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EVALUATION OF PARAMETERS AFFECTING THE GENETIC ENGINEERING OF RECALCITRANT SWITCHGRASS (*PANICUM VIRGATUM* ROXB. EX STEUD.) USING GUS REPORTER GENE SYSTEM

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Abstract

Switchgrass *(Panicum virgatum*) is used as a fodder for livestock due to its high nutritional value; it has potential to grow in cool and warm conditions as well as in low fertile soil. Switchgrass breeding is difficult due to its high degree of self-incompatibility; hence, grass production can be increased through cell and tissue culture. The present research focused on developing protocols for the regeneration and gene transformation of the forage grass to enhance the callus and regeneration efficiency through *Agrobacterium*-mediated transformation. The switchgrass callus was transformed with the pCAMBIA1302 vector carrying beta-glucuronidase (GUS) reporter gene, and 42% transformation efficiency was attained. To ensure a successful transformation, explants were infected with *Agrobacterium* for five minutes, following co-cultivation for two days. To enhance the process, 100 µM acetosyringone was added in co-cultivation media. Pre-selection of transformed callus was carried out for seven days with 150 mg/L of cefixime. The switchgrass transgenic callus was shifted to a selection medium containing hygromycin (50 mg/L). Histochemical staining was used to evaluate the presence of the GUS gene in the hygromycin-resistant transgenic plants. The successful transformation of switchgrass opens opportunities for gene function analysis and germplasm improvement through the application of advanced biotechnological tools.

Key words: Switchgrass, Gene transformation, Forage grass, *Agrobacterium tumefaciens*.

Introduction

Switchgrass (*Panicum virgatum*) is a type of perennial grass that shows a potential as a lignocellulosic feedstock for bioenergy (Mann *et al*., 2011). It thrives in cool and warm climates and can grow in low-fertility soils. With its deep root system, switchgrass offers soil conservation benefits and can be easily integrated into conventional farming practices (Burris *et al*., 2009). Switchgrass possesses numerous agricultural benefits, including climate and disease tolerance, cost-effective harvesting, and high biomass yield (Wang *et al*., 2021). It serves as both livestock fodder and biomass production for renewable fuel. As a perennial grass, it also holds superior agronomic traits compared to other species of grasses, such as disease resistance, efficient usage of water, and low fertilizer requirements (Lin *et al*., 2017). In the United States, the average annual biomass yield of switchgrass is approximately 12.9 ± 5.9 Mg ha^{-1} for lowland varieties and 8.74.2 Mg ha-^{1} for upland varieties. Additionally, switchgrass is considered as a versatile crop, fulfilling the dual purposes of providing both bioenergy and forage applications (McLaughlin & Kszos, 2005).

Overcoming the challenges associated with conventional breeding, such as self-incompatibility, and the utilization of cell and tissue culture techniques enable increased productivity and genome analysis in switchgrass (Lin *et al*., 2017). Its genome characteristics including size, allopolyploidy, and extended biological cycle make switchgrass a potential candidate for advanced genetic engineering research. Tissue culture and transformation systems have significantly advanced switchgrass biotechnology, with recent improvements in transformation efficiency and transgenic modifications (Nageswara-Rao *et al*., 2013; Ribeiro *et al*., 2019). *Agrobacterium* gene transformation is an extensively utilized technique for transformation of genes due to its cost-effectiveness, high transformation efficacy, increased probability of single gene copy insertion, ease of use, high production of transgenic plants, and ability to transform large DNA fragments (Somleva *et al*., 2002). Establishing a dependable and efficient plant transformation system is crucial for successful genetic transformation, involving plant regeneration, gene delivery, and selection of regenerated tissues expressing the desired gene (Gelvin, 2003). Successful transformation of switchgrass could be achieved through particle bombardment and *Agrobacterium*-mediated approaches, providing alternatives for producing transgenic plants.

This study aimed to evaluate and optimize key parameters, including the regeneration protocol of switchgrass and *Agrobacterium*-mediated transformation, to establish a reliable protocol for generation genetically modified switchgrass to increase biofuel and forage production.

Material and Methods

Plant material: Switchgrass (*Panicum virgatum*) seeds were obtained from the National Agricultural Research Centre, Islamabad, and subsequently stored at 25ºC under standard atmospheric humidity conditions.

