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Agrobacterium-mediated transformation of Nang Thom Cho Dao, an indica rice variety

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ABSTRACT

Agrobacterium-mediated transformation is the most efficient method to transfer gene of interest into the plant genome. However, the transformation efficiency of this method with indica rice varieties is still low and needs to be improved. In this study, the Agrobacterium-mediated method was used to transfer reporter genes into the Nang Thom Cho Dao, an indica rice variety. Different transformation parameters, such as selection to used hygromycin at concentrations of 0, 25, 30, 40, and 50 mg/L, bacterial density, and infected duration, were tested and optimized. The results showed that 30 mg/L of hygromycin was the most appropriate concentration for selecting transgenic callus in Nang Thom Cho Dao. In addition, the highest transformation efficiency of this Nang Thom Cho Dao rice cultivar was observed at the bacterial suspension density OD_{600nm} of 0.1, the infected duration of 20 minutes, and the 3-day co-culture period. The presence and expression of transgenes were confirmed by gus staining and PCR with specific primers for hptII and gus genes. This transformation procedure should be used for further studies in genetic engineering of Nang Thom Cho Dao and other indica rice varieties.

1. INTRODUCTION

Traditional breeding methods are still popular, based on the recombination of genotypes and chromosomes through sexual reproduction according to Mendelian principles of heredity. This approach requires 7-8 generations of self-pollination in a row to generate a pure line (4-5 years). The duration of a pure line selection is depending upon the crops. After numerous generations of self-pollination, the selection mechanism will reject some valuable and good genotypes, allowing some undesirable genotypes to be expressed. Because we only need to transfer a single gene into crops, scientists have created a transgenic technique to overcome this issue since the first transgenic rice was published in 1988 (Toriyama et al., 1988). This provides the foundation for future gene transfer research in rice.

According to published studies on rice gene transformation, *Agrobacterium tumefaciens*-mediated gene transfer has been known as the most preferred approach. Regarding regeneration, indica rice varieties typically have low regeneration efficiency (Chu & Croughan, 1990; Zuraida et al., 2010; Thao et al., 2021), resulting in low gene output in transgenic plants. Regarding gene transfer, in addition to the difficulty of different types of species, the efficacy of gene transfer depends on the kind of tissue and infectious strain. Due to the regeneration process of indica rice varieties, studies in Vietnam on gene transfer and gene modification are limited. Therefore, it is important to establish sufficient procedures for regeneration and

transformation for the indica rice varieties in Vietnam. The density of bacteria is one of the most important factors for plant transformation via *Agrobacterium*-mediated method. The low bacterial cells could reduce the effectiveness of the contact of bacteria with the plant samples, while the high bacterial density may cause bacterial over-growth at the later stages of co-cultivation and selection (Wu et al., 2003). The other important factor is the infection time required for the bacteria to come into contact with plant cells at the site of injury. In addition, the infected duration also affects plant regeneration and transformation efficacy (Wu et al., 2003). During this time, gene transfer from the bacteria to the host cell will occur, such as transferring T-DNA into the host cell and inserting T-DNA into the host cell genome. Therefore, optimizing these parameters is very important to have successes in Agrobacterium-mediated transformation of selected crops.

In addition, in gene transfer, it is necessary to use bactericidal antibiotics to inhibit or kill bacteria after co-cultivation (Ratnayake & Hettiarachch, 2010), as well as to select transgenic plants (Quisen et al., 2009).

The aim of this study is to optimize main factors affecting transformation efficacy and establish a sufficient procedure for *Agrobacterium* -mediated transformation of Nang Thom Cho Dao, an indica rice variety.

2. MATERIAL AND METHODS

2.1. Material

Nang Thom Cho Dao belongs to *Oryza Sativa* L. subsp. India, is an aromatic rice landrace and is geographically restricted to the Can Duoc district in the Long An province of Vietnam. This variety was collected and stored at the Faculty of Agriculture - Can Tho University. The *Agrobacterium tumefaciens* strain AGL1 carrying the binary vector pCAMBIA1301 containing the *gus* gene and Hygromycin resistance gene (*hptII*) was provided by the Institute of Biotechnology, VAST (Figure 1).

