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Factors influencing the efficiency of *Agrobacterium*-mediated transformation in *Eucalyptus camaldulensis* × *Eucalyptus urophylla* hybrid variety CT4

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Abstract: Genetic transformation of *Eucalyptus* remains challenging due to the species' complex genomic structure and limited breeding methods. This study provides critical insights into transformation efficiency of a commercially important hybrid. We aimed to optimize *Agrobacterium*-mediated transformation protocols for the commercial *Eucalyptus* hybrid variety CT4 by systematically investigating factors influencing gene transfer efficiency. Using a callus-mediated transformation system, we employed GUS histochemical assays and PCR analysis to quantify transformation success under varying experimental conditions. Key parameters including bacterial cell density, infection duration, acetosyringone concentration, and antibiotic selection were comprehensively evaluated. Bacterial cell density significantly impacted callus survival, with lower densities (OD_{600} = 0.01-0.05) maintaining higher survival rates. Prolonged infection periods (60 minutes) enhanced β-glucuronidase (GUS) expression without compromising callus viability. Acetosyringone at 50 μM concentration optimized transformation efficiency, while a combination of cefotaxime (200 mg/L) and vancomycin (100 mg/L) effectively controlled *Agrobacterium* contamination. The final transformation efficiency reached 4.3%, representing one of the first successful genetic transformation protocol specifically optimized for the commercially important CT4 hybrid variety. Molecular confirmation through PCR validated successful gene transfer. This genotype-specific protocol addresses limitations in traditional breeding, offering a promising approach for genetic improvement of *Eucalyptus* hybrid varieties. The findings contribute to advancing biotechnological strategies for forest tree improvement, highlighting the importance of tailored transformation protocols for specific plant genotypes.

Key words: *Agrobacterium*; CT4; Genetic optimization; Hybrid Eucalyptus; Transformation.
Abbreviation: BAP_6-benzylaminopurine; BSM_bacterial suspension medium; CCM_co-cultivation medium; CIM_callus induction medium; CSM_callus selection medium; CT4_ Eucalyptus hybrid variety CT4; GA3_gibberellic acid; GUS_ β-glucuronidase; HPT_ hygromycin phosphotransferase; SD_standard deviation; LB_Luria-Bertani; MS_ Murashige and Skoog; NAA_1-Naphthaleneacetic acid; SRM_shoot regeneration medium.

Introduction

Eucalyptus, a genus of significant economic and ecological importance, has become a crucial resource in global forestry due to its rapid growth, high yield, and adaptability. Despite these valuable attributes, genetic improvement of *Eucalyptus* through biotechnology remains challenging, primarily due to its long breeding cycle, high genetic heterozygosity, and complex genome (Zhao et al., 2022).

Among the commercially significant Eucalyptus hybrids, the CT4 variety (*Eucalyptus camaldulensis* × *E. urophylla*) has gained prominence in Vietnamese forestry due to its superior growth characteristics, adaptability to diverse environmental conditions, and high-quality wood properties suitable for pulp and timber production. Despite its economic importance, genetic improvement of CT4 through conventional breeding faces significant limitations, highlighting the need for biotechnological approaches tailored to this specific hybrid.

Genetic transformation offers a promising approach to overcome these breeding limitations, allowing for the introduction of specific traits across genera and even kingdoms (Girijashankar, 2011). However, forest species like *Eucalyptus* present unique challenges for gene transfer technology, with research on transgenic varieties remaining limited due to high costs, patent issues, and concerns about transgene release risks.

Early achievements in *Eucalyptus* transformation were reported by Moralejo et al. (1998) for *E. globulus* and Chen et al. (2001) for *E. camaldulensis* (Moralejo et al., 1998; Chen et al., 2001). Subsequently, Brasileiro et al. (2007) documented successful transformation in *E. urophylla × E. grandis* hybrids, while Fernando et al. (2016) achieved genetic modification in *E. polybractea* (Brasileiro et al., 2007; Fernando et al., 2016). More recently, Wang et al. (2022) advanced *Agrobacterium*-mediated transformation of *Eucalyptus urophylla × E. tereticornis* hybrids, and Wang et al. (2023) established protocols for the commercially important *E. urophylla × E. grandis* hybrid DH32-29 (Wang et al., 2022; Wang et al., 2023). These studies collectively demonstrate significant progress in *Eucalyptus* transformation, yet also highlight the genotype-specific nature of successful protocols, with Wang et al. (2024) emphasizing the need for customized approaches for different *Eucalyptus* varieties and hybrids (Wang et al., 2024).