Seed sterilization: Switchgrass seeds were sterilized by immersing in 70% ethanol for 5 minutes. Subsequently, surface sterilized using Clorox bleach (40%) for 10 minutes with gentle agitation. Following this, the seeds

were rinsed with double-distilled water and subjected to second round of sterilization with 40% Clorox for an additional 10 minutes, followed by rinsing with sterilized distilled water and dried.

Callus induction: Switchgrass sterilized seeds were cultured in tissue culture media which includes 4.3 g/L of MS (Murashige and Skoog) augmented with sucrose (3%), 0.3 g of Casein hydrolysate, 100X of vitamin B12 solution, 2.8 g/L of Gelrite. For callus induction, MS media was fortified with different doses of 2,4-D growth hormones (0.5 mg/L, 1 mg/L and 1.5 mg/L) and without growth hormone as control (Chutipaijit *et al*., 2018). The media pH is maintained at 5.8 afterward subjected to autoclaving at temperature of 121ºC for 20 minutes. Culture was maintained at 28°C within growth chamber under a light-dark cycle of 16 hours of light and 8 hours of dark. Embryogenic callus was obtained after four weeks and media was refreshed every two weeks. Callus induction percentage was calculated by utilizing the given formula:

$$\text{Callus induction (\%)} = \frac{\text{No. of callus obtained through explant}}{\text{No. of explants inoculated}} \text{ x } 100$$

Regeneration of callus: After four weeks, the callus was transferred to regeneration media and to test for both friable and solid nature of calluses. The regeneration media were augmented with NAA (1 mg/L) and BAP (2 mg/L), vitamin B12 (100X), Casein hydrolysate (0.3 g), and 2.8 g/L Gelrite. The culture tubes were placed in 16 h photoperiod for four weeks in 25±2.2. The percentage of regenerated calluses and plants were calculated after eight weeks. The regeneration frequency was determined after twelve weeks of inoculation.

$$\text{Callus regeneration efficiency (\%)} = \frac{\text{No. of callus regenerated}}{\text{No. of callus inoculated}} \text{ x } 100$$

To induce root formation, the regenerated shoots approximately 1-2 cm in length, were excised from the base of shoots and shifted into the rooting media (RM. The regenerated grass was cultures on MS media with with indole 3-butyric acid (IBA, 1.5 mg/L). The culture tubes were placed at 25±2°C for a 16 h light period. Fully established plantlets were shifted into a test tube for acclimatizing subsequently regenerated plants were shifted into pots having sand for three to four days and kept at 25 ±2°C.

Vector construction and Agrobacterium transformation: Transformation of switchgrass was conducted utilizing *Agrobacterium tumefaciens* EHA101 strain containing GUSA reporter gene, hygromycin phosphotransferase as a selectable marker gene driven by cauliflower mosaic virus (CaMV) 35S promoter sequence possessing pCAMBIA1301 vector backbone (Fig. 1).

Preparation of Agrobacterium culture: The glycerol stock of the EHA101 strain containing GUS reporter gene was refreshed by streaking on LB media containing 50 mg/L of kanamycin in combination with 25 mg/L of rifampicin. The petri plates were placed in an incubator for two days at 28ºC. Subsequently, a single colony was picked and grown on LB liquid at 28°C with agitation at 200 rpm. The overnight Agrobacterium grown culture was then shifted to 50 ml LB media containing selection antibiotics and optical density of 0.6-0.8 was achieved.

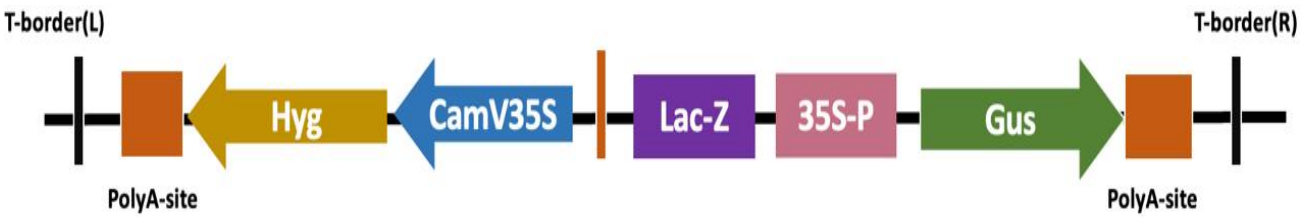

Fig. 1. Schematic diagram of pCAMBIA1302 vector T-DNA region carrying GUS reporter gene.