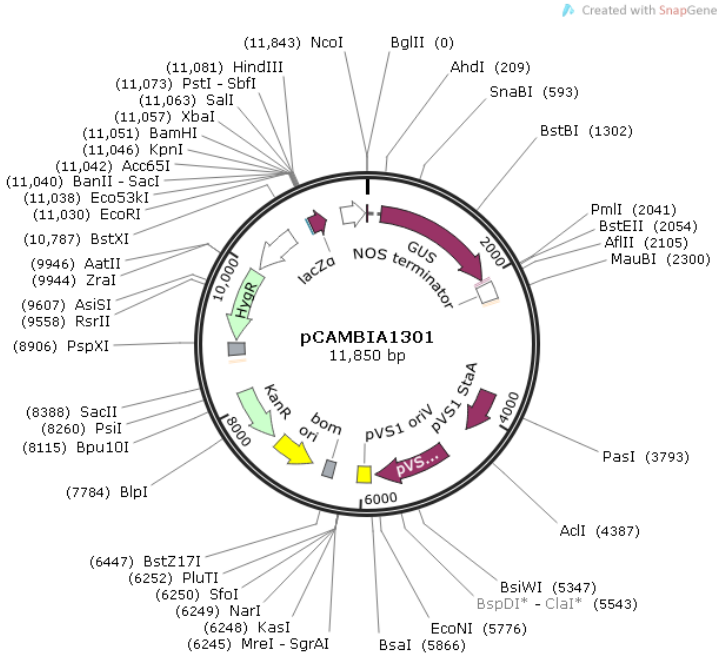

Figure 1. The map of pCAMBIA1301 vector

2.2. Methods

2.2.1. Optimizing gene transfer conditions

Identification of hygromycin concentrations transgenic selection

The study tested 4 different concentrations of Hygromycin, 25 mg/L, 30 mg/L, 40 mg/L, and 50 mg/L, for different stages including calli induction, pre-regeneration, and plant regeneration. Each treatment has 3 replicates with 240 samples.

Bacterial suspension density

To determine the optimal bacterial density for transformation, a test for calli infection in bacterial

suspension was performed with OD_{600nm} densities of 0.05, 0.1, 0.3, and 0.6. For each treatment, there will be 3 replicates with 240 samples of calli in one repeat.

Infection time

Infection time periods of 10 minutes, 20 minutes, and 30 minutes were examined. For each treatment, there will be 3 replicates with 240 samples of calli in one repeat.

Co-cultivation time

Co-cultivation periods of 2, 3, and 4 days were tested, with replications as described above

2.2.2. Genetic transformation by agrobacterium tumefaciens

Implementation of gene transfer process into Nang Thom Cho Dao rice variety

The gene transfer procedure was performed in 3 replicates, each time with 400 rice grains. Mature healthy rice seeds were de-husked without damaging embryos and surface sterilized in 70% ethanol for 1 minute, then in 60% Javel bleach solution supplemented with 1- 2 drops of Tween 20 with intermittent shaking for 45 minutes, followed by five rinses in sterile water. Sterilized seeds were dried on sterile filter paper and cultured on calli induction medium (N6D) in the dark at 26°C (Table 1). After 2 weeks, pale yellow crumb calli were derived from mature embryos and were sub-cultured to a fresh N6D medium for 5 days to serve as transgenic material.

Preparation of bacteria for gene transfer: bacteria were grown on solid YEP medium supplemented with 50 mg/L kanamycin antibiotic for 3 days in the dark, at 28°C (Table 1). Bacteria dissolve in a dilute N6 medium supplemented with 150 µM AS with an optimized concentration of bacteria used for gene transfer of OD_{600nm}.