Various applications of *Eucalyptus* genetic modification have been reported, including alterations to lignin content (Chen et al., 2001; John et al., 2022), pest resistance (Harcourt et al., 2000), disease resistance (Ouyang et al., 2016) and stress tolerance (Yamada-Watanabe et al., 2003; Dibax et al., 2010; Navarro et al., 2011; Matsunaga et al., 2012). However, optimizing transformation protocols for specific *Eucalyptus* varieties remains a significant challenge. The efficiency of *Agrobacterium*-mediated transformation, a widely used method for plant genetic engineering (Wang et al., 2024), can vary considerably depending on numerous factors, including bacterial strain, plant genotype, and transformation conditions (Luo et al., 2022).

This study aimed to identify and optimize key factors influencing the transformation efficiency of the commercial *Eucalyptus* hybrid variety CT4. Specifically, we investigated the effects of antibiotics, bacterial infection processes, and acetosyringone concentration on transformation success. By systematically evaluating these parameters, we sought to develop an efficient, reproducible protocol for genetic modification of CT4, potentially accelerating the development of improved *Eucalyptus* varieties for commercial forestry applications.

Our research addresses a critical gap in the current understanding of *Eucalyptus* transformation by focusing on genotype-specific optimization of *Agrobacterium*-mediated gene transfer. The results of this study contribute to the broader field of forest biotechnology, offering insights into the complex interplay of factors affecting transformation efficiency in woody plant species.

Results and Discussions

Effect of bacterial cell density and bacterial infection

Bacterial cell density significantly affected callus survival rate but had minimal impact on GUS expression (Fig 1A). At lower densities (OD_{600} = 0.01 and 0.05), callus survival rates were highest (70-75%). Increasing bacterial density (OD_{600} = 0.25 and 1.00) resulted in a significant decrease in survival rates to approximately 30% and 15%, respectively ($p < 0.05$). This inverse relationship between bacterial density and callus survival rate may be attributed to cellular stress induced by high *Agrobacterium* concentrations, potentially triggering defense responses or overwhelming cellular machinery. Ziemienowicz (2014) reported that excessive bacterial densities can be toxic to callus tissues and reduce infection efficiency, which aligns with our observations (Ziemienowicz, 2014).

Interestingly, GUS expression remained relatively consistent (10-15%) across all bacterial densities, with no statistically significant differences observed (Fig 1A). This stability suggests that even at lower concentrations, sufficient *Agrobacterium* cells are present to facilitate gene transfer. Our findings contrast with studies by Wang et al. (2023) and Nguyen et al. (2023), who used bacterial densities with an OD_{600} of 0.3 for infection. This difference highlights the importance of optimizing transformation protocols for specific *Eucalyptus* genotypes (Wang et al., 2023, Nguyen et al., 2023).

The infection period demonstrated a distinct pattern of effects (Fig 1B). Callus survival rates remained stable (70-75%) across all infection times (5, 15, 30, and 60 minutes), with no statistically significant differences. However, GUS expression increased with prolonged infection periods. At 5 and 15 minutes, GUS expression was low (approximately 5%). It increased to about 10% at 30 minutes and reached its maximum (approximately 20%) at 60 minutes ($p < 0.05$). This positive correlation between infection time and GUS expression, without significant impact on callus survival, indicates that longer co-cultivation times allow for more efficient T-DNA transfer and integration. Gelvin (2010) suggested that this could be due to increased opportunity for bacterial attachment and initiation of the type IV secretion system responsible for T-DNA transfer (Gelvin, 2010).

Optimal infection times appear to vary among different studies. Nguyen et al. (2023) achieved best results after a 20-minute infection, while Wang et al. (2023) found 30 minutes to be optimal (Nguyen et al., 2023; Wang et al., 2023). Our investigation of *Eucalyptus* hybrid variety CT4 showed that infection times less than 30 minutes did not differ significantly in GUS expression, with 60 minutes yielding the highest expression rate. These discrepancies may be due to specific genetic characteristics of each *Eucalyptus* variety or the *A. tumefaciens* strain used, further emphasizing the need for genotype-specific optimization.