Co-cultivation of callus: The *Agrobacterium* culture with optical density (0.6-0.8) was centrifuged at 4000 rpm for 10 minutes, followed by resuspension in a liquid media. The callus samples were immersed in the Agrobacterium suspension followed by incubation for 4-10 min at 80 rpm at 30°C. Furthermore, the callus was gently dried on filter paper (90 mm) and transferred into co-cultivation media (MS solid, 1 mg/L of 2,4-D, sucrose, pH 5.8). To increase transformation efficiency various concentrations of Acetosyringone (25, 50, 75 and 100 µM) were incorporated into co-cultivation media. The co-cultivated explant was incubated for 2, 3, and 4 days at 26°C in dark. Transformation efficiency was assessed after a period of four weeks.

Selection of infected calluses: The co-cultivated and desiccation, calluses were subjected to washing with varying concentrations of cefixime (25, 50, 100, 150, 200, and 250 mg/L) in liquid MS media (pH 5.8) and callus were dried on sterilized filter paper. The transformed calluses were transferred to regeneration media for seven days prior to antibiotic selection. Antibiotic. Afterward, callus samples were transferred into the selection medium (MS media augmented with 2 mg/L of BAP and 1 mg/L of NAA) and three different levels of hygromycin (25, 50, and 75 mg/L) were used for selection. These selected explants were maintained in darkness and incubated at 25°C for three to four weeks on the selection medium.

Regeneration of transformed callus: Subsequently, calluses showing hygromycin resistance were shifted to regeneration media and allowed to undergo shoot regeneration for 4-6 weeks. The regenerated shoots, approximately 1-1.5 cm in length, were then transferred to rooting media consisting of MS solid with 1.5 mg/L of IBA.

Histochemical GUS assay: The GUS gene encoded β-glucuronidase, which catalyzes the cleavage of the X-Gluc (5-bromo-4-chloro-3-indoly-β −D-glucuronide) substrate. Transformed cells were subjected to X-Gluc incubation, resulting in the formation of a visible blue precipitate. GUS activity in the hygromycin-resistant callus was assessed through histochemical staining. Following the staining, the callus was washed with ethanol (70%) three times, after that immersed in a solution of lactic acid, glycerol, and phosphate buffer (1:1:3) in a dark condition for four hours. GUS activity was visualized as indigo, blue-colored spots on the callus.

Statistical analysis

All the experiments were conducted in triplicates and data were analyzed using SPSS Statistics 20. The significance of the data was evaluated using a one-way ANOVA with least significance difference (LSD) test at a significance level of 0.05 for comparing the means of three or more samples analysis of variance.

Results

Effect of 2,4-D on callogenesis: Various media hormonal combination and concentrations were employed to induce and regenerate callus. The impact of 2,4-D on the callus induction was investigated, and results are depicted in (Table 1). The different doses of 2,4-D were (0.5 mg/L, 1 mg/L and 1.5 mg/L) tested and at 1 mg/L concentration, there was a significant increase (63%) in callogenesis efficiency compared to the control, which showed no callus induction. Whereas, at 1.5 mg/L concentration, explants showed a maximum callogenesis of 83%. The data reflect the affirmative response of 2,4-D on switchgrass callus growth.

Table 1. The impact of varying concentrations of 2, 4-D on callus induction in MS media.

Concentration (2,4-D)	Callus induction (%)
1 mg/L	$38.1 + 0.58^{c}$ (63%)
1.5 mg/L	50.06 ± 2.65^{c} (83%)
2.5 mg/L	13.14 ± 0.01^{b} (45%)
Control	0%

Regeneration of callus: The switchgrass callus was shifted to regeneration media and the callus was regenerated with green spots within a few weeks (Fig. 2). The regeneration potential of all switchgrass seeds was increased as they interact with different hormonal combinations, triggering modifications in plant physiology and morphology. The best regeneration efficiency, reaching 76%, was observed with a concentration of 2 mg/L BAP and 1 mg/L of NAA, while while control plants exhibited poor regeneration.

The least significant difference (LSD) for the media was calculated to be 4.3920, while the LSD for the explants was 2.5227. The statistical significance was indicated by $p<0.05$. Lowercase letters represent the mean values obtained from three replicates.