Calli infection with bacterial suspension: Was done by soaking the calli in the bacterial suspension and shake gently for 20 minutes. Blotting the bacterial solution with sterile blotting paper and transferring the calli to 2N6-AS co-culture medium with blotting paper (Table 1). After 3 days of co-culture, calli will be infiltrated and transferred to N6D medium supplemented with antibiotics 500 mg/L Cefotaxime and 50 mg/L Timenti n.

Table 1. Compositions of different media used in the study

Medium	Label	Compositions
Calli induction	N6D	N6 (Chu et al., 1975) + 2 mg/L 2.4-D + 100 mg/L myo-inositol + 300 mg/L casamino acid + 2.878 g/L L-proline + 30 g/L sucrose + 7 g/L agarose, pH 5.8
Co-cultivation	2N6-AS	N6 + 2 mg/L 2,4-D + 100 µM AS + 100 mg/L myo-inositol + 300 mg/L casamino acid + 10 g/L glucose + 30 g/L sucrose + 3 g/L gellan, pH 5.2
Calli differentiation	MSNK+Hygromycine	MS (Murashige & Skoog, 1962) + 0,2 mg/L NAA + 2 mg/L kinetin + 100 mg/L myo-inositol + 2 g/L casamino acid + 30 g/L sucrose + 30 g/L sorbitol + 7 g/L agarose, pH 5.8 + Hygromycine
Regeneration	MS+B+Hygromycine	MS + 1 mg/L BAP + 30 g/L sucrose + 7 g/L agarose, pH 5.8 + Hygromycine
Rooting	MS	MS + 30 g/L sucrose + 7 g/L agarose, pH 5.8
Bacterial culture	YEP	10 g/L yeast extract + 10 g/L bacto peptone + 5 g/L NaCl + 15 g/Lbacto agar, pH 7.0
Suspension	N6 liquid	N6 salts + 68 g/L sucrose + 36 g/L glucose + 3 g/L KCl + 4 g/L MgCl2 +100 µM AS, pH 5.2

After 7 days, calli were transferred to selective medium N6D with Hygromycin at the selected concentration, supplemented with 400 mg/L cefotaxime and 30 mg/L timentin (Table 1). Following 7-10-day incubation in the dark, calli were transferred to selective medium II (similar to selective medium I). Calli were placed on selective medium II for 7 days before transferring to MSNK medium (supplemented with 400 mg/L Cefotaxime and Hygromycin the selected concentration) (Table 1).

Plant regeneration: After 9 days post transfer to MSNK, Hygromycin-resistant calli were transferred to regeneration medium MS+B containing 400

mg/L cefotaxime, and the selected Hygromycin concentration under the light at 26℃ condition for 10-14 days. Then, regenerated shoots were transferred to rooting medium MS supplemented with 250 mg/L cefotaxime (Table 1).

Examination of transgenic plants by PCR and X-Gluc staining method

To assess the transgenic plants, the *gus* and Hygromycin genes in the construct were targeted through PCR. Each PCR (Polymerase Chain Reaction) reaction had a total volume of 15 ul, containing 5X MyTaq Buffer, Taq DNA Polymerase, additional MillQ water, primer pairs, and DNA. Mixed well before being put into the GeneAmp PCR System 2700 thermal cycler. Following these procedures, the PCR program was established. The samples were subjected to 35 cycles of 30 seconds of melting at 94°C, 30 seconds of annealing at primer temperature (Table 1), and 30 seconds of synthesis at 72°C, followed by five minutes of final extension at 72°C. The PCR products were detected on 2% agarose gels, stained with ethidium bromide, and visualized and photographed under UV light to be scored.

Table 2. List primer sequences used in the study

Primers	Sequences (5'-3')	Temperature (℃)	Size (bp)
Gus F	5'ACCGTTTGTG TGAACAACGA3'	56	1003
Gus R	5'GGCACAGCAC ATCAAAGAGA3'		
Hyg F	5'GCGAAGAATC TCGTGCTTTC3'	56	605
Hyg R	5'GATGTTGGCG ACCTCGTATT3'		

Embryogenic shoot and roots were incubated in X-Gluc solution (Tris/NaCl pH 7.2 + 0.1% X-Gluc + 10% Triton X-100) at 37°C for 48 hours. Then the stained shoots and roots were rinsed and soaked in 70% ethanol to remove the chlorophyll completely. The frequency of transient or stable *gus* expression was based on positive shoots and roots.

