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Establishment of Callus Induction, Cell Suspension Culture, and *Agrobacterium*-mediated Transformation system for Iranian Rice Cultivars

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ABSTRACT

Up to now, a large number of optimized plant systems have been established for various purposes in *Japonica* and *Indica* rice. Based on genetic diversity in rice cultivars, this study established highly efficient protocols for *in vitro* callus induction, cell suspension cultures, and genetic transformation for some Iranian rice cultivars using mature embryos. In this study, the effect of different concentrations of 2,4-D (0.0, 1.0, 1.5, and of 2.0 mg L^{-1}) on callus induction were investigated in 10 cultivars. Regarding higher callus induction frequency and mean weight of fresh calli, Hashemi, Binam, and Kazemi cultivars were selected for the experiments. The effects of kinetin (0.0, 1.0, and 2.0 mg L^{-1}) and sucrose (30.0 and 60.0 g L^{-1}) concentrations were tested to improve the biomass yield of a cell suspension culture. The MS medium supplemented with 0.5 mg L^{-1} 2,4-D, 2.0 mg L^{-1} kinetin, and 60.0 g L^{-1} sucrose exhibited the maximum cell growth in the selected cultivars. The transformation efficiency for different bacterial strains (EHA105, LBA4404, and AGL-1), OD_{600} (0.1, 0.3, 0.6), the concentration of acetosyringone (50, 100, 200 µM), and co-cultivation period (1, 2, 3 days) were evaluated. The presence and expression of *gusA* gene in transgenic cultivars were determined by GUS histochemical assay, PCR, and RT-PCR analysis. The result showed that Hashemi cultivar had the highest cell biomass and efficiency of genetic transformation (58%) with EHA105 at bacterial OD_{600}= 0.3, in 100 (µM) acetosyringone and two days of co-culture time. The findings offer insights for genetic transformation studies in Iranian rice cultivars.

Keywords: Callus induction, Genetic transformaion, GUS expression, MS media, Rice.

INTRODUCTION

Rice (*Oryza sativa* L.), which belongs to the Poaceae family, is the most important food crop in the world, following wheat and maize (Khush, 1997). Due to its high biosafety and low production cost, the rice cell suspension culture system is appropriate for producing recombinant human proteins (Liu *et al*., 2018). The friable calli production and subsequently fine cell culture in rice are affected by several factors, including genotypes, Plant Growth Regulators (PGR), temperature, light, sucrose concentration, and other culture medium components. Several studies on callus induction of rice genotypes (Tariq *et al*., 2008) showed that a mature embryo has more potential for callogenesis in comparison to other explants. However, the lack of an efficient protocol applicable for all genotypes is one of the main barriers in

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biotechnological studies (Binte Mostafiz and Wagiran, 2018). Therefore, establishing a dedicated callus induction system for each cultivar to develop a suspension cell culture protocol is an essential primary step. Suspension cultures have been established for different purposes in various cultivars, such as growth kinetics in *Indica* rice (Sathish *et al*., 2018), response to environmental conditions in *Japonica* rice (Wang *et al*., 2013), and agronomic improvement of Indonesian black rice (Susanto *et al.,* 2020). The plant cell culture systems offer more advantages in producing recombinant protein, such as scalability, controlled process conditions, cost-effectiveness and human pathogens-free (Schmale *et al*., 2006), the maximal yield of target proteins (Zagorskaya and Deineko, 2017), and no regeneration of transgenic plant (Fischer *et al.*, 2013). The systems are extensively used to produce a wide range of pharmaceutical proteins, including recombinant human acid α-glucosidase (Jung *et al*., 2016) and glucocerebrosidase (Nam *et al*., 2017) in transgenic rice cell suspension culture. Additionally, Jung *et al*. (2017) suggested the acid α-glucosidase with high-mannose glycans in *gnt1* rice for treating Pompe disease.

A large number of reports on the *Agrobacterium*-mediated transformation of rice has shown that the transformation efficiency is influenced by several factors, including plant genotype (Indurker *et al*., 2010), explant type and age (Koetle *et al*., 2017), *A. tumefaciens* strain (Nyaboga *et al*., 2015), bacterial cell density (Maleki *et al*., 2018), acetosyringone concentration (AS) (Maleki *et al*., 2018), and *Agrobacterium* infection methods (Zhao *et al*., 2011). Although *Agrobacterium tumefaciens*-mediated transformation system for *Indica* and *Japonica* rice cultivars has been extensively established (Slamet-Loedin *et al*., 2014), scant studies have examined the efficient tissue culture and transformation systems in Iranian rice cultivars (Naseri *et al.*, 2012; Ebrahimi *et al.*, 2019).

Adopting a long-run perspective, we intended to evaluate the potential of ten selected Iranian rice genotypes for recombinant protein production and pharmaceutical application using a cell suspension culture method**.** In this regard, we aimed to: (1) Optimize the callus induction system in the cultivars, (2) Select three cultivars with higher callus induction frequency and fresh calli mean weight, (3) Investigate the effects of different concentrations of kinetin and sucrose on the calli quantity and quality for initiating cell suspension cultures, and (4) Evaluate the main factors affecting the genetic transformation, including bacterial strain and density, co-cultivation period, and AS concentration.

MATERIALS AND METHODS

Plant Materials

Ten Iranian rice cultivars, namely, Hashemi, Kazemi, Domsiah, Binam, Fajr, Shafagh, Khazar, Hasani, Gohar, and Tarom were provided by Rice Research Institute of Iran, Rasht, Guilan.

Calli Induction Medium

MS basal media (Murashige and Skoog, 1962) containing B5 vitamins, 30 g L^{-1} sucrose, and 6 g L^{-1} plant-agar was used in all experiments. All PGR were added before autoclaving at 121°C for 20 minutes. The pH of the media was adjusted to 5.8 before autoclaving. The cultures were incubated under culture room conditions, including 25±2°C with 55% relative humidity in the dark.

Establishment of Callus Cultures

Seeds were carefully and manually dehusked and then were sterilized in 70% ethanol for 90 seconds and 50% commercial bleach (5% sodium hypochlorite) containing Tween 20 as the wetting agent, for 20 minutes. The sterilized seeds were washed three times with sterile double distilled water and then blotted dry on a Whatman filter paper. To induce callus, sterile seeds were placed on MS medium containing different concentrations of 2,4-Dichlorophenoxy acetic acid (2,4-D) (0.0, 1.0, 1.5, and 2.0 mg L^{-1}). The calli formed from the scutellum region were transferred to a new fresh media at every 14 days intervals. Subculturing was performed within four cycles in order to select fast-growing callus lines.

