IMAM KHOMEINI INTERNATIONAL UNIVERSITY
IJGPB
Iranian Journal of Genetics and Plant Breeding
Print ISSN : 2251-9610
Online ISSN : 2676-346X

Efficient induction of embryogenic callus and biolistic-mediated transient transformation in the CP57-614 sugarcane cultivar (*Saccharum officinarum* L.)

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ABSTRACT INFO

Research Paper

Received: 08 Jan 2024

Accepted: 14 Jul 2024

ABSTRACT

This study aimed to optimize embryogenic callus formation and transformation of the CP57-614 sugarcane variety using particle bombardment. Young leaf explants were cultured on modified Murashige and Skoog (MS) medium containing different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D; auxin) (1, 2, and 4 mg L^{-1}) and two Dicamba concentrations (4 and 6 mg L^{-1}). The highest callus induction rate was achieved with 2 mg L^{-1} of 2,4-D. Subsequently, particle bombardment was used to introduce genetic constructs containing the uidA reporter gene driven by three promoters (CaMV35S, ubiquitin, and actin1) into the embryogenic calli. We assessed the impact of various biological, physical, and DNA parameters, including bombardment pressure, target distance, particle size, DNA amount, and osmotic pretreatment, through histochemical GUS assay to quantify blue spots. Results showed that the most effective combination involved bombardment at 1100 psi, 6 cm target distance, 1 µm gold particles coated with 1 µg DNA, under a vacuum of 25 inHg. Additionally, the best osmotic pretreatment utilized a solution containing 0.2 M sorbitol and 0.2 M mannitol. Notably, the construct driven by the ubiquitin promoter resulted in the highest transient expression of the uidA gene, suggesting its superiority for gene expression in this system.

***Key words*:** Calli, Gene expression, Particle bombardment, Sugarcane.

How to cite this article:
Matroodi S., Motallebi M., Mousavi A., and Jourabchi E. (2024). Efficient induction of embryogenic callus and biolistic-mediated transient transformation in the CP57-614 sugarcane cultivar (*Saccharum officinarum* L.). *Iranian Journal of Genetics and Plant Breeding,* 13(1): 1-9.
DOI: 10.30479/ijgpb.2024.19760.1364

INTRODUCTION

Sugarcane, a monocotyledonous plant in the Poaceae family, is a vital crop cultivated in tropical and subtropical regions worldwide. It accounts for about 75% of global sugar production (Wang *et al*., 2010) and is extensively grown in South America, the USA, Asia, and Australia (Cheavegatti-Gianotto *et al*., 2011). Commercial sugarcane varieties are complex hybrids of *Saccharum officinarum* and *S. spontaneum*, contributing significantly to the world economy (Irvine, 1999). Given its agricultural significance, efforts are underway to improve sugarcane through biotechnology, focusing on genetic manipulation and commercialization.

An efficient tissue culture system offers a pathway for the mass production of disease-free planting materials, utilizing either direct or indirect somatic embryogenesis, which is recognized as a reliable method for plant regeneration. The microprojectile approach has become favored for sugarcane transformation due to its broad applicability and simplicity (Khatri *et al*., 2002; Santosa *et al*., 2004; Nagai *et al*., 2020; Mbambalala *et al*., 2021; Mohanan *et al*., 2021; Oz *et al*., 2021). Various agronomic genes have successfully been integrated into sugarcane through particle bombardment, resulting in stable transgene expression (Mbambalala *et al*., 2021; Mohanan *et al*., 2021; Oz *et al*., 2021).

Multiple factors influence the efficiency of biolistic gene transfer (Joyce and Sun, 2020), prompting researchers to modify standard protocols to enhance DNA delivery and reporter gene expression, thereby improving transformation efficiency. Literature highlights the need to optimize parameters involved in particle bombardment-based transformation (Mohanan *et al*., 2021). Among these, the promoter is crucial, serving as a key regulatory element in transformation studies (Liu *et al*., 2003). Gallo-Meagher and Irvine (1993) demonstrated that the maize ubiquitin1 promoter led to higher GUS activity in sugarcane leaf explants compared to rice actin1 and CaMV35S promoters.

