



Optimization of *Agrobacterium tumefaciens*-Mediated Transformation Conditions in *Coffea arabica* cv. Catimor CIFC 7963

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ABSTRACT

Agrobacterium-mediated plant genetic transformation is one of the established methods for plant breeding. In this study, conditions for *Agrobacterium*-mediated delivery of the *GUS* reporter gene into *Coffea arabica* cv. Catimor CIFC 7963 calli were optimized. The calli were co-cultivated with *Agrobacterium tumefaciens* and cultured in a liquid medium for 20 and 24 hours. The *Agrobacterium* suspensions consisted of three treatments: undiluted (control), 1:5 dilution, and 1:10 dilution. The calli were transferred to MS/2 medium containing 500 mg/L cefotaxime for 1 month. Subsequently, they were moved to MS/4 medium with 17.76 μ M BA, 50 mg/L hygromycin, and 250 mg/L cefotaxime for another month. Finally, they were subcultured on MS/4 medium with 17.76 μ M BA, 50 mg/L hygromycin, and 125 mg/L cefotaxime for 1 month. The results showed that the survival rate of calli co-cultivated with *Agrobacterium* cultured for 20 hours was better than that of the 24-hour condition. The friable callus was then selected to co-cultivate with *Agrobacterium* cultured for 20 hours. The callus survival rates for the undiluted, 1:5 dilution, and 1:10 dilution treatments were 0%, 62%, and 64%, respectively. Notably, somatic embryos developed from the friable callus after the 1:5 dilution treatment. Additionally, *GUS* expression varied between compact and friable calli. Therefore, co-cultivation of friable calli with a 1:5 dilution of *Agrobacterium* cultured for 20 hours was considered the optimal treatment, as it enhanced the frequency of putative transformation. This transformation system offers an advantage for improving the development of desired traits in coffee.

Keywords: *Agrobacterium tumefaciens*; *Coffea arabica*; Friable callus; *GUS* gene; Putative transformation

1. Introduction

Coffea arabica L., commonly known as Arabica coffee, belongs to the family Rubiaceae. The allo-tetraploid *C. arabica* is a self-fertile species with 44 chromosomes ($2n=4x$), derived from the diploid progenitor species *C. canephora* (Robusta) and *C. eugenoides* [1, 2]. *C. arabica* is of significant economic importance and is widely used for making beverages globally. Compared to Robusta, characterized by stronger bitterness and higher caffeine content, *C. arabica* is preferred for its higher beverage quality, aromatic characteristics, and lower caffeine content. Moreover, coffee is rich in polyphenols and other beneficial compounds, particularly in the beverage form [3]. More than 100 million people in coffee-growing areas worldwide depend directly or indirectly on the crop for income. Arabica coffee is grown in subtropical countries with warm temperatures and high humidity, making it a significant economic crop in northern Thailand, providing employment and income for farmers. Due to their perennial growth and limited genetic diversity, coffee plants are vulnerable to various diseases and pests [2]. Currently, *C. arabica* plants have limited genetic diversity, and global production relies on traditional cultivars or their descendants [4, 5].

However, Arabica coffee cultivation faces challenges such as leaf rust disease caused by the fungus *Hemileia vastatrix*. This disease can significantly damage coffee plants, affecting their growth, yield, quality, and lifespan. Applying chemical measures accounts for 30%-40% of field management costs, making coffee growing, particularly for smallholder farmers, unprofitable [6, 7]. Currently, *C. arabica* cv. Catimor CIFIC 7963 has been reported to have characteristics of leaf rust resistance in Thailand [8]. However, genetic resistance to the coffee white stem borer (*Xylotrechus quadripes*), coffee berry borer (*Hypothenemus hampei*), other diseases, drought and cold tolerance, and herbicide resistance are features not easily available in

the coffee gene pool or difficult to incorporate using traditional breeding [9].