Establishment of Cell Suspension Cultures

The two-month-old calli (0.5 g) from the Hashemi, Kazemi, and Binam brands were transferred to 100-mL Erlenmeyer flasks that contained 25 mL MS liquid medium supplemented with 2,4-D (2.0 mg L^{-1}), kinetin (0.0, 1.0, 2.0 mg L^{-1}), and sucrose (30 and 60 g L^{-1}). The cells were transferred to a fresh medium every 14 days with a 5% inoculum. The cell suspension flasks were put on a gyratory shaker at 26±2°C and shaken at 120 rpm in the dark.

Analysis of Growth Parameters

The callus induction was obtained according to the following equation:

Callus induction frequency= (No. of seeds produced calli)/(No. of seeds cultures)×100

The growth index of calli was determined by measuring fresh callus weight, dry weight, and packed cell volume after one month. Dry weight was measured through vacuum filtration of 10 mL cell suspension culture with a Whatman filter (No. 5), and the cells were dried at 60 °C for 24 hours (Schmale *et al.*, 2006). The growth curve of suspension cultures was obtained by recording the fresh weight every three days in a month. Cell viability was estimated using Triphenyltetrazolium Chloride (TTC) (Chen *et al.*, 1994). The cells were collected with centrifuge and then incubated with 2.5 mM TTC for 8 hours at room temperature in the dark. After 50% methanol extraction, OD_{485} was measured with a Spectrophotometer System (Smart Spec Plus, BIO-RAD). Three cultivars (Hashemi, Binam, and Kazemi) were selected for the next step based on the biomass in their cell culture.

Preparation of Bacterial Cultures and *Agrobacterium*-Mediated Transformation

Calli transformation was conducted by using different *Agrobacterium tumefaciens* strains, including EHA105, LBA4404, and AGL_1, harboring the binary vector pCAMBIA1304, carrying the *β-glucuronidase (gusA)* and *hygromycin phosphotransferase (hpt)* genes under the control of CaMV35S promoter, and employing a nos terminator. The *A. tumefacient* strains were grown on LB agar medium supplemented with 50 mg L^{-1} of Kanamycin and 25 mg L^{-1} Rifampicin. A single fresh colony of *Agrobacterium* strains was transferred to 20 mL of LB medium supplemented with the similar antibiotics and incubated at 28°C on a shaker at 150 rpm for 48 hours. An aliquot of 500 µL of bacterial suspension was added to a 50 mL LB medium with the additional antibiotics and was grown overnight at 28°C. The bacterial cells were harvested from the overnight grown culture by centrifuging at 3,000×g for 15 minutes. The bacterial cell pellets were resuspended in inoculation medium (MS medium containing different concentrations of AS and bacterial density). The resuspended bacterial cells were shaken at 150 rpm at 28°C for 60 minutes before being used. The calli were pre-cultured for 48 hours and then infected by different A. tumefaciens strains to harbor pCAMBIA1304 binary vector. The *Agrobacterium*-treated calli were blotted on a sterile filter paper and placed onto co-cultivation agar medium overlaid with a single piece of sterile filter paper in the dark at 25±2°C. After a co-cultivation period, the calli were first washed by 250 mg L^{-1}

cefotaxime three times and then transferred to selection agar medium supplemented with 250 mg L^{-1} cefotaxime and 50 mg L^{-1} hygromycin. The cultures were sub-cultured four times every ten days.

Optimization of Effective Parameters in the Transformation

The effects of genotype (Hashemi, Binam, and Kazemi), bacterial strain (AGL1, EHA105, and LBA4404), bacterial density (OD= 0.1, 0.3, and 0.6), AS concentrations (50, 100 and 200 μM), and co-cultivation time (1, 2, and 3 days) were investigated on the transformation in separate experiments. Only the candid parameter for optimization was variable in all experiments, and the other factors were applied at an optimized or average level. The transformed calli were harvested 21 days after the inoculation.

Histochemical GUS assay

According to the established method, the histochemical analysis of *gusA* gene expression was performed on the calli (Jefferson, 1987). Hygromycin-resistant callus that proliferated after four weeks in selection media was subjected to GUS staining. The agroinfiltrated calli were incubated for 72 hours at 37°C in a buffer containing 50 mM $NaPO_{4\leq}$ (pH 7.2), 20 mM EDTA (pH 8.0), 0.1% Triton X-100, 500 mg L^{-1} of 5-Bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc), and 20% methanol.

Transgene Analysis for GUS Presence and Expression

The Polymerase Chain Reaction (PCR) method was used to confirm the presence of *gusA* gene in transgenic rice callus according to the method described by Japelaghi *et al*. (2011). The PCR was performed under the following conditions: 1× (95°C for 3 minutes), 35× (95°C for 30 seconds, 58°C 1 minute, 72°C for 1 minute), 1× (72°C for 5 minutes). The used primer sequences were as follows: F-primer (*5′-ATACCGAAAGGTTGGGCAGG-3′)* and reverse primer sequence R-primer (*5′-ATAACGGTTCAGGCACAGCA-3′).* RT-PCR method was also used to evaluate cDNA Synthesis, as described by Sambrook and Russell (2001). To test the quality of the obtained RNA, 3 µg of total RNA treated with DNase I (Fermentas) was used for 5 minutes at 70°C as a template using Oligo$(dT)_{18}$ primer (1 µg µL^{-1}, Qiagen). Reaction products and DNA size markers (100 bp DNA ladder) were resolved on the 1.2% TBE-agarose gels and visualized under UV light following EtBr staining.

Statistical Analysis

All experiments were undertaken in a factorial form based on a completely randomized design and were repeated three times. The results were analyzed statistically by SPSS ver. 16 (SPSS Inc., Chicago, IL, USA), and mean comparisons were made using the Duncan multiple range test with a confidence level of $P \leq 0.05$. The variability of the data was expressed as mean±Standard Deviation (SD).

RESULTS AND DISCUSSION

Effect of Different Concentrations of 2, 4-D on Callus Induction

It has been reported that 2,4-D is a commonly employed auxin to induce friable callus induction in rice (Mohammed, 2020; Rima *et al*., 2020). The effect of 2,4-D concentrations on different cultivars for callus induction is shown in Table 1. The proliferation of callus was initiated from the basal (mesocotyl or coleoptile) region of the germinated seeds one week after the cultivation, and was continued until the fourth week of culture (Figure 1). No callus formation was found on MS medium without 2,4-D. The rice seeds produced only roots and shoots. When the

Table 1. Effect of different concentrations of 2,4-D on callus induction of different rice cultivars.