3. RESULTS AND DISCUSSION

3.1. Hygromycin concentration for transgenic selection medium

It is known that Hygromycin, an aminoglycoside, suppresses the creation of scar tissue and is therefore considered as a marker of calli death (Canino et al., 1998; Gonzales et al., 1978). So Hygromycin was used as a selection marker for this study. The results showed that 100% of calli died at concentrations from 30 mg/L to 50 mg/L Hygromycinon (Table 3). However, survival was found at 25 mg/L Hygromycin. After 14 days on a rooting media treated with 30 mg/L hygromycin, 100% of regenerated plants became black and died (Figure 2). Similar observations were found by Tee et al. (2011) for the hygromycin concentration range from 25 mg/L to 30 mg/L. Therefore, the hygromycin concentration at 30 mg/L will be used for transgenic plant selection in further experiments of Nang Thom Cho Dao transformation.

Table 3. Effect of hygromycin concentrations on selection stages

Concentration (mg/L)	Total calli	Sample survival rate (%)		
		Selection stage	Shooting stage	Rooting stage
0	720.0	100.0	100.0	100.0
25	720.0	28.4	0.0	7.9
30	720.0	0.0	0.0	0.0
40	720.0	0.0	0.0	0.0
50	720.0	0.0	0.0	0.0

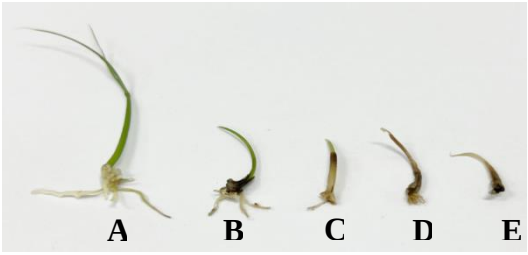

Figure 2. Effect of Hygromycin concentrations on rice growth at the rooting stage

A: 0 mg/L hygromycin: B: 25 mg/L hygromycin; C: 30 mg/L hygromycin; D: 40 mg/L hygromycin; E: 50 mg/L hygromycin.

3.2. Bacterial suspension density

The concentration of the bacterial suspension is one element influencing the efficacy of transgene integration. According to the data, the maximum percentage of calli with GUS was 75.81% as OD_{600} of 0.1 bacterial suspension density was used for infection (Table 4). With an OD_{600} bacterial suspension density of 0.1, the highest levels of shoots with GUS were also observed (11.30%).

Compared to the results of the previous study, which employed the GV3101 *Agrobacterium* strain with an OD_{600} of 0.1 to infect the calli of the Sri Lankan

rice variety (Ratnayake & Hettiarachchi, 2010), the current study's results are more favorable. With a high percentage of GUS-positive samples and a high ratio carrying the *gus* gene at a 0.1 OD_{600} bacterial density, it was concluded that a bacterial concentration of 0.1 OD_{600} would be optimal for *Agrobacterium tumefaciens*-mediated transformation of calli.

Table 4. Bacterial cell density on transformation efficiency

OD_{600}	Total calli	Calli with GUS (%)	Shoots with GUS (%)	Shoots carrying the *gus* gene (%)
0.05	720.0	65.7±2.3	9.2±0.9	8.1±1.0
0.1	720.0	75.8±1.9	11.3±0.7	9.4±0.8
0.3	720.0	58.2±2.4	5.3±1.2	5.1±1.2
0.6	720.0	37.6±2.8	3.7±1.1	3.3±1.2

3.3. Bacterial inoculation time

Different plant species have different ideal bacterial inoculation durations in their transgenic processes, such as watermelon, which had an infection time of approximately 30 minutes, resulting in a range from transgene expression rates between 86.67 to 93.33% (Nga et al. 2012). Similarly, in soybean, Thao et al. (2021) discovered that the optimal duration for bacterial inoculation of soybean is approximately 30 minutes, which results in the best transgenic rate.