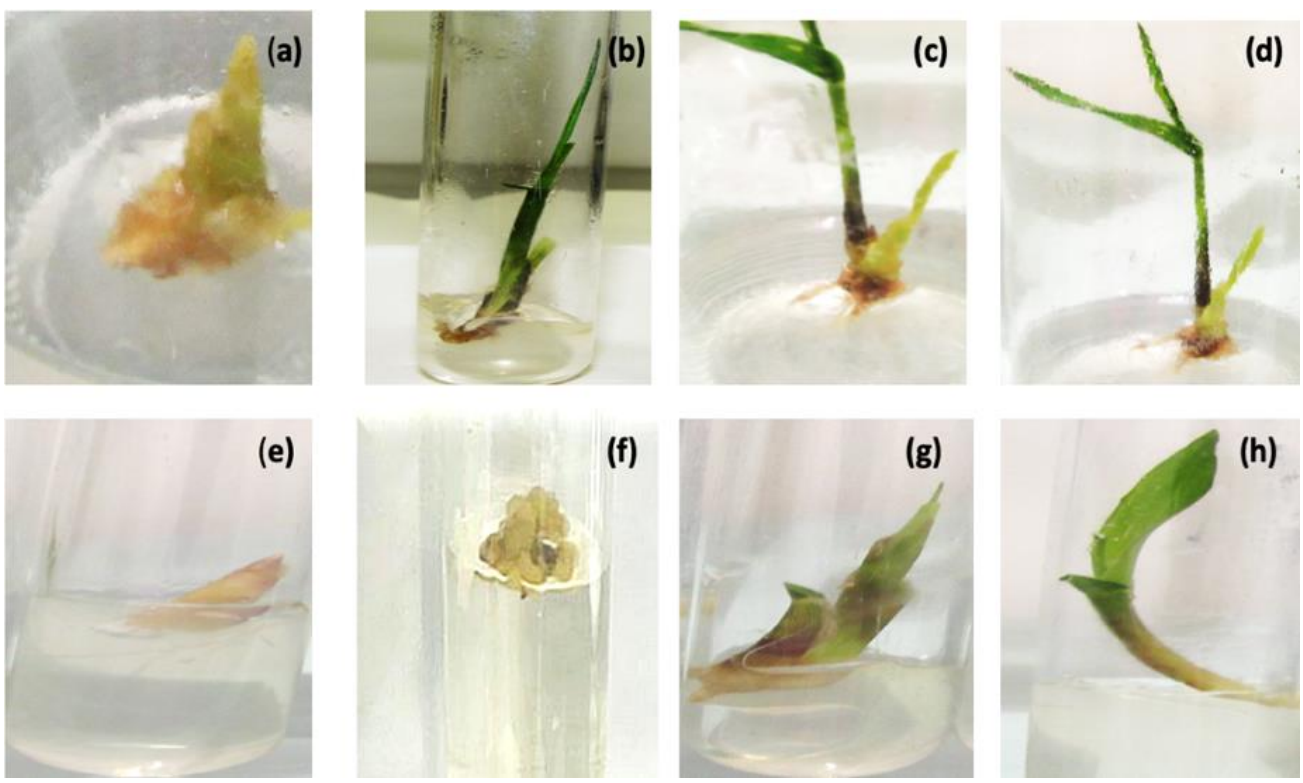

Fig. 2. Callus induction and regeneration frequency of switchgrass. (a) Callus induction was observed at 1.5 mg/L concentration of 2,4-D (b) regeneration of switchgrass at BAP (2 mg/L) and NAA (1 mg/L), (c) and (d) root development was observed at 1.5 mg/L concentration of IBA (e) and (f) callus formation was observed a concentration of 1 mg/L of of 2,4-D, (g) and (h) shows shoot regeneration of switchgrass.

Factors affecting Agrobacterium-mediated transformation: In the present study, several factors that impact efficient delivery of T-DNA, which includes co-cultivation time and period, impact of acetosyringone, and the utilization cefixime dose to conclude the co-cultivation stage, was thoroughly investigated.

Effect of co-cultivation time: Switchgrass callus was co-cultivated in the dark at 26ºC for 2, 3, and 4 days. It was observed that the transformation efficiency decreased with longer co-cultivation periods. Specifically, a two-day co-cultivation period increased transformation efficiency. However, extending the co-cultivation period from 3 to 4 days led to excessive proliferation of Agrobacterium. Consequently, the co-cultivation time was optimized, and it was noticed varying the time of co-cultivation also influenced the efficiency of transformation. Callus were co-cultivated for 7-8 minutes and experienced cell death upon transfer to the selection medium. In contrast, explants co-cultivated for 5 minutes exhibited the highest transformation efficiency. However, when the co-cultivation time was extended to 10 minutes, the transformation efficiency dropped to zero due to the excessive proliferation of Agrobacterium.