This study aimed to optimize tissue culture conditions and examine the effects of biological and physical parameters on embryogenic callus induction and transformation via microprojectile bombardment of the CP57-614 sugarcane cultivar, a major variety in southern Iran. Additionally, the research assessed the transient expression of the uidA reporter gene in embryogenic calli, ultimately striving to establish an efficient and stable transformation system for the CP57-614 cultivar through parameter optimization.

In this study, donor plants of the CP57-614 sugarcane cultivar were provided by the Iranian Sugarcane Research and Training Institute. Leaf cylinder explants were decontaminated using various treatments. This included surface disinfection with 70% ethanol, followed by hot water treatment at 50 °C for 2 hours for the top stem segments. The explants were then immersed in different concentrations (0.2%, 0.5%, and 0.7%) of mercuric chloride ($HgCl_2$) for 10 minutes, followed by three rinses with sterile distilled water for 10 minutes each. After drying on sterile filter paper, the aseptic leaf cylinders were placed on Murashige and Skoog (MS) medium supplemented with Dicamba (4 mg L^{-1}; 6 mg L^{-1}), 2,4-Dichlorophenoxyacetic acid (1 mg L^{-1}; 2 mg L^{-1}; 4 mg L^{-1}), and 30 g L^{-1} sucrose. The medium's pH was adjusted to 5.8 with NaOH (1N) before autoclaving for 20 minutes at 121 °C, with all media containing 8 g L^{-1} agar. Each Petri dish held five explants (~0.5 cm), and the cultures were incubated in the dark at 28 °C. Callus induction percentage was assessed after two weeks, and the number of embryogenic calli was recorded after one month of culture.

MATERIALS AND METHODS

Plant and callus preparation

In this study, donor plants of the CP57-614 sugarcane cultivar were provided by the Iranian Sugarcane Research and Training Institute. Leaf cylinder explants were decontaminated using various treatments. This included surface disinfection with 70% ethanol, followed by hot water treatment at 50 °C for 2 hours for the top stem segments. The explants were then immersed in different concentrations (0.2%, 0.5%, and 0.7%) of mercuric chloride ($HgCl_2$) for 10 minutes, followed by three rinses with sterile distilled water for 10 minutes each. After drying on sterile filter paper, the aseptic leaf cylinders were placed on Murashige and Skoog (MS) medium supplemented with Dicamba (4 mg L^{-1}; 6 mg L^{-1}), 2,4-Dichlorophenoxyacetic acid (1 mg L^{-1}; 2 mg L^{-1}; 4 mg L^{-1}), and 30 g L^{-1} sucrose. The medium's pH was adjusted to 5.8 with NaOH (1N) before autoclaving for 20 minutes at 121 °C, with all media containing 8 g L^{-1} agar. Each Petri dish held five explants (~0.5 cm), and the cultures were incubated in the dark at 28 °C. Callus induction percentage was assessed after two weeks, and the number of embryogenic calli was recorded after one month of culture.