Cultivar	2,4-D (mg L^{-1})	Callus induction (%)[A]	Mean weight of fresh callus (mg)[A]	Morphology of callus
Hashemi	0.0	0.00 ± 0.00	0.00 ± 0.00	Production of roots and shoots without callus formation
	1.0	76.67 ± 5.77^{a-d}	150.00 ± 8.88^{ij}	White, nodular and compact
	1.5	86.67 ± 5.67^{a}	168.33 ± 4.16^{h}	White, friable and sticky
	2.0	86.67 ± 5.57^{a}	374.33 ± 7.09^{a}	Yellowish white, friable and sticky
Binam	0.0	0.00 ± 0.00	0.00 ± 0.00	Production of roots and shoots without callus formation
	1.0	73.33 ± 5.74^{b-e}	143.00 ± 4.58^{i-l}	White, nodular and compact
	1.5	83.33 ± 4.77^{ab}	153.00 ± 4.58^{hij}	White, friable and sticky
	2.0	86.67 ± 4.78^{a}	336.00 ± 6.08^{b}	Yellowish white, friable and sticky
Kazemi	0.0	0.00 ± 0.00	0.00 ± 0.00	Production of roots and shoots without callus formation
	1.0	66.67 ± 5.37^{de}	136.33 ± 6.80^{j-m}	White, nodular and compact
	1.5	76.67 ± 5.77^{a-d}	149.00 ± 5.00^{ijk}	White, friable and sticky
	2.0	83.33 ± 3.55^{ab}	314.67 ± 5.03^{c}	Yellowish white, friable and sticky
Domsiah	0.0	0.00 ± 0.00	0.00 ± 0.00	Production of roots and shoots without callus formation
	1.0	70.00 ± 10.12^{cde}	144.00 ± 9.16^{i-l}	White, nodular and compact
	1.5	80.00 ± 9.25^{abc}	160.33 ± 4.04^{hi}	White, friable and sticky
	2.0	83.33 ± 6.67^{ab}	331.67 ± 25.89^{b}	Yellowish white, friable and sticky
Tarom	0.0	0.00 ± 0.00	0.00 ± 0.00	Production of roots and shoots without callus formation
	1.0	63.33 ± 7.78^{e}	113.33 ± 11.59^{no}	Whitish and nodular
	1.5	73.33 ± 7.88^{b-e}	126.67 ± 6.65^{lmn}	White, globular and compact
	2.0	76.67 ± 5.22^{a-d}	251.00 ± 10.00^{d}	White, friable and sticky
Khazar	0.0	0.00 ± 0.00	0.00 ± 0.00	Production of roots and shoots without callus formation
	1.0	66.67 ± 9.32^{de}	113.33 ± 6.80^{no}	Whitish and nodular
	1.5	76.67 ± 6.23^{a-d}	131.33 ± 9.50^{k-n}	White, globular and nodular
	2.0	76.33 ± 5.55^{a-d}	213.67 ± 23.86^{ef}	White, globular and friable
Hasani	0.0	0.00 ± 0.00	0.00 ± 0.00	Production of roots and shoots without callus formation
	1.0	66.67 ± 11.54^{de}	128.00 ± 4.58^{lmn}	White and nodular
	1.5	76.67 ± 11.55^{a-d}	144.67 ± 3.78^{i-l}	White, globular and nodular
	2.0	73.33 ± 5.77^{b-e}	220.00 ± 11.5^{e}	White, globular and friable
Fajr	0.0	0.00 ± 0.00	0.00 ± 0.00	Production of roots and shoots without callus formation
	1.0	63.33 ± 7.74^{e}	124.67 ± 4.16^{mno}	Whitish and nodular
	1.5	76.67 ± 4.57^{a-d}	147.00 ± 9.00^{ijk}	White, globular and nodular
	2.0	80.00 ± 10.56^{abc}	226.67 ± 30.86^{e}	White, friable and sticky
Gohar	0.0	0.00 ± 0.00	0.00 ± 0.00	Production of roots and shoots without callus formation
	1.0	63.33 ± 7.79^{e}	106.33 ± 8.08^{o}	White and nodular
	1.5	73.33 ± 8.43^{b-e}	123.33 ± 3.21^{mno}	White, globular and compact
	2.0	73.33 ± 5.77^{b-e}	203.00 ± 9.64^{fg}	White, globular and friable
Shafagh	0.0	0.00 ± 0.00	0.00 ± 0.00	Production of roots and shoots without callus formation
	1.0	63.33 ± 5.77^{e}	113.67 ± 11.06^{no}	White and nodular
	1.5	73.33 ± 6.59^{b-e}	139.00 ± 6.24^{j-m}	White, globular and compact
	2.0	76.67 ± 3.28^{a-d}	192.00 ± 6.55^{g}	White, globular and friable

[A] The values represent the means±SD. Different letters in a column indicate a significant difference at $P< 0.05$ with Duncan's multiple range test.

Figure 1. The callus induction of rice cultivars on culture media supplemented with 2.0 mg L^{-1} 2,4-D. The proliferation of callus was initiated from the basal (mesocotyl or coleoptile) region of the germinated seeds. (a) Mature seeds of the callus induction medium, (b) Callus induction after two weeks of culture, (c) Callus subculture in same media after four weeks. Scale bars= 1 cm.