These findings highlight the complex interplay between bacterial density, infection period, and transformation efficiency in the *Eucalyptus* hybrid variety CT4. Our results suggest that using lower bacterial densities (OD_{600} = 0.01-0.05) and longer infection periods (60 minutes) may achieve high transformation efficiency while maintaining callus viability. This information is crucial for optimizing *Agrobacterium*-mediated transformation protocols for this *Eucalyptus* hybrid variety.

Table 1. Transformation efficiency stages in *Eucalyptus* hybrid variety CT4.

Stage	Efficiency (%)
Explant	100 ± 0
Callus induction	79.0 ± 4.2
Co-cultivation	58.0 ± 3.3
Selection	28.0 ± 2.9
Regeneration	13.7 ± 1.2
PCR analysis	4.3 ± 1.2

Data represent the mean of three independent experiments ± standard deviation. Efficiency (%) calculated as: (Total number of surviving or positive samples / Total number of initial samples) × 100%.

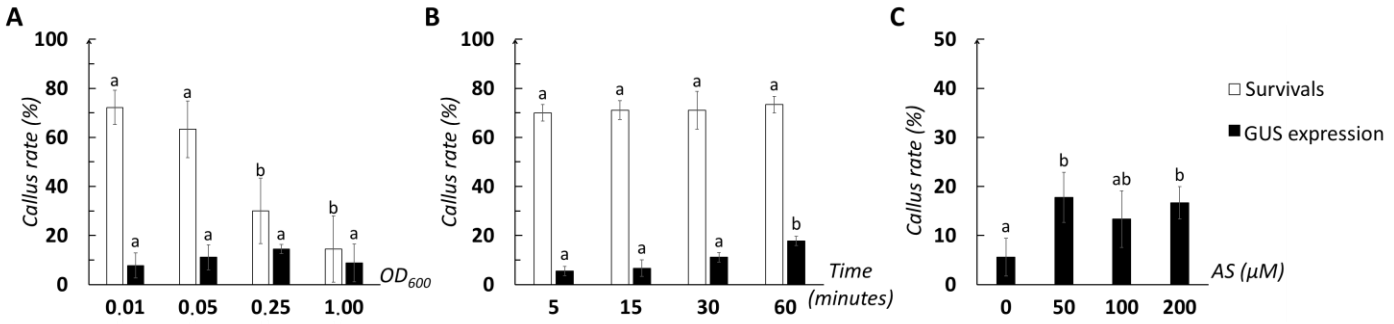

Fig 1. Effect of factors on callus survival and GUS expression in *Eucalyptus* hybrid variety CT4. (A) *A. tumefaciens* cell density (OD_{600}). (B) *A. tumefaciens* infection period. (C) Acetosyringone concentration. White bars represent callus survival rate, and black bars indicate GUS expression rate. Data are presented as means ± SD (n = 3). Different letters above the bars denote statistically significant differences ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's post-hoc test.

Effect of acetosyringone concentration

The influence of acetosyringone concentration on GUS expression in Eucalyptus callus was investigated to optimize *Agrobacterium*-mediated transformation efficiency (Fig 1C). The addition of acetosyringone significantly enhanced GUS expression, indicating improved transformation efficiency. The lowest tested concentration (50 μM) resulted in the highest GUS expression, with no further significant improvements at higher concentrations (100 μM and 200 μM).

The mechanism behind this effect likely relates to the role of acetosyringone in inducing *Agrobacterium* virulence genes. At low concentrations, acetosyringone effectively triggers the bacterial type IV secretion system, facilitating T-DNA transfer. The plateau effect at higher concentrations suggests a saturation point in gene transfer efficiency, potentially due to maximal induction of virulence gene expression, possible cellular stress at elevated concentrations, or limited binding sites for acetosyringone-induced proteins.