Effect of acetosyringone concentration: The acetosyringone has been incorporated into the co-cultivation media to enhance the transformation efficiency. After co-infection with Agrobacterium suspension for five minutes, the explants underwent a two-day co-cultivated on media having different concentrations (25, 50, 75, 100 µM). Results, summarizing the transformation efficiency achieved with different acetosyringone concentrations are shown in (Table 2). The 42% transformation efficiency was observed at 100 µM concentration. The discrepancies in transformation efficiency at different acetosyringone concentrations may be attributed to variations in genotypes and bacterial strain.

Determination of cefixime concentration: Following co-cultivation, the co-cultivation explants were shifted to a pre-selection medium. Cefixime was selected as a broad-spectrum antibiotic to eliminate the remaining Agrobacterium after co-cultivation step. Various concentrations of cefixime (25, 50, 100, 150, 200, and 250 mg/L) of cefixime were incorporated into the regeneration media. After a four-weeks period, it was noticed that the cefixime enhanced transformation efficiency (Table 3). Bacterial growth was solely observed in the control treatments where no antibiotics were applied, leading to decreased transformation efficiency and explant mortality. Notably, the addition of cefixime up to a concentration of 150 mg/L gradually enhanced the transformation efficiency of 39.6%. However, a further increase to 250 mg/L resulted in a rapid decline in transformation efficiency; with only 2% transformation efficacy was observed.

Effect of pre-selection: Explants were placed on Pre-selection media with cefixime (150 mg/L) for different durations of 3, 5, and 7 days. Subsequently, the explants were transferred to the selection medium and data was recorded after a six-weeks. The results showed a highly significant difference in transformation efficiency based on the pre-selection time, as shown in (Table. 4). Increasing the pre-selection time resulted in an increase in transformation efficiency. Notably, the maximum efficiency of transformation (42.5%) was achieved after a pre-selection period of seven days. This finding suggests that a pre-selection period of seven days is optimal for maximizing the efficiency of transformation, surpassing the results obtained with direct selection.

Table 2. The impact of various concentrations of acetosyringone on transformation efficiency.

Concentration (µM)	Transformation efficiency (%)
25	15.33 ± 0.33^{c}
50	28.00 ± 0.57^{b}
75	34.00 ± 0.57^{d}
100	42.1 ± 0.88^{a}

LSD values was determined to be 2.75. Values shared the same letters are not significantly different at $p<0.01$. Data represents the average of three replicates

Table 3. Effect of different concentrations of cefixime in transformation efficiency in the medium.

Cefixime concentrtaion (mg/L)	Transformation efficiency (%)
25	12.5 ± 0.45^{f}
50	24.5 ± 1.25^{e}
100	32.2 ± 0.07^{d}
150	39.6 ± 0.75^{c}
200	21.5 ± 0.32^{e}
250	2.0 ± 0.98^{g}

LSD = 2.16; Values shared identical letters are not statistically different at $p<0.01$. The presented data represents the mean of three replicate measurements

Table. 4. Effect of pre-selection time on the efficiency of transformation.

Pre-selection days	Efficiency of transformation
3	35.7 ± 0.577^{b}
5	37.3 ± 0.33^{b}
7	42.5 ± 0.33^{a}

LSD = 3.15; data presented average of three replicates and values followed by identical letter are significantly different ($p<0.01$)

Selection: Pre- selected explants were shifted into selection media and effect of hygromycin (50 mg/L) on shoot regeneration was assessed, and the results showed that 42.5% of switchgrass shoots were successfully regenerated on the selection medium containing hygromycin within six weeks. The percentage was lower compared to the untransformed control explants grown on a medium without hygromycin (80.5%). It was observed that in the absence of hygromycin, the explants remained green, and regeneration occurred in over 80% of the explants. However, the addition of hygromycin had an adverse effect on the growth of the explants, and an increase in hygromycin concentration halted their regeneration. When 25 mg/L of hygromycin was added, only 20% of the explants showed regeneration, while the remaining explants exhibited a pale appearance (Fig. 3). Increasing the hygromycin concentration to 75 mg/L resulted in no regeneration, and the explants turned pale yellow, indicating complete necrosis. Statistical analysis showed that the interaction between hygromycin concentration and regeneration efficiency was significant at p=0.01 (Table. 5). These findings suggest that variations in bacterial strain, plasmid construct, and cultivar used may contribute to the observed differences in transformation efficiency.