In this study, the rate of calli with GUS was found at 71.2% from the treatment of 20 minutes of infection. Also, at 20 minutes, the approximately shoot induction rate (9.7%) was found (Table 5).

In addition, the experiment revealed that transformation times shorter or longer than 20 minutes resulted in a significantly lower infection rate. Similar to several prior investigations, a 20-minute infection period was used on rice types such as Tai Chung 65 (Giang et al., 2015), and Khang Dan 18 (Thao et al., 2021). Therefore, a 20-minute infection time permits the greatest transfer of genes and has the least impact on calli formation.

Table 5. Effect of infection time on gene transfer efficiency

Time	Total calli	Calli with GUS (%)	Shoots with GUS (%)	*Gus* positive (%)
10 minutes	720.0	57.8±1.4	6.2±1.1	6.3±0.6
20 minutes	720.0	71.2±1.6	12.3±0.9	9.7±0.7
30 minutes	720.0	49.7±2.1	4.6±1.3	4.8±1.0

3.4. Co-cultivation time

During the co-culture period, the bacteria continue to develop, penetrate the calli and incorporate the T-DNA fragment into the plant genome. In this study, a co-culture time of 3 days resulted in about 88.2% of the calli samples stained with X-Gluc were green. At 2 days and 4 days, the *gus* staining rate was 78.5 and 73.6 percent, respectively (Table 6).

With 3 days of co-culture, also had a greater proportion of green samples at the budding stage (13.6%) than in the other two periods. This outcome is comparable to recent research on the transformation of Indica rice variety Bac Thom 7 (Cao et al., 2019). Three days (72 hours) on an N6-AS medium with blotting paper was judged to be the optimal co-culture time for the gene transfer process based on the findings of the culture time experiment. It helps prevent the growth of microorganisms in the environment.

Table 6. Effect of time of co-culture on transgenic efficiency

Time	Total calli	Cali with GUS (%)	Shoots with GUS (%)	Rate shoots carrying the *gus* gene (%)
2 days	720.0	78.5±1.2	5.7±1.1	5.1±0.7
3 days	720.0	88.2±1.3	13.6±0.8	9.2±0.7
4 days	720.0	73.6±1.1	5.9±1.0	3.8±0.9

3.5. Transformation procedure

The stage of calli generation from seed embryos with a regeneration rate of 94% calli was used as genetic material (Table 7). After the transformation phase, co-cultured on 2N6-AS medium for 3 days in the dark and 7 days on N6D+C4+T5 medium in the bacteriostatic stage, the calli were transferred to the 7-day stage on selection medium 1 (N6D+H30+C4+T5) and 7-day selection period 2. After two stages of selection, black, shrunken, and infected calli will be eliminated because of their

inability to carry trangenes, as they cannot survive on a medium containing the selective antibiotic Hygromycin. Those calli that continue to develop and turn yellow will advance to the subsequent phase. (Figure 3), 74.60 and 54.60% of calli from selection stages 1 and 2, respectively, continued to develop and could carry genes to the next stage.