seeds were cultivated on MS medium supplemented with 2,4-D, callus induction occurred, indicating that 2,4-D has a determinable role in calli induction. When calli were transferred in a fresh medium containing 2.0 mg L^{-1} 2,4-D, their size increased two times almost in two weeks. At higher or lower than 2.0 mg L^{-1} concentration, all cultivars showed a similar amount of decrease in calli initiation. The culture medium supplemented with 2.0 mg L^{-1} 2,4-D revealed the maximum callus induction frequency (79.33%), followed by the supplementation with 1.5 mg L^{-1} 2,4-D (77.67%) and 1.0 mg L^{-1} 2,4-D (67.33%). Our study exhibited that 2,4-D at 2.0 mg L^{-1} produces maximum callus from mature rice seeds. Similar outcomes have been observed for aromatic rice (Mannan *et al*., 2013), Turkish upland rice (Yilmaz *et al*., 2018), and Popular *Indica* Rice Genotypes (Aananthi and Anandakumar, 2020). However, some researchers showed that a great amount of callus for various cultivars was induced from MS medium under different auxin treatments. For instance, Shweta *et al*. (2020) reported 2.5 and 3 mg L^{-1} as optimum 2,4-D concentrations for CO 51 and Binte and Wagiran (2018) in Malaysian wetland rice, respectively. Therefore, it appears that the optimum concentration of 2,4-D for maximum callus induction depends on the explant source and genotype. Among the ten cultivars, Hashemi, Binam, and Kazemi showed 86.67, 86.67 and 83.33% of callus induction frequency at 2.0 mg L^{-1} 2,4-D, respectively. The maximum weight of fresh callus was achieved in the cultivar Hashemi (374.33 mg weight), and Gohar exhibited the lowest (106.33 mg weight) callus biomass (Table 1). Different responses may happen due to the different genotypic efficiency of cultivars. The physiological and morphological potentials of rice genotypes play important roles in callus formation. Several authors have already reported these varietal differences (Rima *et al*., 2020; Paul and Roychoudhury, 2019). Morphology of the generated calli varied depending on the 2,4-D concentration. However, callus induction on MS medium supplemented with 2.0 mg L^{-1} 2,4-D seemed yellowish-white, big-sized, and friable in nature, compared to other 2,4-D concentrations.

Effects of Kinetin and Sucrose Concentrations on Cell Suspension Cultures

Different kinetin (0.0, 1.0, and 2.0 mg L^{-1}) concentrations besides the 2 mg L^{-1} 2,4-D and sucrose (30.0 and 60.0 g L^{-1}) were evaluated for initiating cell suspension cultures from friable calli of the three rice cultivars, including Hashemi, Binam, and Kazemi (Table 2). To establish suspension culture, 0.5 g of the friable calli were transferred to the same basal calli induction medium (MS medium supplemented with 2.0 mg L^{-1} 2,4-D and 30.0 g L^{-1} sucrose) without a gelling agent. After the 4th subculture, cells were transferred to a liquid MS medium with a reduced concentration of 2,4-D (0.5 mg L^{-1}). Decreasing the concentration of 2,4-D in the MS medium caused proliferating suspension cells without any sign of cell browning. Frequent subculture to fresh medium with different strengths of MS medium and low 2,4-D (auxin) concentration has been previously reported as affecting factors in good cell growth without cell browning (Tiwari *et al*., 2002). Moreover, adding antioxidants like ascorbic acid and citric acid has produced whitely yellowish and vigorous suspension cell cultures without browning (Bushra *et al*., 2009).

A significant difference in cell growth quantity was observed among the cultivars and between the kinetin and sucrose concentrations. The culture medium supplemented with 2.0 mg L^{-1} kinetin and 60.0 g L^{-1} sucrose exhibited the maximum cell growth (Figure 2). This indicates that using kinetin and sucrose with 60.0 g L^{-1} was adequate for producing a high number of cells in rice. The cultivars Hashemi, Binam, and Kazemi showed 3.97, 3.92, and 2.71 g mean weight of fresh cells from cell suspension cultures, respectively. The presence of 2,4-D

Table 2. Effect of different concentrations of kinetin and sucrose on cell cultures of different rice cultivars.

Cultivar	Kinetin (mg L^{-1})	Sucrose (g L^{-1})	Mean fresh weight of cells (g)[A]	Mean dry weight of cells (mg)[A]	Packed cell volume (%)[A]
Hashemi	0.0	30.0	1.88 ± 0.01[g]	94.23 ± 6.22[g]	25.66 ± 2.45[g]
	0.0	60.0	2.12 ± 0.01[fg]	106.33 ± 8.96[fg]	27.66 ± 3.71[fg]
	1.0	30.0	2.93 ± 0.02[de]	146.50 ± 12.50[de]	35.72 ± 5.23[de]
	1.0	60.0	3.14 ± 0.05[cd]	157.26 ± 15.69[cd]	37.68 ± 5.64[cd]
	2.0	30.0	3.62 ± 0.02[b]	181.54 ± 20.98[b]	43.44 ± 5.23[b]
	2.0	60.0	3.97 ± 0.02[a]	198.5 ± 18.64[a]	47.64 ± 6.59[a]
Binam	0.0	30.0	2.33 ± 0.02[f]	116.50 ± 14.23[f]	29.42 ± 3.66[f]
	0.0	60.0	2.69 ± 0.02[e]	134.50 ± 12.56[e]	32.42 ± 4.77[e]
	1.0	30.0	3.22 ± 0.57[c]	161.23 ± 20.32[c]	38.64 ± 4.78[c]
	1.0	60.0	3.10 ± 0.01[cd]	155.47 ± 15.32[cd]	37.20 ± 6.42[cd]
	2.0	30.0	3.60 ± 0.02[b]	180.25 ± 21.35[b]	43.20 ± 6.78[b]
	2.0	60.0	3.92 ± 0.01[a]	196.35 ± 12.69[a]	47.50 ± 6.88[a]
Kazemi	0.0	30.0	1.13 ± 0.01[j]	56.50 ± 3.58[j]	19.42 ± 2.69[j]
	0.0	60.0	1.36 ± 0.01[i]	68.55 ± 4.51[i]	21.33 ± 2.44[i]
	1.0	30.0	1.61 ± 0.02[h]	80.50 ± 9.89[h]	19.32 ± 1.28[h]
	1.0	60.0	2.13 ± 0.03[fg]	106.50 ± 10.22[fg]	25.56 ± 3.45[fg]
	2.0	30.0	2.31 ± 0.02[f]	115.49 ± 11.87[f]	27.72 ± 3.45[f]
	2.0	60.0	2.71 ± 0.02[e]	135.55 ± 14.36[e]	32.52 ± 4.12[e]

[A] The values represent the means±SD. Different letters in a column indicate a significant difference at $P< 0.05$ with Duncan's multiple range test.