Table. 5. An average number of calluses regeneration at various hygromycin concentrations.

Hyg concentration (mg/L)	No. of regenerated calluses	Morphological characteristics
0	80.5 ± 0.45	Callus was green
25	20.0 ± 1.25	80% callus became yellow
50	42.5 ± 0.97	Callus became yellow in color
75	0.00 ± 0.00	All calluses became pale in color

LSD for concentration average number of explants regenerated interaction = 2.573 Values followed by the same letters are not significantly different at $p<0.01$

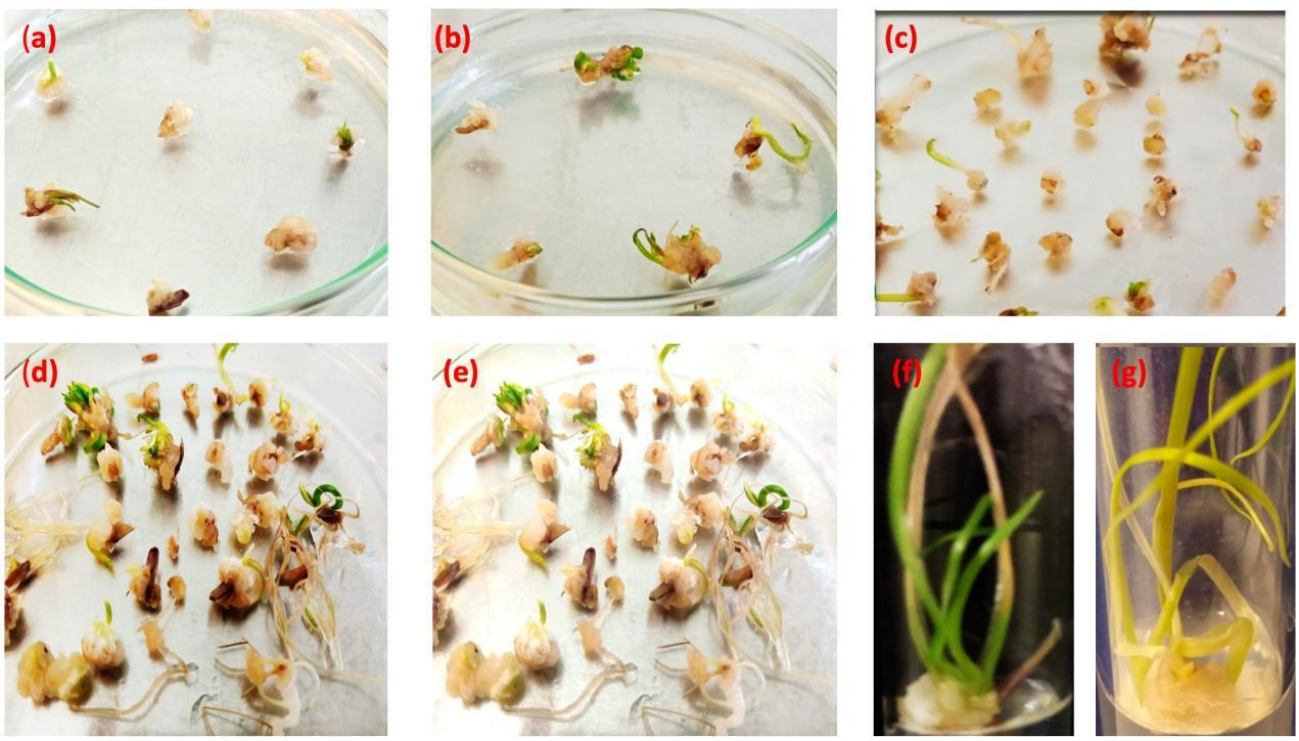

Fig. 3. *Agrobacterium* gene transformation of switchgrass embryogenic callus**.** (a) Co-cultivation, (b) Pre-selection, (c) Selection (20 mg/L of hygromycin), (d) and (e) Selection (50 mg/L of hygromycin), (f) and (g) Regeneration of callus on rooting media.