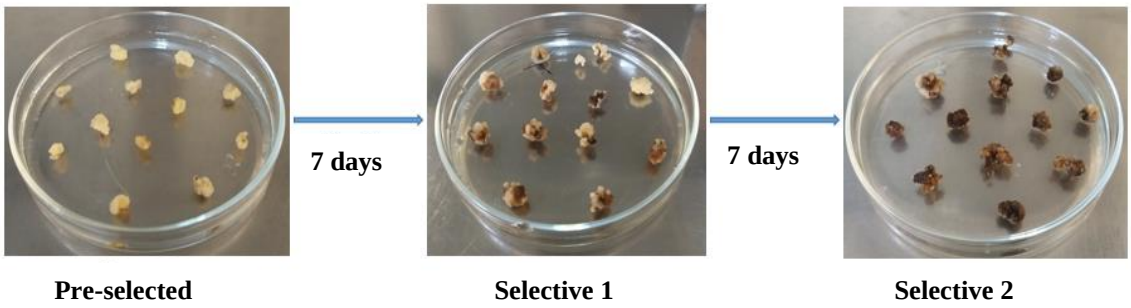

Figure 3. Calli selection on N6D Hygromycin medium

Table 7. Results of the gene transfer process into the rice variety NTCD

Stages	Total calli	Infection rate (%)	Death rate (%)	Rate of scar tissue growth (%)
Calli	1200.0	0.0	5.90	94.08
Selective 1	1129.0	10.32	15.07	74.60
Selective 2	855.0	16.70	28.75	54.60
Shoot induction	153.0	3.35	14.37	82.30
Plant regeneration	125.0	0.00	0.85	99.10
Total				124

In the pre-regeneration stage on MSNK medium for 14 days in the dark, the calli continued to grow spongy vigorously and spongy with a rate of 82.30%, and the rate of calli death and infection was low. Then transplanted to the regeneration stage on MS+1B medium for 14 days under the light, the calli developed with small green shoots at 99.10% (Table 7, Figure 4) .

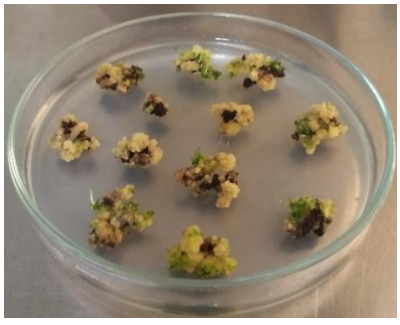

Figure 4. Rice somatic embryo development on selection MS+1B medium

Figure 5. Transgenic plants regenerated on selection MS medium

From the produced shoot clusters, the samples were transferred to MS medium to develop the plant's roots (Figure 5). Upon completion of the gene transfer procedure, the NTCD rice variety produced 124 transgenic lines that grew at a rate of 10.33%. The plants were reproduced further and the presence and expression of the *gus* gene were examined .

3.6. Evaluation of transgenic plants by molecular biology techniques and X-Gluc staining method

The results showed that *gus* and *hptII* gene fragments, 1005 bp and 605 bp, respectively, were successfully amplified from the transgenic plants (Figure 6 a, b). In contrast, the wild type (control) did not show any amplification. The result showed that transgenic plants carried the genes.

To evaluate *gus* gene expression, the X-Gluc staining technique was used. The results showed that 9.89% of transgenic plants expressed *gus* (Figure 7). Upon X-Gluc staining, the wild-type control calli remained yellowish white. Based on the identification of transgenes by X-Gluc staining, the initial estimation of the effectiveness of the gene transfer method was up to 9.89% of the total samples (Figure 8)