In breeding programs worldwide, the main goal is to develop disease-resistant coffee cultivars [10]. This helps reduce the need for pesticides, which benefits the environment and human health [7]. However, the genetic improvement of coffee through traditional breeding is slow due to the perennial nature of the plant. Additionally, the long generation time of the coffee tree and the process involving several different techniques—namely selection, hybridization, and progeny evaluation over five generations—contribute to this slowness [11]. Genetic transformation has enormous potential for developing improved coffee varieties with desired agronomic traits that are difficult to achieve through traditional breeding. The main objectives of using genetic engineering techniques in coffee are to introduce new traits into elite coffee genotypes, improve cup quality, and to develop new cultivars with desirable traits such as pest and disease resistance, herbicide resistance, drought tolerance, and frost tolerance; accomplishing is not possible using traditional breeding techniques [9]. Overall, it takes at least 12 months to produce regenerated transgenic plants in soil conditions, though it may take as much as 18 to 27 months, as reported in other publications [12-15].

The *Agrobacterium*-mediated genetic transformation method uses *Agrobacterium tumefaciens*, a natural plant pathogen. It has several advantages over physical transformation methods, including the generation of only a single or a low copy number of transgenes with defined ends as well as its ability to preferentially integrate into transcriptionally active regions of the chromosomes [16]. An initial report of *Agrobacterium*-mediated transformation of coffee involved the co-cultivation of protoplasts with different *Agrobacterium* strains carrying *nptII* and *GUS* genes [17]. This method involves several factors, such as the type of explants (leaves, embryogenic calli,

somatic embryos, protoplasts), co-cultivation culture, and *Agrobacterium* concentration. Ribas et al. [15] suggested that the method of genetic transformation in *Arabica* coffee using *Agrobacterium* involved concentration during co-cultivation. The optical density (OD₆₀₀) of 0.6 with undiluted and diluted *Agrobacterium* conditions affected the genetic transformation efficiency. The results showed that using diluted conditions resulted in significantly higher genetic transformation efficiency compared to the undiluted treatment. This research provides valuable insights for optimizing genetic transformation protocols and improving the development of desired traits in *Arabica* coffee varieties.

However, genetic transformation efficiency in coffee is strongly influenced by genotype, often necessitating the development of cultivar-specific protocols. While transformation methods have been reported for several *C. arabica* cultivars, no optimized *Agrobacterium*-mediated transformation protocol has been established for *C. arabica* cv. Catimor CIFC 7963. In addition, coffee transformation remains a labor-intensive process due to the limited availability of suitable embryogenic tissues and generally low transformation efficiencies [18]. Genetic transformation has become an important tool for the development of disease-resistant cultivars and the improvement of agronomically important traits in coffee. In this study, *A. tumefaciens* strain EHA105 was used to introduce the *GUS* reporter gene into leaf-derived calli of *C. arabica* cv. Catimor CIFC 7963. The objective was to optimize *Agrobacterium*-mediated gene delivery and to evaluate putative transformation responses based on callus survival under selective conditions and transient *GUS* expression detected by histochemical staining.

2. Materials and Methods

2.1 Callus induction from the leaves of *Coffea arabica* cv. Catimor CIFC 7963

Leaves of *C. arabica* cv. Catimor CIFC 7963 were obtained from the Chiang Mai

Royal Agricultural Research Center, Chiang Mai, Thailand, and used as explants for callus induction and subsequent *Agrobacterium*-mediated transformation. *In vitro*-grown leaves of *C. arabica* cv. Catimor CIFC 7963 were cut into 1×1 cm segments and cultured on MS medium supplemented with 1 µM 2,4-D, 5 µM BA, and 30 mg/L cysteine. The cultures were incubated at 28°C in the dark for 1 week.

2.2 Freeze-thaw transformation of pCambia 1305.1 into *Agrobacterium tumefaciens* EHA 105

A. tumefaciens strain EHA105 was cultured on LB medium at 28°C for 2 days. A single colony was inoculated into LB broth and grown overnight. The bacterial culture was then centrifuged at 4,000 rpm for 10 minutes, and the cell pellet was resuspended in 20 mM CaCl₂. The plasmid pCambia1305.1, containing the hygromycin phosphotransferase (*hptII*) gene driven by the CaMV 35S promoter and 35S poly(A) terminator, and the β-glucuronidase (*GUS* plus) reporter gene under the control of the CaMV 35S promoter and NOS terminator, was introduced into *A. tumefaciens* EHA105 using the freeze-thaw method. The transformation mixture was subjected to alternating liquid nitrogen and ice treatments, followed by incubation at 37°C for 15 minutes and on ice for 5 minutes. LB medium was then added, and the culture was incubated at 28°C in the dark for 2 hours for recovery. The transformed cells were plated on LB medium supplemented with 50 mg/L kanamycin and incubated at 28°C in the dark for 2 days. Individual colonies were subsequently screened by polymerase chain reaction (PCR) to confirm the presence of the *GUS* plus gene.

