanced callus induction and *in vitro* plantlets regeneration in rice. Mohammad et al. (2005) found that the percentage of callus formation increased at a high concentration of 2,4-D (2.5 mg L^{-1}). All nine Japonica cultivars produced the highest callus rate on media supplemented with a low concentration of BAP (0.3 mg L^{-1}), whereas indica type showed best on a medium containing a combination of 2.0 mg L^{-1} BAP, 0.5 mg L^{-1} kinetin and 1.0 mg L^{-1} NAA, and hybrid types showed the best results on media supplemented with a combination of kinetin (1.0 mg L^{-1}) and BAP (1.0 mg L^{-1}). In a study by Yadav et al. (2023), it was showed that a combination of 2.5 mg L^{-1} 2,4-D and 0.25 mg L^{-1} BAP resulted in maximum embryogenic callus induction (92%) for the MTU1010 indica rice variety. The highest frequency of friable calli was induced from *Oryza sativa* cv. Fujisaka 5 seeds on MS medium supplemented with 2.0 mg L^{-1} 2,4-D. In contrast, embryogenic calli were produced from *Brachiaria decumbens* seeds using a similar culture medium and remained embryogenic even after repeated subculturing (Duad et al., 2023). Establishing an *in vitro* regeneration protocol is very important for applying other techniques, especially genetic transformation or gene editing. Shoot regeneration

from callus depended on genotypes and growth regulators. To enhance callus induction, Pawar et al. (2015) supplemented the medium with proline and glutamine along with 2.0 mg L^{-1} 2,4-D, resulting in a significant increase in the callus formation rate (85.3%) compared to the medium without supplementation of proline and glutamine (78.4%). Additionally, a regeneration test revealed that four rice genotypes exhibited regeneration rates ranging from 32.0% to 83.2% on the medium containing 2.0 mg L^{-1} BAP, 0.5 mg L^{-1} NAA and 500 mg L^{-1} each of proline, and glutamine. However, the regeneration frequency significantly decreased (32.0%–59.4%) when 2.0 mg L^{-1} TDZ was used as a replacement (Pawar et al., 2015). In this study, the five rice cultivars exhibited the highest callus induction rate on medium supplemented with 2.5 mg L^{-1} 2,4-D, ranging from 73.67% to 84.67% across cultivars. It increased significantly with the addition of L-proline, L-glutamine, and casein hydrolysate (Table 2). Regeneration frequency was highest on medium supplemented with a combination of 2.0 mg L^{-1} BAP, 0.5 mg L^{-1} Kinetin, 0.5 mg L^{-1} NAA and 500 mg L^{-1} each of proline, glutamine, casein, resulting from 80.23% to 90.77% across cultivars.

Previous studies have shown that several factors influence transformation efficiency in rice (Li et al., 1992; Sundararajans et al., 2017). Japonica rice varieties are generally exhibits higher transformation efficiency than indica varieties due to differences in their tissue culture responses (Toki et al., 2006; Sahoo et al., 2011). Previous transformation methods have performed well in japonica rice cultivars such as ZH11 and Nipponbare (Hiei & Komari, 2008). In contrast, indica rice displays lower callus induction rates, higher levels of phenolic compound release, reduced regeneration efficiency and low transformation efficiency, which have hindered funcional stuies and application of indica cultivars (Thakur et al., 2022; Yadav et al., 2023). Chen et al. (2023) compared the transformation efficiency of five indica cultivars using the pCAMBIA1300 plasmid, indicating that the frequency of resistant calli ranged from 40.6% to 71.5% and transformation rate varied from 2.6% to 10.4%. In addition to the genetic factors, other physiological factors such as bacteria, callus quality, and phytohormone, also significantly influence the plant tissue culture process and transformation efficiency (Chen et al., 2004; Chen et al., 2023). Based on GUS gene expression and PCR analysis, the five tested cultivars in this study exhibited different transformation efficiencies, ranging from 13.67% to 29.0%.

To establish an efficient gene transformation process, it is crucial to develop a stable *in vitro* plant regeneration system. Therefore, although numerous studies have been conducted to establish gene transformation protocols in rice, optimizing these for specific rice varieties remains essential. This is also the primary objective of our research, which aims to identify suitable rice varieties that can be used for gene transformation and gene editing studies. Taken together, the BT7 cultivar exhibited the highest callus induction, plantlet regeneration, and responsiveness to genetic transformation. These results indicate that the BT7 cultivar is the most suitable for use as an explant in transformation techniques.

CONCLUSIONS

In this study, callus induction and shoot regeneration were investigated in five indica rice cultivars (BC15, BT7, KD18, OM5451, TBR225) to optimize the process for genetic transformation. Callus induction was most effective with 2.5 mg L^{-1} 2,4-D

combined with 500 mg L^{-1} L-proline, 500 mg L^{-1} L-glutamine, and 500 mg L^{-1} casein, yielding callus formation rates between 83% to 93.67% across the cultivars. Either lower (2.0 mg L^{-1} or higher (3.0 mg L^{-1}) concentrations of 2,4-D reduced callus formation in all five cultivars. Shoot regeneration was maximized on media supplemented with a combination of 0.5 mg L^{-1} kinetin, 2.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA, achieving average rates up to 84.47%. Among them, the BT7 cultivar exhibited the highest regeneration (90.77%). High transformation efficiency was recorded in the BT7 cultivar with 44.0% hygromycin-resistant calli and 29.0% GUS expression. PCR analysis confirmed a high percentage (87.5%) of transgenic plants in BT7. These results indicate that the BT7 cultivar is the most responsive for *in vitro* regeneration and genetic transformation.

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