Comparative studies highlight the genotype-specific nature of acetosyringone optimization. da Silva et al. (2012) found 100 μM effective for *E. saligna*, while Guo et al. (2012) reported 150 mg/L as optimal for *E. grandis* clone Eg5 (da Silva et al., 2012; Guo et al., 2012). Our finding of 50 μM as the most effective concentration for the CT4 hybrid underscores the importance of tailored transformation protocols for specific *Eucalyptus* varieties.

These results contribute to our understanding the delicate balance required in *Agrobacterium*-mediated transformation, emphasizing that a universal approach is ineffective in genetic modification of woody plant species. The use of a lower acetosyringone concentration (50 μM) offers potential advantages in terms of reduced costs and simplified procedures without compromising transformation efficiency.

Impact of antibiotics on survival and regeneration of Eucalyptus hybrid variety CT4 callus

The effects of antibiotics on *Agrobacterium* contamination, callus survival, and shoot regeneration of *Eucalyptus* hybrid variety CT4 callus were investigated (Fig 2).

Cefotaxim and vancomycin significantly impacted *Agrobacterium* contamination and callus survival (Fig 2A). The control treatment without antibiotics (T1) resulted in *Agrobacterium* contamination reaching 100% with negligible callus survival. Cefotaxim alone (T2 and T3) markedly reduced contamination and improved callus survival. Combining cefotaxim and

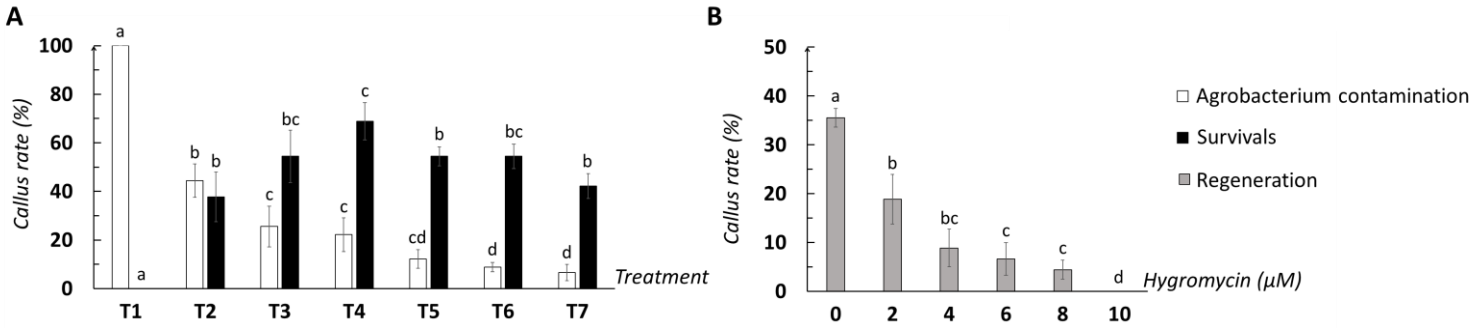

Fig 2. Effect of antibiotics on callus survival and shoot regeneration in *Eucalyptus* hybrid variety CT4. (A) Cefotaxim and vancomycin treatment of *Agrobacterium*-infected calli. (B) Hygromycin treatment of non-transformed calli. White bars represent *Agrobacterium*-contaminated callus rate, black bars indicate survival rate, and grey bar shows shoot regeneration rate. Data are presented as means ± SD (n = 3). Different letters above the bars denote statistically significant differences ($p < 0.05$) as determined by one-way ANOVA followed by Tukey's post-hoc test.

vancomycin (T4-T7) further decreased contamination, with T6 and T7 showing the lowest rates. Callus survival peaked in treatments T3-T6 (55-70%). Based on these results, we selected the T4 formulation (200 mg/L cefotaxim + 100 mg/L vancomycin) for the final transformation protocol, as it optimally balanced *Agrobacterium* control (reduced to ~25%) and callus viability (~70%).