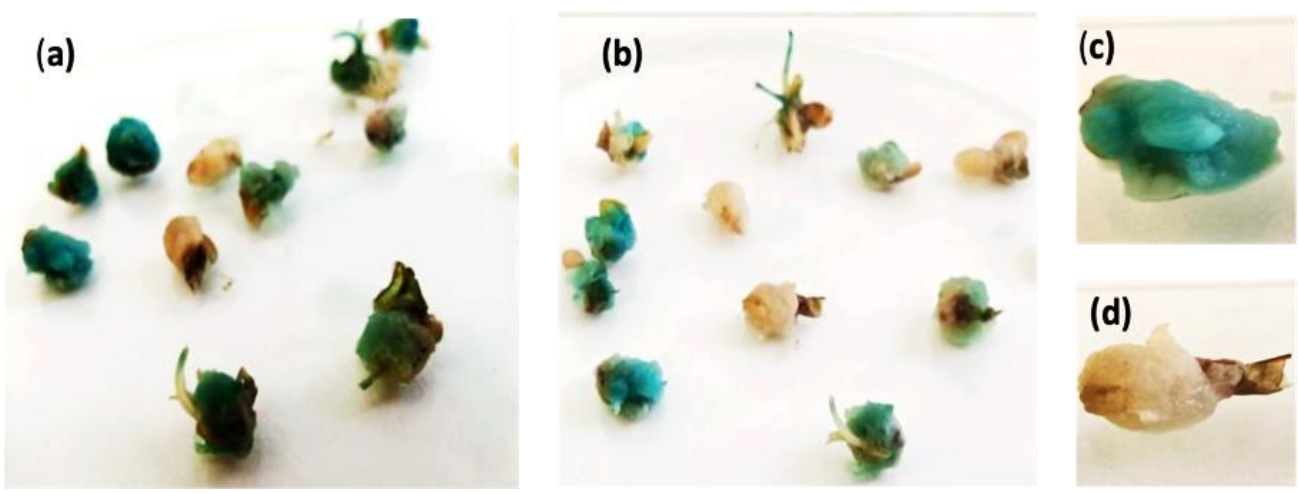

Fig. 4. GUS assay staining showing *Agrobacterium* transformed switchgrass callus. The non-transformed callus (negative control) showing cream white color, whereas the transformed callus indigo-blue color for GUS expression.

Analysis of histochemical GUS transformed callus: The genetic transformation of switchgrass callus was confirmed using a histochemical GUS assay and the callus that exhibited resistance to hygromycin underwent staining with X-Gluc. Positive transformants displayed a characteristic indigo-blue color, indicating successful gene expression, whereas non-transformed cells did not exhibit any blue coloration (Fig. 4). This observation suggests successful transformation and gene expression in a significant portion of the switchgrass callus.

Discussion

Switchgrass is highly recalcitrant towards genetic transformation due to its inadequate capability to regenerate under laboratory conditions (Song *et al*., 2012), creating an obstacle to apply modern genetic engineering techniques (Ogawa *et al*., 2016). However, we have optimized an effective protocol for gene transformation in switchgrass by optimizing callus regeneration and *Agrobacterium*-mediated transformation by expressing the GUS reporter gene. To increase callus induction, various concentrations of growth hormone 2,4-D were tested, and the addition of 2,4-D (1 mg/L) showed 63% increase in induction frequency of callus, while, of 1.5 mg/L resulted in a maximum callogenesis of 83%. Subsequently, the callus was shifted to a regeneration media, and the highest regeneration efficiency of 76% was attained with 2 mg/L BAP + 1 mg/L of NAA concentration. Previously, (Liu *et al*., 2015) successfully developed highly regenerative callus in switchgrass cultivars with 7.5% of transformation frequency. By optimizing the conditions for co-cultivation and pre- culture, the frequency of genetic transformation of switchgrass cultivars were enhanced (Merrick & Fei, 2015). Switchgrass exhibits a high level of heterozygosity and heterogeneity as a result of being derived from open-pollinated synthetic cultivars (Casler *et al*., 2011). Consequently, the co-cultivation time was optimized, and it was noticed that varying the time of co-cultivation also influenced efficiency of transformation. However, when the co-cultivation time was extended to 10 minutes, the transformation efficiency dropped to zero due to the excessive proliferation of *Agrobacterium*. These findings align with previous reports on switchgrass transformation. Liu *et al*. (2015) showed that extending the co-cultivation period to 3 and four days led to excessive growth of *Agrobacterium*. Following the optimization of the co-cultivation time various concentrations were tested.