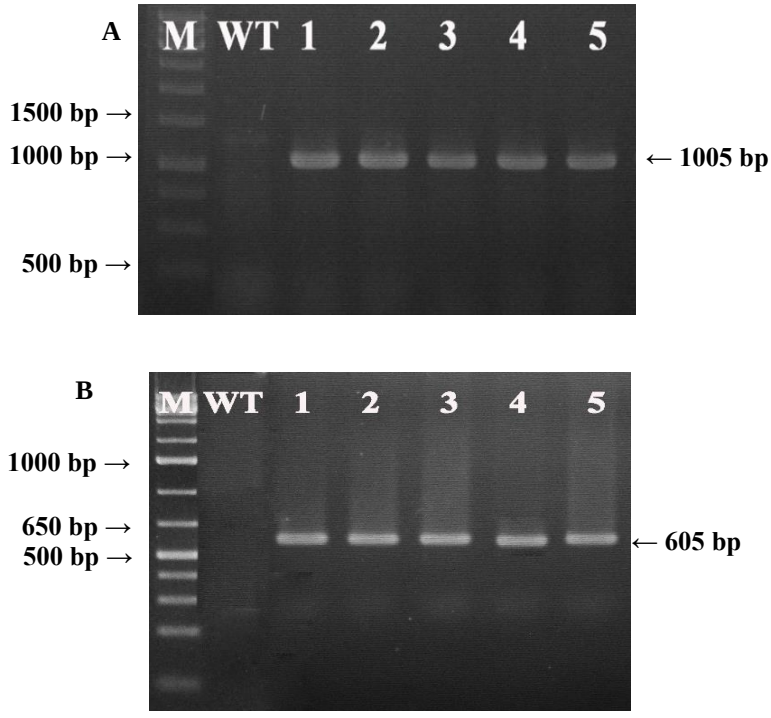

Figure 6. Gel electrophoresis with PCR products of transgenes (A) PCR products of the *gus* gene; (B) PCR products of the *hptII* gene

(M: ladder 1kb plus – Invitrogen - US, WT: non-transgenic control plants, 1-5: transgenic plant lines)

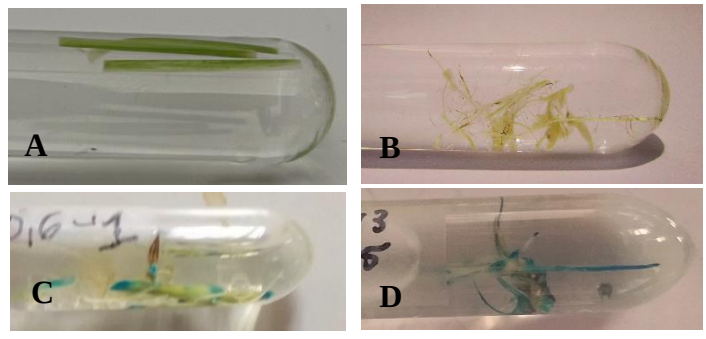

Figure 7. GUS staining of rice tissue

(A: Control shoot; B: Control root; C: Shoot; D: Root)

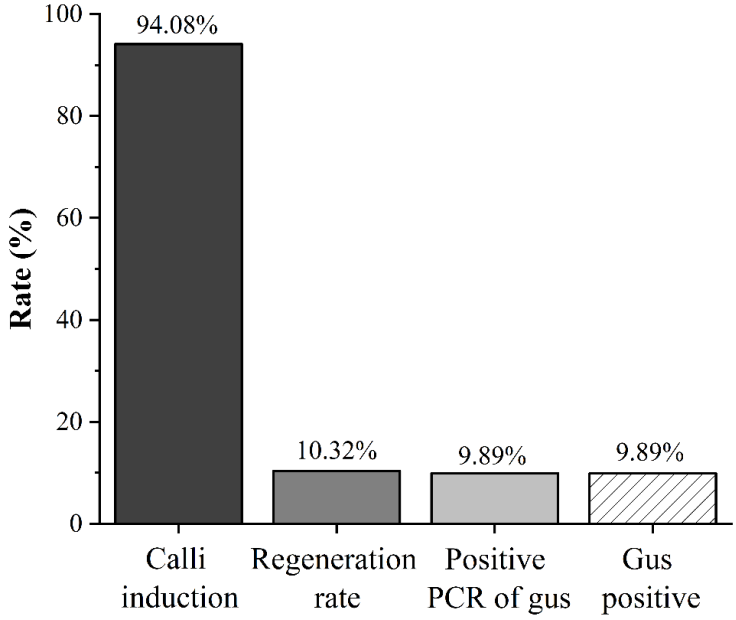

Figure 8. Efficiency of the gene transfer process into the rice variety NTCD

4. CONCLUSION

In this study, different parameters were tested and optimized for *Agrobactrium*-mediated transformation of NTCD, an Indica rice variety. On the large scale, the transformation efficiency could reach 9.89% using a microbiological concentration of OD_{600} of 0.1 in combination with the selective antibiotic hygromycin with a threshold of 30 mg/L. This procedure should be potential for further research on genetic engineering of NTCD and other Indica rice varieties.

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