The combination of cefotaxim and vancomycin proved effective in controlling *Agrobacterium* growth while maintaining high callus viability, consistent with previous studies using antibiotic concentrations ranging from 200 to 1000 mg/L (Pollock et al., 1983; Shackelford and Chlan, 1996). Our lower concentrations may reduce toxicity to plant cells, potentially improving transformation efficiency and plant regeneration (Nauerby et al., 1997; Mayolo et al., 2003). Notably, our optimal antibiotic combination differs from single antibiotic approaches reported for other *Eucalyptus* species and hybrids. Quisen et al. (2009) investigated various cefotaxime concentrations for *E. camaldulensis*, with some studies using up to 500 mg/L, while Ha et al. (2019) employed 400 mg/L cefotaxime for the eucalyptus hybrid variety UP (Quisen et al., 2009; Ha et al., 2019). Our use of a lower cefotaxime concentration combined with vancomycin demonstrates the potential benefits of antibiotic synergy in controlling *Agrobacterium* growth while minimizing phytotoxicity. This variation in antibiotic strategies underscores the importance of optimizing protocols for specific *Eucalyptus* genotypes, considering both single and combination antibiotic treatments to balance effective *Agrobacterium* control with minimal impact on plant tissue viability and regeneration capacity.

Hygromycin concentration had a dramatic effect on shoot regeneration efficiency (Fig 2B). The control treatment (0 mg/L hygromycin) showed the highest regeneration rate (~38%). A sharp decline occurred at 2 mg/L hygromycin (regeneration ~20%), with further increases leading to progressively lower rates. At 10 mg/L, shoot formation was completely inhibited. We chose 4 mg/L hygromycin for selection, which decreased regeneration to ~10%, providing sufficient selection pressure while allowing transformed cell regeneration.

These results indicate that *Eucalyptus* hybrid variety CT4 is highly sensitive to hygromycin, consistent with other *Eucalyptus* studies noting the genus's high hygromycin sensitivity (Fernando et al., 2016; Wang et al., 2023). Our optimal concentration (4 mg/L) aligns closely with Wang et al. (2023), who used 5 mg/L for selecting transgenic callus tissue (Wang et al., 2023). This sensitivity, attributed to hygromycin's mechanism of inhibiting protein synthesis by binding to ribosomal subunits, allows effective selection of transformed cells at low concentrations, potentially reducing stress on plant tissue and improving regeneration rates.

Our study highlights the delicate balance required in antibiotic use for *Agrobacterium*-mediated transformation of *Eucalyptus*. The optimized protocol, using cefotaxim and vancomycin for bacterial control and low-concentration hygromycin for selection, provides an effective approach for transforming the *Eucalyptus* hybrid variety CT4. This work contributes to the growing knowledge on species- and genotype-specific optimization of transformation protocols in *Eucalyptus* and may serve as a foundation for future studies on related species or hybrids.

Efficiency of Agrobacterium-mediated transformation in Eucalyptus hybrid variety CT4

The genetic transformation of *Eucalyptus* hybrid variety CT4 was evaluated through a comprehensive protocol that revealed significant challenges in gene transfer. The transformation efficiency (Table 1) progressively decreased from 79.0% during callus induction to a final 4.3% of original explants, highlighting the complex biological barriers in the transformation process. The transformation protocol exhibited stepwise efficiency decreases through critical stages: co-cultivation (reducing efficiency to 58.0%), selective antibiotic treatment (further declining to 28.0%), and shoot regeneration (ultimately yielding only 13.7% of the initial explant population). These stages demonstrate the stringent requirements for successful gene transfer in *Eucalyptus*, with each step presenting significant biological barriers that progressively reduce transformation potential. The final PCR analysis (Fig 3) confirmed gene integration, with the 0.26 kb *hygromycin phosphotransferase* (*HPT*) marker gene fragment detected in selected regenerated plants, providing molecular evidence of successful gene transfer.

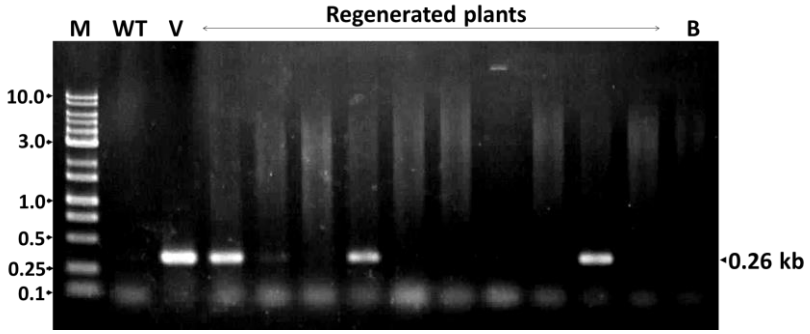

Fig 3. PCR analysis of CT4 regenerated plants. The presence of T-DNA was detected through amplification of *HPT* fragment (selectable marker gene, 260 bp). WT: negative control (DNA from wild-type CT4 plant); V: positive control (pCAMBIA1301 vector); B: blank control (no DNA template).