Plasmid constructs

The transient expression experiments utilized various

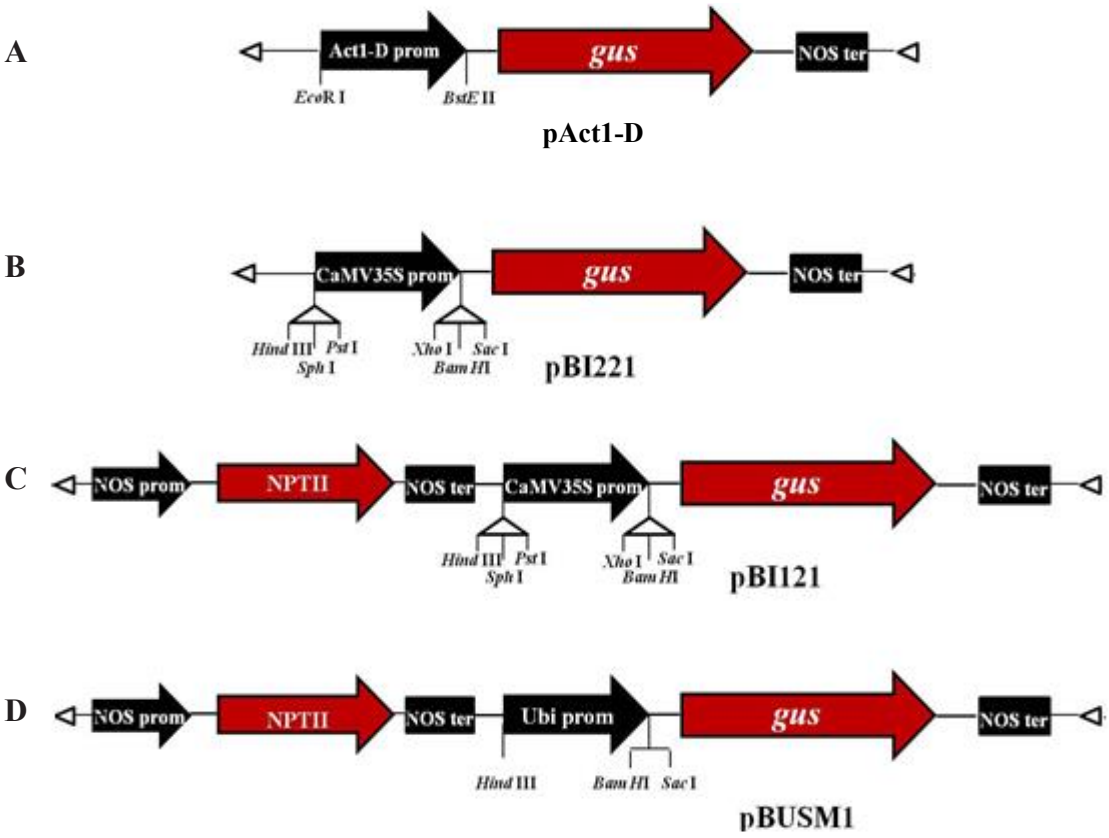

Figure 1. Schematic representation of four vectors used in optimizing the expression of *gus* gene in sugarcane plant; **A:** pAct1-D, **B:** pBI221, **C:** pBI121 and **D:** pBUSM1.

plasmid constructs, including pBI121 (Clontech, USA), pAct1-D (McElroy *et al*., 1990), pBI221 (Clontech, USA), and pBUSM1, each containing different genes under specific promoters (Figure 1). Antibiotic selection was not used in this experiment, as the focus was on transient gene expression rather than cell selection based on resistance.

DNA Preparation and particle bombardment

Preparation of DNA and particle bombardment (using PDS-1000/He, BioRad) followed Matroodi's method (Matroodi *et al*., 2013). GUS activity was assessed by counting blue spots post-treatment with a Zeiss Stemi 2000-C binocular microscope (Germany) and documented through photography. Statistical analysis was conducted using SAS PROC GLM with a minimum of three replicates per treatment. One-way ANOVA was used to analyze trait variances. Significant results (p<0.05) were further analyzed using Tukey's test at a 5% probability level.

RESULTS AND DISCUSSION

Sugarcane transformation success relies heavily on the tissue culture adaptability of plant materials. After decontaminating the leaf sheath explants and inducing embryogenic callus, we utilized these cells as the gene recipient tissue to establish a transformation system for sugarcane cultivar CP57-614. Significant differences (p<0.05) were consistently observed among treatments and variables, as evidenced by the number of blue spots observed (Figure 2).

Decontamination of explants and callus induction

It was found that using 0.7% $HgCl_2$ for 10 minutes resulted in the lowest rate of contaminations. Badawy *et al*. (2008) also used 0.03% mercuric chloride with Tween 80 for surface sterilization in their research.