2.3 *Agrobacterium*-mediated gene delivery into coffee calli

A. tumefaciens strain EHA105 harboring the binary vector pCambia1305.1 was used for transformation experiments. A single PCR-confirmed colony (AM2) was inoculated into 5 mL SOB medium

supplemented with 50 mg/L rifampicin and 50 mg/L kanamycin and incubated overnight at 28°C in the dark. The culture was subsequently expanded into 200 mL SOB medium containing the same antibiotics and incubated at 28°C in the dark for either 20 or 24 hours prior to transformation. Co-cultivation conditions were compared by assessing subsequent putative transformation frequency using three *Agrobacterium* suspension concentrations: undiluted (control), diluted 1:5, and diluted 1:10 with SOB medium. The putative transformation frequency was compared between compact and friable calli, with a total of 70 samples under the same culture conditions. From this comparison, we further selected the 50 friable calli that exhibited the best results for co-cultivation with the optimal *Agrobacterium* concentration, incubating for either 20 or 24 hours. Each treatment was conducted in triplicate.

The calli were immersed in *Agrobacterium* suspension for 10 minutes without shaking. The suspension was removed, and the inoculated calli were co-cultivated on solid MS/2 medium containing 200 µM acetosyringone (AS) at 20 °C in the dark for 5 days. After this period, the calli were rinsed twice with sterile water, then incubated in 20 mL of MS/2 medium containing 1.2 g/L cefotaxime in a shaker at 30 rpm for 30 hours. The liquid was removed, and the calli were blotted on sterilized filter paper to remove excess *Agrobacterium* solution, then rinsed with MS/2 medium for 15 minutes. They were subsequently transferred to Petri dishes containing MS/2 medium with 500 mg/L cefotaxime and placed in the dark for four weeks at 28 °C, after which *GUS* reporter gene expression was assessed.

2.4 Evaluation of putative transformation efficiency by the *GUS* assay

Ten calli of 1-month-old embryogenic callus were vacuum infiltrated with X-Gluc assay buffer (2 mM X-Gluc, 100 mM phosphate buffer [pH 8.0], 10 mM EDTA, 1 mM K₄Fe(CN)₆, 1 mM K₃Fe(CN)₆, and 20%

methanol) for 5 minutes. The calli were left to stain at 37 °C overnight, cleared in 70% ethanol, and observed using a stereomicroscope at 3.5× magnification.

2.5 Regeneration of the putative transgenic callus

After 1 month, the calli were subcultured on selective medium (solid MS/4 medium supplemented with 17.76 µM BA, 50 mg/L hygromycin, and 250 mg/L cefotaxime) for four weeks at 28 °C in the dark. The putative transformation frequency was calculated as the proportion (p) of putative transformed calli ($p = x/n$), where x is the number of putative transformed calli and n is the number of co-cultivated calli. The putative transformed calli were cultured on solid MS/2 medium supplemented with 17.76 µM BA under a 16-hour photoperiod (20–30 µmol m⁻² s⁻¹ light intensity) at 28 °C until embryo development.

2.6 Statistical analysis

Data were analyzed using SPSS software version 29 (IBM Corp., Armonk, NY, USA). Differences among treatments were evaluated by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) at $p < 0.05$. All treatments were conducted in triplicate, and the data are presented as mean ± standard deviation (SD).

3. Results and Discussion

3.1 PCR confirmation of transformed *Agrobacterium* harboring pCambia1305.1

Five independent *Agrobacterium* transformants were screened by PCR amplification of the *GUS* plus expression cassette. One positive clone, designated AM2, produced the expected 1.82 - kb amplicon generated by primers located in the *GUS* plus regions (Fig. 1A and 1B). The AM2 clone was subsequently used for transformation of coffee calli.