This efficiency aligns with previous *Eucalyptus* transformation studies. Brasileiro et al. (2007) reported transformation efficiencies ranging from 0.07% to 20% in *E. urophylla × E. grandis*, while Fernando et al. (2016) and Wang et al. (2022) documented transformation rates of 2-8% in various *Eucalyptus* hybrids (Brasileiro et al., 2007; Fernando et al., 2016; Wang et al., 2022).

Multiple factors contribute to these variations, including genotype-specific transformation potentials (Wang et al., 2024), *Agrobacterium* strain selection (Moralejo et al., 1998; Wang et al., 2022), and strain-specific interactions between bacteria and plant tissues (Wang et al. 2022). These complex interactions highlight the intricate nature of genetic transformation in *Eucalyptus* species, underscoring the need for tailored approaches that consider the unique characteristics of each hybrid and bacterial strain.

The developed protocol addresses critical limitations in traditional breeding methods for hybrid varieties. While the current transformation efficiency is modest, it represents a significant breakthrough in Eucalyptus biotechnology, offering promising opportunities for genetic improvement through tailored transformation approaches. Future research should focus on comprehensive strategies including exploring novel *Agrobacterium* strains, developing sophisticated co-cultivation techniques, investigating molecular mechanisms of *Agrobacterium* infection, and creating more sensitive screening methods for transformed cells. These targeted research directions aim to overcome current technological barriers, potentially revolutionizing genetic modification approaches for *Eucalyptus* and other woody plant species by enhancing our understanding of the complex interactions between bacterial vectors and plant cellular systems.

Materials and Methods

Plant materials

The *Eucalyptus* hybrid variety CT4 (*Eucalyptus camaldulensis × E. urophylla*) was obtained from the Vietnam High-Tech Agricultural Seeds and Materials Joint Stock Company. Callus was generated following the protocol described by Nguyen et al. (2024), with minor modifications (Nguyen et al., 2024). Stem segments (0.5 cm) from 21-day-old in vitro-grown plants were placed on callus induction medium (CIM) comprising Murashige and Skoog (MS) basal medium supplemented with 30 g/L sucrose, 100 mg/L myo-inositol, 1.0 mg/L 1-Naphthaleneacetic acid (NAA), 1.5 mg/L zeatin, and 10 g/L agar. Cultures were maintained at 28°C in darkness for 4 – 5 weeks, with explants being subcultured every 14 days.

Agrobacterium strain and plant transformation vector

The binary vector pCAMBIA1301 (Addgene, USA), containing the reporter gene *GUS*, was introduced into *Agrobacterium tumefaciens* strain EHA105 via freeze–thaw transformation. Recombinant colonies were selected on Luria-Bertani (LB) agar with rifampicin and kanamycin. Bacterial cultures were prepared by growing cells in overnight culture to OD_{600} of 1.0 followed by resuspension in bacterial suspension medium (BSM) containing MS basal medium supplemented with 30 g/L sucrose, 100 mg/L myo-inositol, 1.0 mg/L 6-benzylaminopurine (BAP), 0.1 mg/L NAA, and 100 µM acetosyringone.

Agrobacterium-mediated transformation

The general procedure for evaluating gene transformation efficiency was conducted systematically. *Eucalyptus* calli were infected with *A. tumefaciens* suspension and cultured on co-cultivation medium (CCM) (BSM supplemented with 10 g/L agar) for 72 hours in darkness at 25°C. Subsequently, the samples were transferred to callus selection medium (CSM) (CIM supplemented with 250 mg/L cefotaxime) and incubated at 28°C in complete darkness for 30 days. Following the selection period, calli were transferred to shoot regeneration medium (SRM) containing MS basal salts, 30 g/L sucrose, 100 mg/L myo-inositol, 1.5 mg/L BAP, 1.0 mg/L gibberellic acid (GA3), and 0.5 mg/L NAA. The cultures were maintained at 28°C under continuous light, with the medium being refreshed every 10 days.