After sterilization, leaf sheath explants were cultured on MS medium with different types and concentrations of growth regulators. The impact of Dicamba (4 mg L^{-1} and 6 mg L^{-1}) and 2,4-D (1 mg L^{-1}, 2 mg L^{-1}, and 4 mg L^{-1}) on embryogenic callus production using leaf-based explants was assessed (see Table 1). Results showed that 2 mg L^{-1} and 4 mg L^{-1} of 2,4-D significantly increased the number of explants producing embryogenic callus compared to other concentrations of 2,4-D (1 mg L^{-1}) and Dicamba-treated explants. Calli formed within two weeks in dark conditions at 28 °C, resulting in compact

Table 1. The effect of different concentrations of 2,4-D and dicamba in MS medium on callus and embryogenic callus induction.

Plant hormone concentration (mg l^{-1})		Callus induction (%)	Embryogenic Callus induction (%)
2,4-D	Dicamba		
1	_	40	26.67^{ab}
2	_	76	46.27^{a}
4	_	65	40.00^{a}
_	4	19	6.67^{b}
_	6	31	20.00^{ab}

Means within a column that were not significantly different (P>0.05) were assigned the same letter.

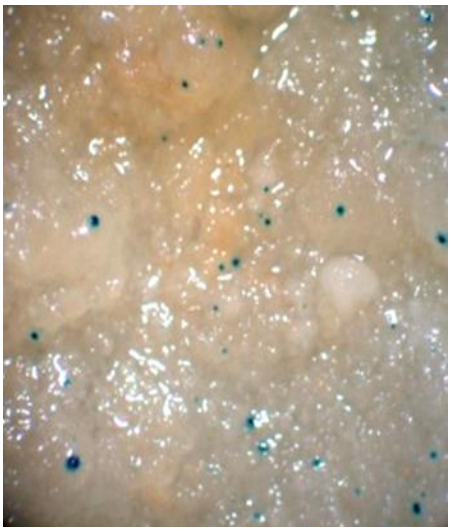

Figure 2. Transient GUS expression in embryogenic sugarcane calli after bombardment with a construct containing the ubiquitin1 promoter.

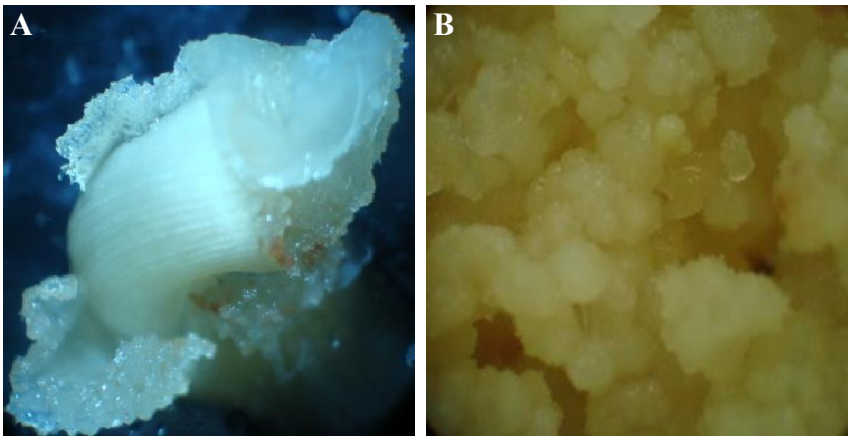

Figure 3. Embryogenic calli induced from sugarcane cv. CP57-614 leaf explants. **A:** Callus induction in sugarcane, **B:** Embryogenic callus development following four weeks of culture on MS medium.

yellow embryogenic calli and yellowish-white soft non-embryogenic callus after one month (see Figure 3). Previous studies have shown varying optimal concentrations for callus induction in sugarcane explants, with different researchers recommending 2,4-D concentrations ranging from 3 mg L^{-1} to 4 mg L^{-1} (Kaur *et al*., 2008; Badawy *et al*., 2008; Mohanan *et al*., 2021; Aksa *et al*., 2023).