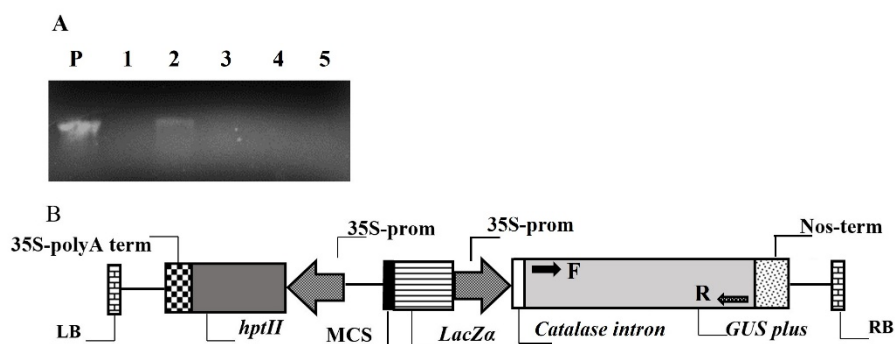


Fig. 1. Verification of the *GUS* plus construct in *Agrobacterium tumefaciens* and schematic representation of the pCambia1305.1 vector. (A) PCR amplification of the *GUS* plus gene from *A. tumefaciens* transformants. Lane P, positive control (pCambia1305.1); lanes 1–5, independent *A. tumefaciens* transformants carrying pCambia1305.1. The expected amplicon size (1.82 kb) was verified by comparison with the positive control plasmid amplified using the same primer pair. (B) Schematic map of the T-DNA region of pCambia1305.1. The *GUS* plus reporter gene containing the catalase intron is driven by the CaMV35S promoter and terminated by the NOS terminator. The hygromycin resistance gene (*hptII*) is controlled by the CaMV35S promoter and the 35S poly(A) terminator. Arrows labeled F and R indicate the locations of the forward and reverse primers used for amplification of the expected 1.82-kb PCR product.

3.2 Effect of *Agrobacterium* concentration and callus type on putative transformation frequency in coffee calli

The success of genetic transformation using *A. tumefaciens* is often limited in many plant species due to the activation of plant defense mechanisms when the pathogen attacks. To enhance the virulence of *Agrobacterium* and increase transformation efficiency, various manipulations involving *Agrobacterium* concentration, plants, and physical conditions have been applied [19]. The concentration of *Agrobacterium* used in genetic transformation is a crucial factor. Using the optimal concentration of the culture can significantly enhance the efficiency of genetic transformation. However, if the long duration of the culturing process leads to a culture concentration that is too high, it can result in plant tissue death due to *Agrobacterium* overgrowth. As a result, this can decrease the survival rate of the plant tissue and the induction of surviving cells into mature plants [20]. In the present study, prior to inoculation, *Agrobacterium* cultures were grown for either 20 or 24 hours. These culture durations were selected because preliminary

observations indicated that they produced bacterial suspensions with OD₆₀₀ values close to 1.0 (20 hours) and greater than 1.0 (24 hours), respectively. Since bacterial physiological status and cell density can influence virulence induction, T-DNA transfer, and transformation outcomes, both culture durations were evaluated. Through a 10-fold serial dilution, it was determined that an OD₆₀₀=1.0 corresponds to a colony count of 1×10⁸ CFU/mL (data not shown) [21]. Following this, three different concentrations of the culture were prepared: undiluted suspension, 1:5 dilution, and 1:10 dilution to adjust the OD₆₀₀ to <1.0. It was observed that culturing the bacteria for 20 hours led to a higher frequency of putative transformation compared to 24 hours, with frequencies of 60.96%, 80.96%, and 99.53% respectively (Table 1). When testing for the optimal duration for culturing the bacteria used for *Coffea canephora*, it differed from that of *A. tumefaciens*, where an OD₆₀₀=0.5–1.0 was used for genetic transformation into the hypocotyl [22]. The results indicated that the 1:10 dilution treatment after 20 and 24 hours of *Agrobacterium* cultivation resulted in a

significant difference in the number of putative transformed calli compared to the undiluted and 1:5 treatments (Table 1).