To evaluate the effects of various factors on callus transformation efficiency, three independent experiments were conducted: (i) bacterial cell density, where calli were submerged in *A. tumefaciens* suspensions with optical densities (OD_{600}) of 0.01, 0.05,

0.25, and 1.0; (ii) infection duration, with calli being agitated in bacterial suspension for 5, 15, 30, and 60 minutes; and (iii) acetosyringone concentration, where 0, 50, 100, and 200 μM acetosyringone were added to both BSM and CCM. *Agrobacterium*-infected calli were cultured on CSM for 10 days prior to GUS assay. Transformation efficiency was calculated as: (Number of GUS-positive calli / Total number of infected calli) × 100.

To validate the optimized transformation protocol, three independent experimental batches were performed, each utilizing 100 explant samples of *Eucalyptus* hybrid variety CT4. Genomic DNA was extracted from leaves of wild-type and regenerated plants using the cetyltrimethylammonium bromide method. PCR analysis was performed using 50 ng of extracted DNA as a template, with primer pairs HPT-F/HPT-R (5'-AAACTGTGATGGACGACACCGT-3'/5′-GTGGCGATCCTGCAAGCTCC-3′) specifically designed to amplify the *HPT* marker gene.

Antibiotic sensitivity of callus

The sensitivity of *Eucalyptus* callus to antibiotics was evaluated through two distinct experiments. For cefotaxime and vancomycin sensitivity, calli were infected and co-cultivated with *Agrobacterium tumefaciens* following an optimized protocol, then transferred to CSM supplemented with various combinations of cefotaxime and vancomycin as follows: no antibiotics (T1), 200 mg/L cefotaxime (T2), 400 mg/L cefotaxime (T3), 200 mg/L cefotaxime + 100 mg/L vancomycin (T4), 200 mg/L cefotaxime + 200 mg/L vancomycin (T5), 400 mg/L cefotaxime + 100 mg/L vancomycin (T6), and 400 mg/L cefotaxime + 200 mg/L vancomycin (T7). For hygromycin sensitivity, non-infected calli were cultured on SRM supplemented with hygromycin at concentrations of 0, 2, 4, 6, 8, and 10 mg/L. Cultures were maintained at 28°C under continuous illumination.

In both experiments, samples were subcultured onto fresh media containing the respective antibiotic concentrations at 10-day intervals, for a total of three subcultures. For the cefotaxime and vancomycin experiment, callus survival rate was calculated as: (Number of surviving calli / Total number of infected calli) × 100%. In the hygromycin experiment, the number of calli exhibiting shoot regeneration was documented. Each experimental condition was evaluated using a minimum of 30 non-contaminated calli per treatment.

GUS histochemical assay

GUS activity was analyzed using a modified protocol based on previous studies. Callus tissues were prepared by carefully selecting 30 randomly chosen samples from each experimental condition after 10 days of selection. These samples were immersed in X-Gluc staining solution. The tissues were incubated at 37°C for 2-4 hours with gentle agitation. Following staining, the tissues were rinsed three times with 70% ethanol for 2 hours each to remove chlorophyll pigments. GUS-positive calli were identified by the presence of blue coloration, with the number of blue-stained calli recorded for quantitative analysis of transformation efficiency.

Statistical analysis

Each experiment was conducted in triplicate with a minimum of 50 samples per replicate. Data are presented as means ± standard deviation (SD). Statistical significance between treatments within each experiment was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test ($\alpha = 0.05$) in SPSS v.20.

Conclusion

In this study, we optimized multiple parameters to establish an efficient Agrobacterium-mediated transformation protocol for Eucalyptus callus tissue of the CT4 variety. Key factors, including bacterial cell density, infection duration, acetosyringone concentration, and the concentrations of cefotaxime, vancomycin, and hygromycin, were systematically evaluated and shown to significantly improve transformation efficiency in callus tissue. These optimized conditions collectively contributed to enhancing the frequency of successful gene transfer, providing a robust framework for future genetic transformation studies in Eucalyptus.

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