Acetosyringone has been reported to enhance transformation efficiency in different crops such as rice, wheat and cucumber (Manfroi *et al*., 2015; Xi *et al*., 2018; Du *et al*., 2022) Previous studies on switchgrass transformation have suggested that inclusion of acetosyringone in the co-cultivation media increase the efficiency (Song *et al*., 2012). The present findings are consistent with those of Lin *et al*. (2017), in which optimal GUS gene expression was reported with 50% regenerated transformants. Li & Qu (2011) also reported that 50 μM acetosyringone was effective in achieving optimal transformation efficiency in switchgrass. To inhibit *Agrobacterium* growth during the transformation, different concentrations of cefaxime were tested. A concentration of 150 mg/L effectively prevented *Agrobacterium* growth and resulted in maximum transformation efficiency. Higher concentrations of cefaxime (250 mg/L) showed antibacterial effects on the surface of the medium but did not effectively inhibit bacterial growth within the inner regions and recesses of cell clusters. It is important to find the right balance in antibiotic concentrations to ensure bacterial suppression without compromising callus proliferation. In previous rice genetic transformation, 250 mg/L of cefotaxime was reported to inhibit bacterial growth (Tran & Sanan-Mishra, 2015). Even though much lower concentrations of these antibiotics were effective against *Agrobacterium* (Naderi *et al*., 2016).

In terms of selection and regeneration, a pre- selection period of seven days was found to be optimal for achieving maximum transformation efficiency. Increasing the pre-selection period correlated with increased efficiency of transformation. Our results align with the studies conducted on switchgrass lowland cultivars, where improved transformation frequencies were achieved with pre- selected calli (Somleva *et al*., 2002; Song *et al*., 2012). However, the success of transformation can vary depending on the bacterial strain, plasmid construct, and cultivar used. In terms of hygromycin resistance, our results demonstrated that the absence of hygromycin allowed for more than 80% to remain green and initiate regeneration. However, the addition of hygromycin had a negative impact on the explant growth. Increasing the hygromycin concentration halted explant regeneration. Specifically, when 50 mg/L of hygromycin was added, only 42.5% of the explants exhibited regeneration, while the remaining explants became pale. Hygromycin has been previously used for selecting transformed explants containing the GUS gene and non-transformed explants in various crops like Arabidopsis (Ee *et al*., 2014), cotton (Meng *et al*., 2007) and rice (Htwe *et al*., 2014). It has also been shown that hygromycin-based selection systems exhibit a low frequency of selection escape and hygromycin was the optimal concentration for selecting transgenic shoots, as untransformed explants showed a brown coloration within one week without any morphogenic response (Greenwood & Glaus, 2022). Regenerated callus exhibited GUS expression on the regeneration medium, and hygromycin-resistant regenerated callus were detected. However, high concentrations of hygromycin resulted in cell necrosis and a significant reduction in GUS expression. The experimental study confirmed the survival of callus and transgenic calli within two to three weeks of culture. Notably, hygromycin concentrations higher than 50 mg/l induced necrosis in the calli, causing complete death within one week, particularly since the somatic embryogenic callus is sensitive to hygromycin antibiotics. This suggests that increasing the hygromycin concentration had a more pronounced effect on the behavior of embryogenic callus. In our study, GUS transgenes expression has been successfully excised transgenic plants. The results demonstrate that by optimizing the transformation protocol, the challenges associated with transformation recalcitrance can be overcome. Moreover, the extensive availability of omics resources for upland switchgrass (Wang *et al*., 2021) combined with the developed transformation protocol in this study will greatly accelerate genomics-related research efforts.

Conclusion

This study demonstrates the improved efficiency of *Agrobacterium* mediated GUS reporter gene transformation in switchgrass through the tissue culture method involving callus induction, and optimization of callus regeneration frequency using varying doses of 2,4-D. The average frequency of callus induction was 83%.

Agrobacterium-mediated gene transformation and GUS expression were also influenced by several different parameters such as infection time, co-cultivation period, acetosyringone concentration, cefixime, and hygromycin dose and showed 42.5% efficiency of transformation, and an average number of hygromycin resistant regenerated calli showed GUS expression by the histochemical GUS assay. This transformation system offers a higher efficiency and simple propagation, making it suitable for generating switchgrass transgenic lines harboring diverse genes, facilitating the study of its unique characteristics. The effective transformation of recalcitrant switchgrass will enable the genetic engineers to carry out genetic modifications to develop edible vaccine and other products for livestock.

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