Additionally, friable calli showed better survival compared to compact calli (data not shown). These results suggest that *Agrobacterium* cultured for 20 hours is suitable for the friable calli of coffee transformation. As a result, this culturing duration was selected for genetic transformation into friable calli, which is more efficient at developing into somatic embryos than compact calli [23]. The type of explants affects the efficiency of genetic transformation. However, in *C. arabica*, embryogenic calli have been reported to be the most suitable target tissues for *A. tumefaciens*-mediated transformation [24]. Calli can be categorized into two types: friable and compact. The explants used for cultivation will develop into either friable or compact calli,

depending on the type of plant and the components of the culture medium. The cream-yellow friable callus is soft, looser, and easily separated, while the green and hard compact callus is tight [25]. Asakura et al. [26] found that the friable calli of *Actinidia kolomikta* developed into somatic embryos more effectively than compact calli. Ribas et al. [24] examined calli from Arabica coffee of different colors - gray, whitish, and yellow - for genetic transformation. They observed that the gray callus was more compact than the yellow callus, while the whitish callus exhibited friability, allowing for rapid multiplication. The genetic transformation efficiency suggests a high success rate (75%) using the yellow friable calli, demonstrating their high suitability for genetic transformation in coffee.

Table 1. The effect of concentration of *Agrobacterium* on compact/friable calli after 3 months of culture on a selective medium.

Culture durations	Treatments	No. of co-cultivated calli	No. of putative transformed calli	Putative transformation frequency(%)
20 hours	1	70	42.67±0.58 ^d	60.96
	1:5	70	56.67±0.58 ^b	80.96
	1:10	70	69.67±0.58 ^a	99.53
24 hours	1	70	35.67±0.58 ^e	50.96
	1:5	70	53.33±0.58 ^c	76.19
	1:10	70	68.67±0.58 ^a	98.10

Note: Values (mean ± SD) with different lowercase superscripts in column indicate significant ($p < 0.05$) differences.

3.3 Survival rate of friable calli co-cultivated with *Agrobacterium* cultured for 20 hours on a selective medium

It is crucial to use the optimal concentration of *Agrobacterium*. An excessive concentration culture makes it challenging to eliminate excess *Agrobacterium*, leading to overgrowth, which can turn plant tissue brown and cause necrosis. On the other hand, too low of a concentration reduces the efficiency of *Agrobacterium* attachment to plant cells and, consequently, the efficiency of genetic transformation [20, 27]. The friable callus was co-cultivated using *Agrobacterium* cultured for 20 hours at different concentrations

(undiluted, 1:5 dilution, and 1:10 dilution). Calli were cultured on MS/2 medium supplemented with 500 mg/L cefotaxime for one month, transferred to MS/4 medium supplemented with 17.76 µM BA, 50 mg/L hygromycin, and 250 mg/L cefotaxime for another month, and finally transferred to MS/4 medium supplemented with 17.76 µM BA, 50 mg/L hygromycin, and 125 mg/L cefotaxime for one more month. The number of putative transformed calli with significant differences showed survival rates of 0%, 60.66%, and 63.34%, respectively (Table 2). These results suggest that the undiluted *Agrobacterium* suspension cultured for 20 hours was not

suitable for use with friable calli, likely due to excessive bacterial growth and its detrimental effects on callus survival and putative transformation responses. Furthermore, the concentration from this dilution resulted in an OD₆₀₀ value of less than 1.0. Previous research indicated that genes were successfully transferred into *C. arabica* by culturing *Agrobacterium* strain LBA 1119 with an OD₆₀₀=0.6 [24]. However, the experiment with undiluted concentration resulted in rapid growth of *Agrobacterium*, making it difficult to eliminate the bacteria and causing *Agrobacterium* overgrowth. The overgrown bacteria caused the callus to turn brown, rot, and eventually die (Table 2).

Moreover, these manipulations include pre-culturing explants before inoculation [19], modifying temperature [19, 28], adjusting medium pH [28], and adding chemicals to the inoculation medium, such as acetosyringone [19,28, 29]. Other approaches involve changing bacterial density and co-cultivation periods [29] and employing vacuum infiltration [30, 31]. In another study on flax, results indicated that applying a magnetic field (MF) strength of 75 millitesla (mT) significantly enhanced both regeneration and transformation rates compared to control treatment. However, at magnetic field strengths exceeding 75 mT, shoot regeneration and transformation were notably hindered. This indicates that culturing explants under an MF strength of 75 mT can significantly enhance both the regeneration capacity and transformation frequency of flax (*Linum usitatissimum* L.) [32].