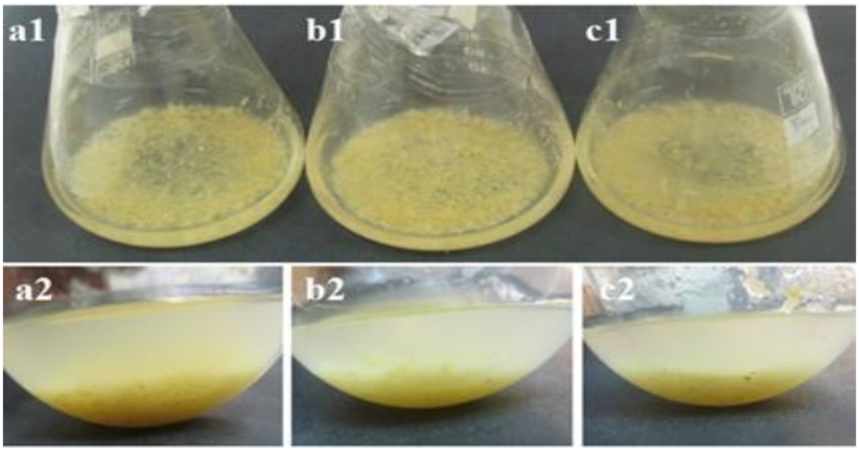


Figure 2. The cell suspension cultures of Iranian rice. (a1), (b1), and (c1) show the first subculture of Cv. Hashemi, Binam, and Kazemi, respectively. (a2), (b2), and (c2) show the final subculture after 4 weeks. In all experiments, the culture media were supplemented with 0.5 mg L^{-1} 2,4-D, 2.0 mg L^{-1} kinetin and 60.0 g L^{-1} sucrose.

in the cell suspension cultures was essentially required to stimulate cell division and strongly suppress organogenesis. Staba (1980) reported that auxin is the most commonly observed potent auxin. Nevertheless, in the current study, the presence of 2,4-D alone with a concentration of 0.5 mg L^{-1} in a liquid MS medium was found to delay cells proliferation. Therefore, kinetin should be added to exert additional physiological effects. The findings revealed that kinetin supplementation at 2.0 mg L^{-1} concentration and its combination with 0.5 mg L^{-1} 2,4-D produced a fine cell suspension culture. The combination of 2,4-D and kinetin could also be effective in calli induction (56.70%) in *Barringtonia racemosa* Lam (Osman *et al.*, 2016).

The maximum growth was obtained when cells were cultured on MS medium containing 60.0 g L^{-1} sucrose. The morphology of cells in this medium was relatively dry and compact compared to those observed on the medium containing 30.0 g L^{-1} sucrose (soft and friable). This type of cells may be produced due to the sucrose effect on the humidity of *in-vitro* culture conditions (Lee *et al.*, 2002).

Assays of Cell Growth and Viability

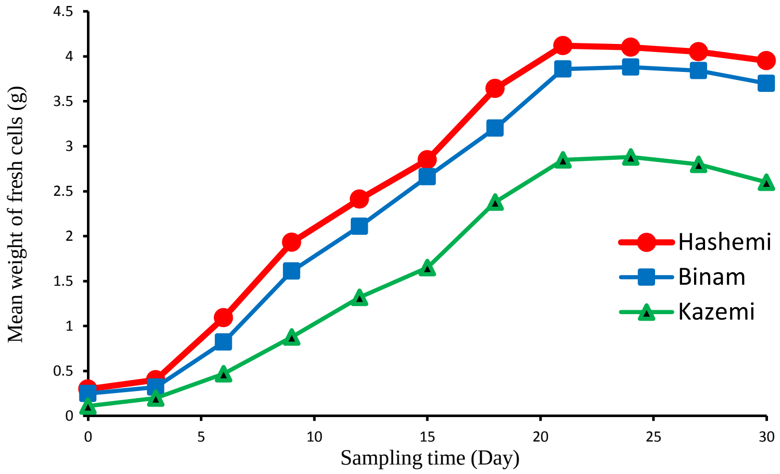
The growth kinetics of cell suspensions of cultivars Hashemi, Binam, and Kazemi were studied on MS media supplemented with 0.5 mg L^{-1} 2,4-D, 2.0 mg L^{-1} kinetin, and 60.0 g L^{-1} sucrose. The growth curves of cell cultures showed a sigmoidal-type pattern with three growth phases (Figure 3-a). The cells started with a three-day lag phase, continued with the log phase and, finally, reached a maximum growth 21 days after initiation, and started to decline seven days after the stationary phase. In the lag phase, cells were prepared for division and energy accumulation. The maximum growth in the exponential growth phase is due to rapid cell division and an increase in cellular volumes (Wang *et al*., 2016). It has also been reported that growth rate decline happened in the medium nutrients due to absorbing cultures and accumulating substances in the culture medium (Lima *et al*., 2008). The growth of cells in suspension cultures has been reported to proliferate more vigorously than cells on the solidified culture medium, probably due to the agitation and loosening of cells and subsequent further cell division (Lima *et al*., 2008). The same growth kinetic patterns were also reported in the cell suspension cultures of different species such as *Orthosiphon stamineus* (Lee and Chan, 2004), and *Nicotiana tabacum* (Schmale *et al*., 2006).

To monitor the cell viability, suspension cells of the three cultivars were prepared with an initial density of 3% (v/v) in MS medium supplemented 0.5 mg L^{-1} 2,4-D, 2.0 mg L^{-1} kinetin, and 60.0 g L^{-1} sucrose. Cell viability was high in about ten days because of residual intracellular sugars and/or supplemental nutrients. Then, the cell viability declined rapidly after the sucrose and almost ended on the18th to 20th days (Figure 3-b). Our data also supports previous reports that the cell viability was maintained in a medium containing sucrose for several days and then decreased progressively due to the lack of a carbon source (Trexler *et al*., 2005; Liu *et al*., 2015).

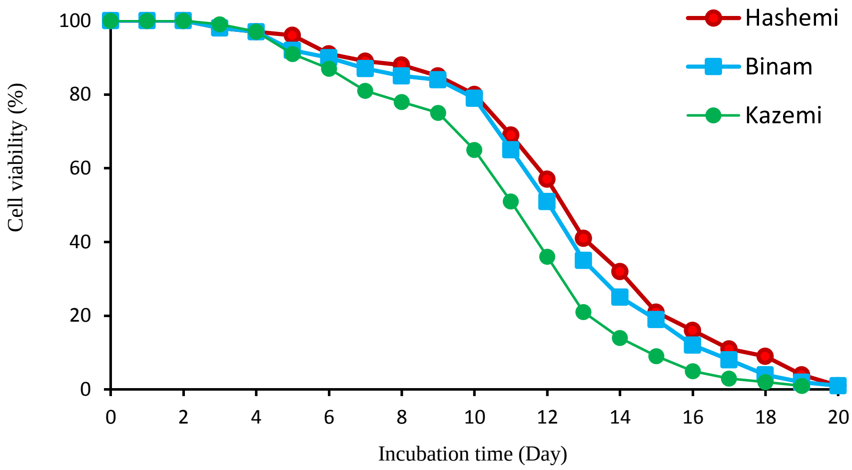
Optimization of the Transformation Parameter