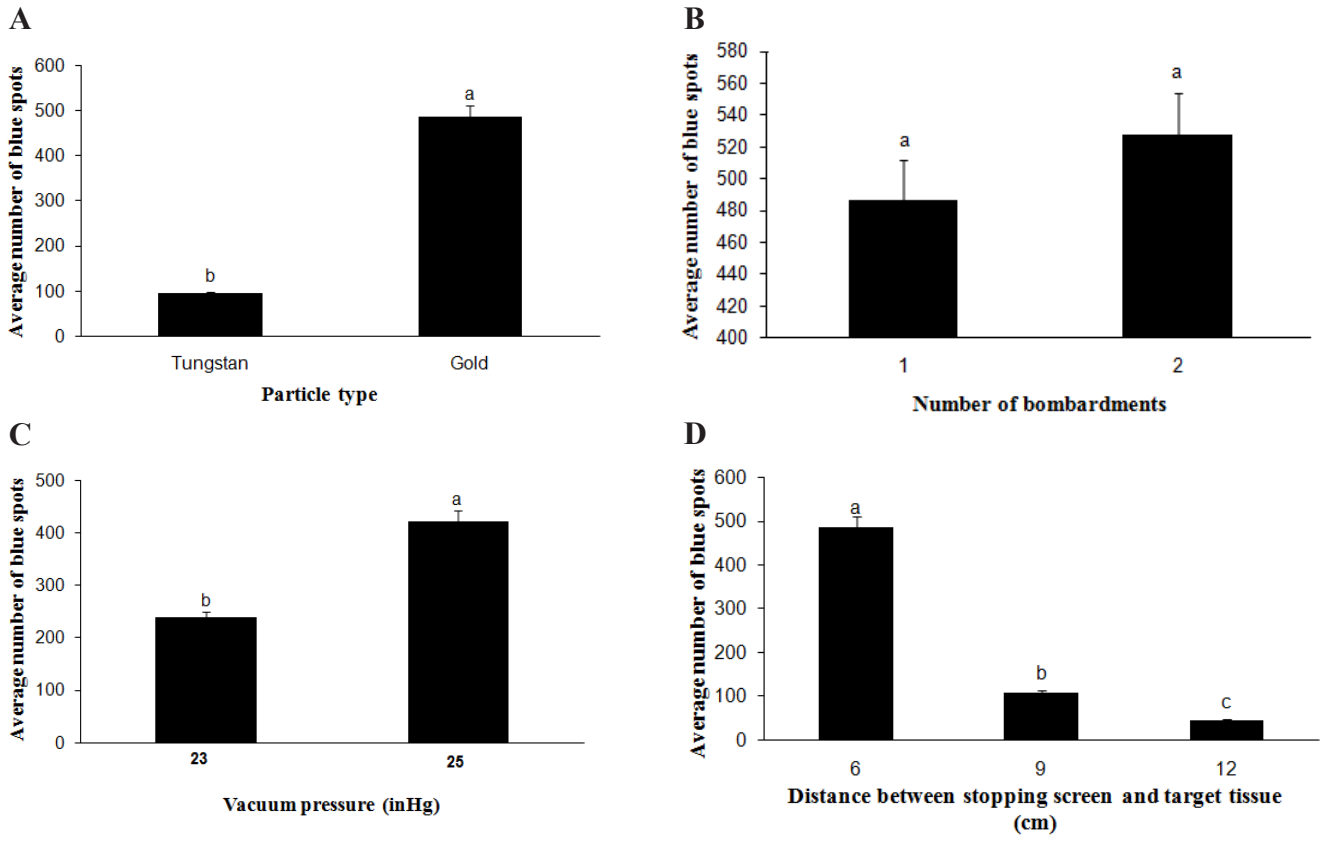

Figure 4. The influence of various physical parameters on the transient expression of the *gus* gene in bombarded sugarcane embryogenic calli. Parameters included: **A:** particle type, **B:** bombardment number, **C:** vacuum pressure and **D:** distance from stopping screen to target tissue. Data are presented as mean±standard deviation from at least three replicates and analyzed using one-way ANOVA (PROC-GLM program of SAS). Different letters denote significant differences at $P<0.05$ for each parameter.

Other studies have also found that the highest percentage of callus induction occurred when using MS medium supplemented with 2 mg L^{-1} 2,4-D (Behera and Sahoo, 2009; Maeda *et al*., 2023) and 4 mg L^{-1} 2,4-D (Kona *et al*., 2019; Joshi *et al*., 2021). These results reinforce the effectiveness of specific concentrations of 2,4-D in promoting callus formation.

Determination of particles type

In this study, 1.1 µm tungsten and 1 µm gold particles were compared. Gold particles resulted in significantly more blue foci per shot (478±14) than tungsten particles (94±15). This suggests that 1 µm gold particles were more effective in enhancing GUS expression in sugarcane callus (Figure 4A). Gold microcarriers have benefits like non-toxicity, biological inertness, and consistent size and shape. Conversely, tungsten particles could be harmful to some cell types by causing acidification and DNA degradation (Parveez *et al*., 1997).

The effect of the bombardment number

In order to improve targeted area coverage, multiple bombardments were used to enhance transformation efficiency. However, this approach may also cause increased damage to target tissues (Lonsdale *et al*., 1990). In this study, two consecutive bombardments were conducted, resulting in increased transient expression (527±47) but no significant difference compared to a single bombardment (487±15) (Figure 4B). Similar findings were reported by Parveez *et al*. (1997) for oil palm, Janna *et al*. (2006) for Dendrobium orchid, and Mousavi *et al*. (2009) for date palm. In contrast, other studies have shown that double bombardment can enhance transient expression efficiency of the *gus* gene in cassava (Schöpke *et al*., 1997), banana (Parveez *et al*., 1997), and Brazilian maize inbred lines (Petrillo *et al*., 2008).

Effect of vacuum pressure

The vacuum pressure used in the bombardment process is crucial for accelerating microcarriers and ensuring their successful delivery into the target tissue (Parveez *et al*., 1997). High vacuum pressure prevents microcarriers from decelerating, enabling

effective delivery to target tissues (Walter *et al*., 1998). In this study, a vacuum pressure of 25 inHg (inch of mercury) resulted in significantly higher efficiency of transient GUS expression (421±15) compared to other pressures, with 23 inHg resulting in 238±16 blue spots (Figure 4C). This finding is consistent with previous studies in Catharanthus roseus cells (Rasco-Gaunt *et al*., 1999) and wheat tissues (Guirimand *et al*., 2009).

Selection of appropriate distance from microcarrier launches assembly to the target tissue

The distance between the macrocarrier and the target tissue is a crucial factor affecting the velocity of microparticles and transformation rates (Petrillo *et al*., 2008). In this study, a distance of 6 cm between the macrocarrier and the calli resulted in a significantly higher number of blue spots (487±15) compared to distances of 9 cm and 12 cm, which showed 108±24 and 43±3 blue spots, respectively (Figure 4D). However, other studies on rice (Ramesh and Gupta, 2005), banana (Subramaniam *et al*., 2005), and date palm calli (Mousavi *et al*., 2009) reported 9 cm as the optimal distance between calli and macrocarrier.

Effect of promoter type

In this study, different promoters were compared for *gus* gene expression levels in sugarcane calli. The effectiveness of monocot-derived promoters, actin1 and ubiquitin, was evaluated with the CaMV35S promoter. Four plasmids were used to bombard sugarcane calli with the *gus* reporter gene. Results showed higher *gus* expression levels with rice ubiquitin and actin1 promoters compared to CaMV35S. Previous studies also support the efficiency of rice ubiquitin promoter. Additionally, genotype effects on transcriptional regulatory factors may influence promoter efficiency.