The factors affecting the transformation efficiency were evaluated based on the frequency of hygromycin-resistant calli that were selected according to their size. The results indicated that the genotype had a significant effect on transformation efficiency. Three *Agrobacterium* strains (AGL1, EHA105, and LBA4404) were also evaluated for their ability to transform (results are shown in Figure 4). The highest rate of transformation efficiency was obtained to be 58% in the *Agrobacterium* strain EHA105 and by 42 and 35% in LBA4404 and AGL1 strains, respectively (Figure 4-a). Previous studies have revealed that variations in transformation efficiency levels among host genotypes may be due to differences in genetic background and susceptibility or compatibility of the genotypes to *Agrobacterium*, as demonstrated in Arabidopsis, tobacco, and rice (Wu *et al*., 2014; Zhao *et al*., 2011). Moreover, the genetic background of the *Agrobacterium* strains can considerably influence the transformation efficiency. For instance, the use of the highly virulent strain, such as EHA105, offered higher levels of transformation than the other tested strains in rice (Zhao *et al*. 2011).

Considering that the EHA105 strain showed the highest transformation efficiency in the Hashemi cultivar, we conducted next experiments to optimize the other influential factors. Therefore, the effect of three AS concentrations (50, 100, and 200 μM) was tested. The highest level of transformation efficiency was obtained with 100 μM AS (about 58.33%) in the co-cultivation medium.



(a)



(b)

Figure 3. The growth parameters of cell suspension cultures of three Iranian rice cultivars. (a) Cell fresh weight and (b) Viability of the cells were determined after sample collection. The error bars represent the standard.

However, less transformation efficiency was obtained in an AS concentration of more than 100 μM (Figure 4-b). Hence, we used the co-cultivation medium with 100 μM AS for further studies. In monocotyledonous plants where phenolic compounds are not naturally synthesized, AS is a key factor in enhancing gene transfer (Sabu *et al*., 2021). Similarly, Yaqoob *et al.* (2017) obtained the highest transformation percentage with 100 μM AS in rice. By contrast, Sawant *et al*. (2018) showed that 350 μM of acetosyringone had the highest transformation frequency.

It has been shown that high *Agrobacterium* concentrations ($OD_{600} \geq 0.4$) resulted in a significant decrease in the number of hygromycin-resistant embryogenic calli in Malaysian rice (Ab Rahman *et al*., 2017). Furthermore, Feng *et al*. (2018) reported that high bacterial density ($OD_{600} > 1.0$) could be toxic, resulting in physiological function disorder in plant cells or necrosis. Therefore,

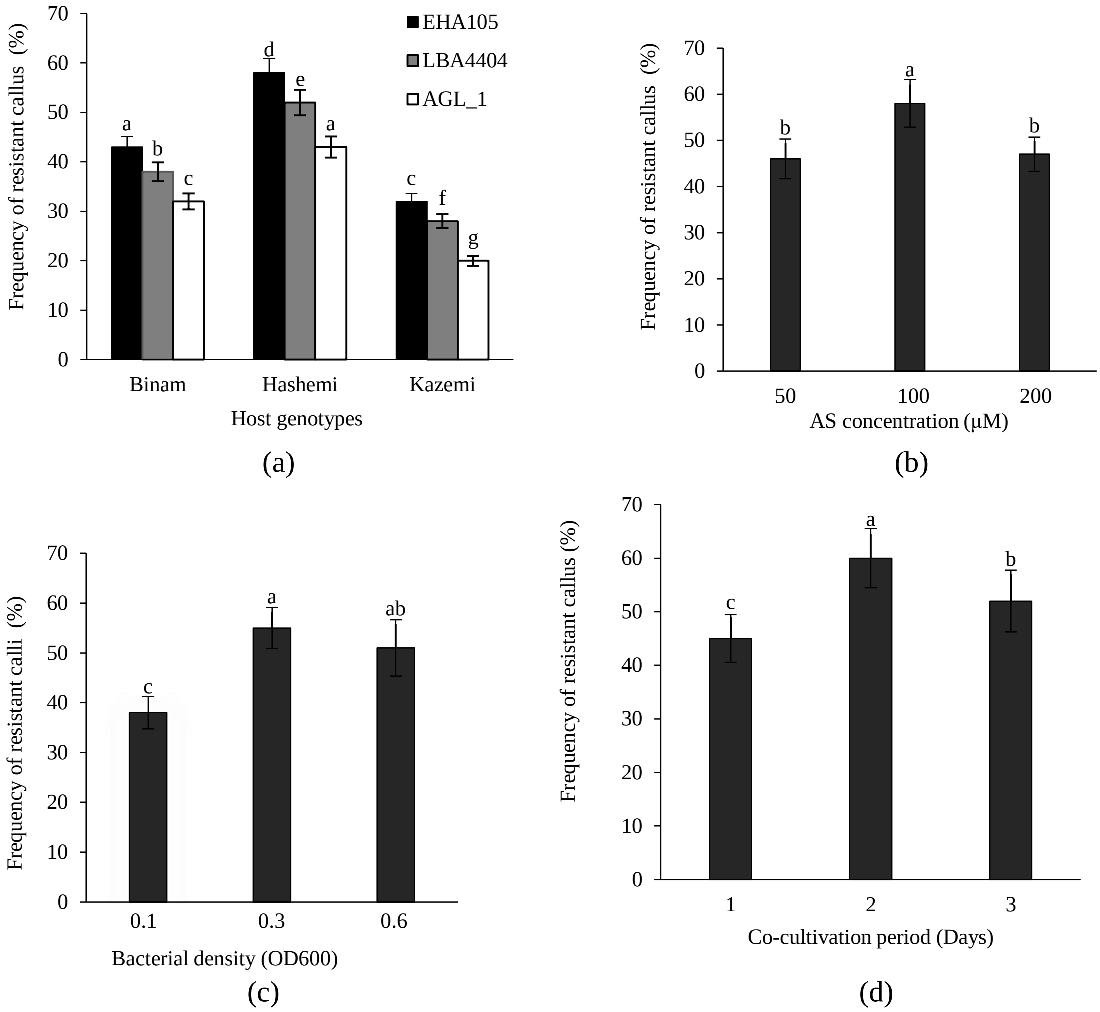


Figure 4. Analysis of factors affecting the transformation efficiency in rice: (a) Frequency of resistant calli (%) to hygromycin in the host genotypes and the bacterial strains, (b) The bacterial density, (c) The concentration of AS, and (d) The time of co-cultivation. Data are mean±SD frequency of resistant callus (%) from three biological replicates. Means followed by different letters are significantly different at P≤ 0.05 with Duncan's multiple range tests.