Ubiquitin 1 promoter from maize was found to have significantly higher GUS activity compared to the CaMV35S promoter in sugarcane calli (Weng *et al*., 2006). In sorghum, ubiquitin1 and actin1 were identified as the most active promoters (Tadesse *et al*., 2003). Similarly, the actin1 promoter led to increased GUS foci in date palm calli (Mousavi *et al*., 2009). The lower number of blue foci in CaMV35S promoter in sugarcane calli may be due to its lower activity in dicotyledons. These findings underscore the potential of the ubiquitin promoter for driving strong gene expression in sugarcane cells. This study's approach enabled the assessment of promoter activity, offering valuable insights into selecting the most effective promoter for gene expression in sugarcane organs. This information is crucial for developing efficient genetic engineering strategies in sugarcane research and biotechnology.

Effect of DNA concentration per bombardment

Various concentrations of pBUSM1 plasmid DNA (0.5, 1, 2.5, 5, 12.5, and 25 µg per shot) were tested to assess the impact of DNA concentration on microcarrier coating and transient GUS expression. The results showed that 1 µg per shot produced the highest expression, with 472±15 blue spots. In contrast, 0.5 µg resulted in significantly fewer blue foci (59±18), indicating that low DNA levels reduce delivery efficiency. The average blue foci for other concentrations were 331±48 for 2.5 µg, 321±48 for 5 µg, 148±8 for 12.5 µg, and 78±8 for 25 µg. This suggests that high DNA concentrations may cause particle aggregation, decreasing cell penetration and potentially causing cellular damage (Rasco-Gaunt *et al*., 1999). Similar optimal DNA concentrations have been observed in other species; for instance, 1.5 µg per shot maximized transient *gus*A and gfp expression in banana (Subramaniam *et al*., 2005). In date palm calli, varying concentrations from 2.5 to 25 µg did not significantly affect gene expression (Mousavi *et al*., 2009). Additionally, 1.5 µg per shot resulted in the highest expression in Arundo donax L. embryogenic calli (Dhir *et al*., 2010), while 0.83 µg was optimal in tomato fruit (Sun *et al*., 2011). These results underscore the necessity of optimizing DNA concentrations to enhance transient gene expression, as both excessive and insufficient amounts can hinder delivery and expression efficiency across plant systems.

Effect of osmoticum type

This study applied osmotic treatments to embryogenic calli of sugarcane to identify the optimal conditions for the transient expression of GUS. The calli underwent osmotic treatments at two time points: four hours before and one day after bombardment. They were cultured on MS medium supplemented with 2 mg L^{-1} 2,4-D and three osmoticum variants: 0.4 M sorbitol, 0.4 M mannitol, and a mix of 0.2 M sorbitol and 0.2 M mannitol. After one day on these media, histochemical GUS staining was conducted to evaluate transient expression. Results showed significant differences among the osmoticum media, with the combination of mannitol and sorbitol (487±15) being more effective than mannitol alone (106±9) or sorbitol alone (93±17) (Figure 5C). Maeda *et al*. (2023) demonstrated that a mixture of 0.25 M sorbitol and 0.25 M mannitol produced efficient osmoticum media. The observed increase in transient expression and stable transformation is likely due to enhanced cytoplasmic concentration, which may have minimized cell damage by preventing protoplasm extrusion from the bombarded cells. Osmotic treatments create an