determining the optimal bacterial inoculation density is important for an efficient transformation system (Koetle *et al*., 2017). In this sense, the bacterial suspensions were adjusted to OD_{600} levels of 0.1, 0.3, and 0.6. The highest percentage transformation was obtained when an optical density (OD_{600}) of 0.3 was used (Figure 4-c). The amounts of percentage transformation for OD_{600} levels of 0.1, 0.3, and 0.6 were 38.33, 54.67, and 51.00%, respectively. In addition, Ozawa (2009) and Zhao *et al*. (2011) obtained the highest transformation efficiency by co-cultivating rice calli in *Agrobacterium* suspension to an OD_{600} of 0.2–0.3. Finally, the transformation efficiency levels were estimated 1, 2, and 3 days after the transformation. The highest level of transformation efficiency (59.67%) was obtained on the 2nd day after transformation and then decreased gradually (Figure 4-d).

GUS Histochemical Assay and Transgene Analysis

GUS expression was visually observed and photographed by using a camera. GUS histochemical assay in calli culture of Hashemi cultivar was inoculated with different strains of *Agrobacterium* containing binary vector pCAMBIA1304. As illustrated in Figure 5, all new calli produced from inoculated calli showed relatively strong and homogenous GUS staining in Hashemi cultivar with EHA105 bacterial strain, OD= 0.3, 100 µM AS and two days after the experiment. Accordingly, a co-cultivation period No GUS stains could be detected in calli transformed with native *Agrobacterium* as a negative control (Figure 5-b). Since the *gusA*-intron gene can be expressed only in plant cells, not in *Agrobacterium*, it is a reliable indicator of plant transformation (Vancanneyt *et al*., 1990). Therefore, in histochemical GUS assay activity, blue spots confirm the expression, transient or stable, of transferred genes in plant transgenic cells (Jefferson, 1987; Subramaniam and Rathinam, 2010; Rahman *et al.*, 2011).

The gene products of the transgenic calli for Hashemi, Binam, and Kazemi cultivars inoculated with different strains of *Agrobacterium* AGL-1, EHA105, and LBA4404 along with positive (binary vector pCAMBIA1304) and negative controls (wild-type *Agrobacterium* without expression) are shown in Figure 6. The *gusA* gene insertion into the transgenic callus genome was confirmed by enhancing PCR in a single-piece length at 1,182 bp and agarose gel (Figure 6-a). The *gusA* gene expression in transgenic rice calli was also investigated using RT-PCR, and a length of 1182 pairs of open pairs was observed on agarose gel (Figure 6-b). The proliferation of the *gusA* gene in the positive control sample and its non-proliferation in the negative control samples in PCR and RT-PCR indicate successful transplantation of transgenic rice calli with this gene.

CONCLUSIONS

In the present research, *in vitro* callus induction was studied on ten selected Iranian rice cultivars with different concentrations of 2,4-D. Higher callus induction frequency was observed with 2 mg L^{-1} concentrations of 2,4-D for all cultivars. Three selected cultivars, namely, Hashemi, Binam, and Kazemi, with higher induction frequency and mean weight of fresh calli were used for cell suspension and transformation. The cell suspension

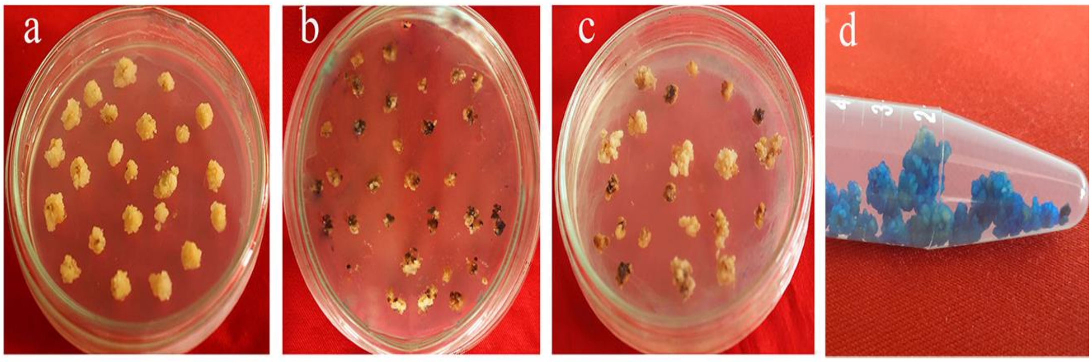


Figure 5. Callus transformation of Iranian rice cultivars via *Agrobacterium*-mediated Transformation system: (a) Callus induction in MS media without hygromycin, (b) The negative control, (c) The frequency of hygromycin resistant calli in the Hashemi genotype transformed by *Agrobacterium* strain (EHA105) harboring pCAMBIA1304 binary vector at four weeks after transformation, and (d) Histological GUS assay: The blue color callus was positive and had transient GUS expression.

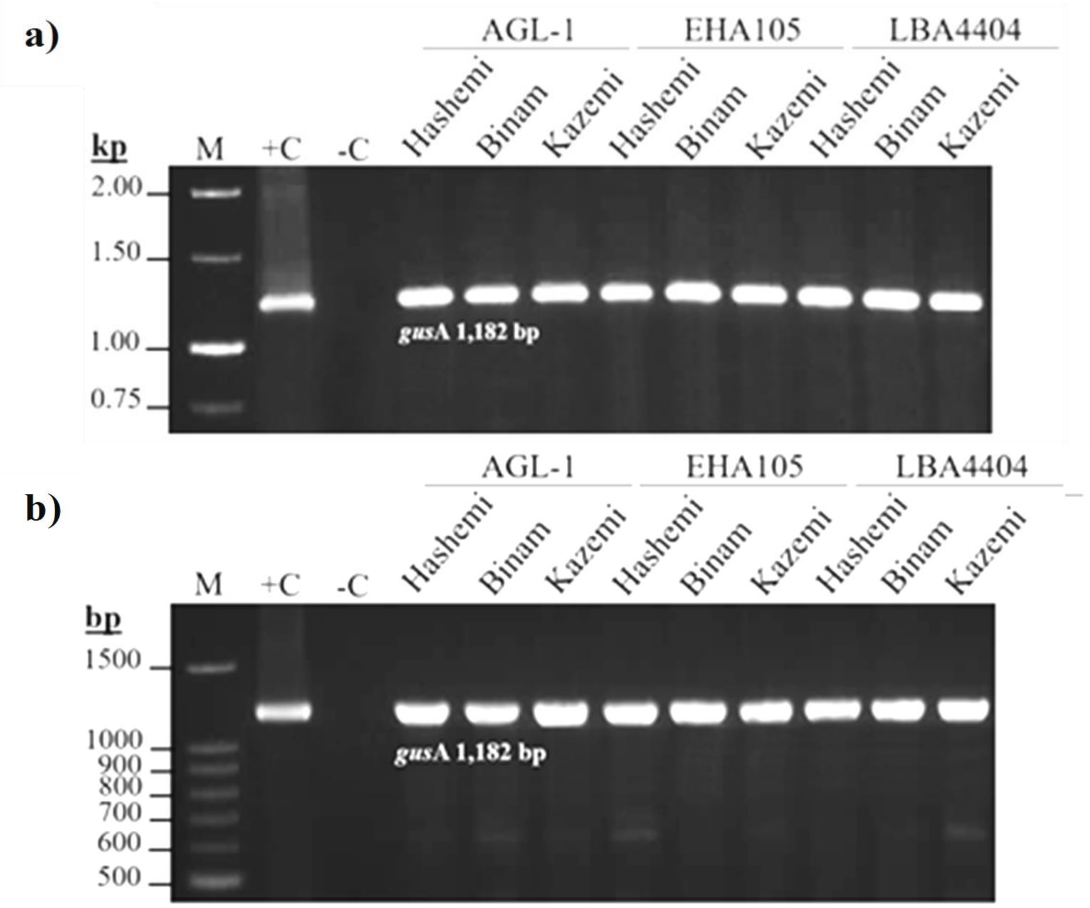


Figure 6. Confirmation of the presence of *gusA* gene in the genome of transgenic rice calli in Hashemi, Binam, and Kazemi cultivars inoculated with different strains of *Agrobacterium* AGL-1, EHA105, and LBA4404 using methods: (a) PCR, and (b) RT-PCR. **M:** Molecular weight of DNA kb 1 or bp 100, **+C:** pCAMBIA1304 binary vector used as a positive control. **C:** Wild-type *Agrobacterium* as a negative control.

cultures were established through inoculating friable calli into MS liquid medium supplemented with 0.5 mg L^{-1} 2,4-D, 2.0 mg L^{-1} kinetin, and 60.0 g L^{-1} sucrose. These friable calli were produced by culturing mature rice seeds on the MS medium containing 2.0 mg L^{-1} 2,4-D. Results showed that adding kinetin in the cell culture media combined with 2,4-D and a high concentration of sucrose significantly increased cell growth. Furthermore, results indicated that the transformation conditions, such as the host genotype, bacterial strain, AS concentration, bacterial density, and co-cultivation time significantly influence the transformation efficiency in rice. The findings of this research could be used for genetic transformation studies in Iranian rice cultivars.

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ایجاد سیستم بهینه القای کالوس، کشت سوسپانسیون سلولی و تراریختی مبتنی بر*آگروباکتریوم* برای ارقام برنج ایرانی

م. فرخنده‌طالع ناوی، ا. دورانی، ر. حدّاد، و ع. ا. عبادی

چکیده

تاکنون، سیستم‌های بهینه شدهء گیاهی متعددی با اهداف مختلف در برنج ژاپونیکا و ایندیکا معرفی شده است. با توجه به تنوع ژنتیکی در ارقام برنج، در این پژوهش، پروتکل‌های کارآمدی به منظور القای کالوس، کشت سوسپانسیون سلولی و انتقال ژن در شرایط آزمایشگاهی، برای برخی ارقام برنج ایرانی با

استفاده از جنین‌های بالغ ایجاد شد. در این مطالعه، اثر غلظت‌های مختلف تنظیم کنندهء رشدی 2,4-D (۰/۰، ۱/۰، ۱/۵ و ۲/۰ میلی‌گرم در لیتر) بر کالوس‌زایی ۱۰ رقم مورد ارزیابی قرار گرفت. در بین ارقام مورد مطالعه، هاشمی، بینام و کاظمی با توجه به بالا بودن فراوانی کالوس‌زایی و وزن‌تر کالوس‌ها، برای مراحل بعدی انتخاب شدند. اثرات غلظت‌های مختلف کاینیتین (۰/۰ ، ۱/۰ و ۲/۰ میلی‌گرم در لیتر) و ساکارز (۳۰ و ۶۰ گرم در لیتر) جهت بهبود عملکرد زیست تودهء حاصل از کشت سوسپانسیون سلولی مورد آزمایش قرار گرفت. محیط کشت پایه MS حاوی ۰/۵ میلی‌گرم در لیتر 2,4-D، ۲/۰ میلی‌گرم در لیتر کاینیتین و ۶۰ گرم در لیتر ساکارز، حداکثر رشد سلولی را در ارقام منتخب نشان داد. کارایی تراریختی برای سویه‌های مختلف باکتریایی (EHA105, LBA4404 and AGL-1)، غلظت‌های مختلف استوسیرینگون (۵۰، ۱۰۰، ۲۰۰ میکرومولار) و مدت زمان هم‌کشتی (۱، ۲، ۳ روز) ارزیابی شد. حضور و بیان ژن gusA در ارقام تراریخته با استفاده از روش‌های هیستوشیمیایی GUS، PCR و RT-PCR مورد تایید قرار گرفت. با توجه به نتایج به دست آمده، بالاترین زیست تودهء سلولی و بازدهی تراریختی ژنتیکی (۵۸٪) با سویه EHA105 (OD600 ۰/۳)، در محیط حاوی ۱۰۰ میکرومولار استوسیرینگون و هم‌کشتی به مدت ۲ روز برای رقم هاشمی مشاهده شد. یافته‌های این پژوهش می‌تواند در مطالعات تراریختی ژنتیکی ارقام برنج ایرانی مورد استفاده قرار گیرد.