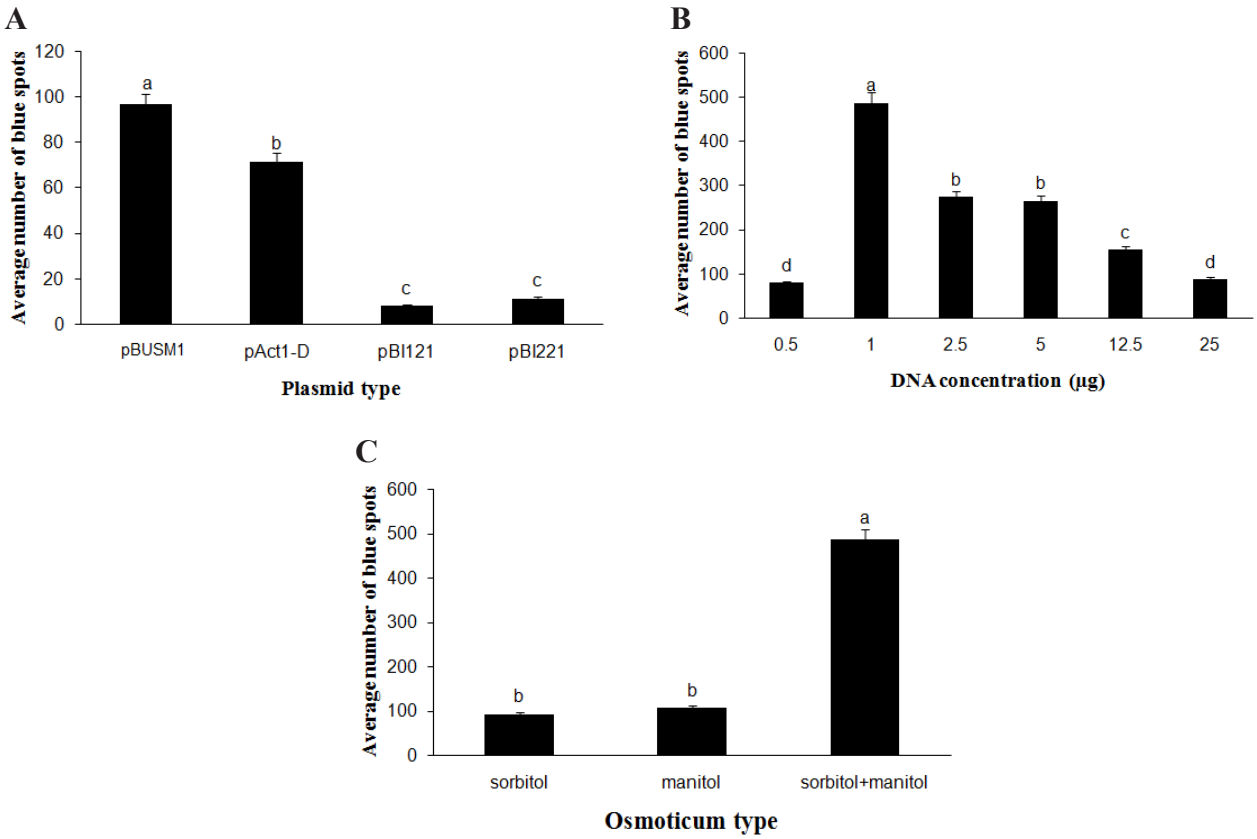

Figure 5. The impact of various biological parameters on the transient expression of the *gus* gene in bombarded sugarcane embryogenic calli. Parameters included: **A:** Plasmid type, **B:** DNA quantity per shot and **C:** osmoticum type target. Data are presented as mean±standard deviation from at least three replicates and analyzed using one-way ANOVA (PROC-GLM program of SAS). Different letters denote significant differences at $P<0.05$ for each parameter.

imbalance that causes water to exit the cells, leading to protoplast shrinkage. The transient expression studies suggest that equimolar ratios of sorbitol and mannitol have a synergistic effect in increasing the cytoplasmic concentration in sugarcane cells.

CONCLUSION

This study induced embryogenic callus in sugarcane using 2 mg L^{-1} 2,4-D. Optimal bombardment conditions were established with the *gus* reporter gene, including a 6 cm distance from target tissue to the stopping plate, single bombardment, a vacuum pressure of 25 inHg, 1 µm gold microcarrier, a combination of 0.2 M sorbitol and 0.2 M mannitol for osmotic treatment, 1 µg DNA concentration, and the use of plasmid pBUSM1. These parameters provide crucial guidance for the genetic transformation of elite sugarcane germplasm. The optimized factors for biolistic delivery may facilitate the transformation of sugarcane genotypes with commercially valuable traits, such as resistance to microbial pathogens and pests, as well as improved quality.

ACKNOWLEDGMENTS

The authors thank the National Institute of Genetic Engineering and Biotechnology (NIGEB) and M. R. Zamani for valuable discussions. We also acknowledge the Iran Sugarcane Research and Training Institute in Ahwaz for providing plant materials.

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