3.4 Histochemical GUS assay of putative transformed callus

In this study, compact and friable yellow calli were evaluated as target tissues for *Agrobacterium*-mediated gene delivery. The results showed that friable calli were more suitable than compact calli, as they exhibited higher frequencies of GUS-positive calli and improved survival under selective conditions. Histochemical GUS staining was observed in

calli cultured on MS/2 medium supplemented with 500 mg/L cefotaxime for 1 month. Blue-stained regions, indicated by arrows, were detected in calli from different treatment groups (Fig. 2). Evaluation of ten calli per treatment revealed that friable calli exhibited higher frequencies of transient GUS expression than compact calli. Histochemical GUS assays were used to identify putative transformed calli of *C. arabica* cv. Catimor CIFC 7963 following 30 days of selection culture after *Agrobacterium* inoculation for 10 minutes. According to Duan et al. [33], *Agrobacterium* transformation with a 25-minute infection period followed by a 3-day co-culture showed the highest GUS expression rate after 45 days on a selective medium. Therefore, the infection time can affect the transformation rate [34].

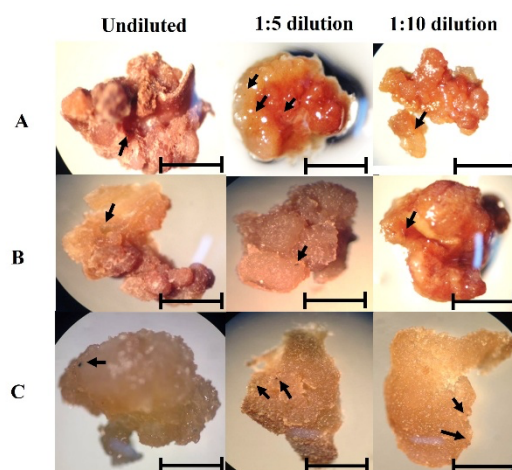


Fig. 2. Histochemical GUS assay of putative transformed coffee calli following *Agrobacterium*-mediated transformation. Columns represent *Agrobacterium* suspension concentrations: undiluted, 1:5 dilution, and 1:10 dilution. Arrows indicate GUS-positive regions observed in the calli. (A) putative transformed compact/friable calli with *Agrobacterium* cultured for 20 hours (B) putative transformed compact/friable calli with *Agrobacterium* cultured for 24 hours (C) putative transformed friable callus with *Agrobacterium* cultured for 20 hours. (scale bar = 2 mm).

Table 2. Concentration of *Agrobacterium* and its effect on friable calli after 3 months of culture on selective medium.

Culture duration	Treatments	No. of co-cultivated calli	No. of putative transformed calli	Putative transformation frequency(%)
20 hours	1	50	—* ^c	0
	1:5	50	30.33±0.58 ^b	60.66
	1:10	50	31.67±0.58 ^a	63.34

Note: * *Agrobacterium* overgrowth

Values (mean ± SD) with different lowercase superscripts in column indicate significant ($p < 0.05$) differences.

3.5 Development of somatic embryos from putative transformed coffee calli

In a recent study, co-cultivation with friable calli involved testing different concentrations of *Agrobacterium*: undiluted, 1:5 dilution, and 1:10 dilution. These results revealed that a 1:5 dilution of *Agrobacterium* was the optimal concentration for achieving the putative transformation [citation]. This concentration resulted in a slightly lower survival rate of callus compared to the 1:10 dilution (Table 2); however, the surviving calli developed into various somatic embryos after 2 months. The development of somatic embryos through five stages: globular embryos, oblong embryos, heart-shaped embryos, torpedo-shaped embryos, and cotyledonary embryos can be seen in Fig. 3. A total of 28 embryos were produced from 31 calli. This result suggests that the optimal conditions for *Agrobacterium*-mediated delivery of the *GUS* reporter gene into coffee calli involved the use of friable calli co-cultivated with *Agrobacterium* cultured for 20 hours and diluted 1:5 prior to inoculation. Although embryogenic calli directly regenerated from leaf explants is the most widely used tissue for coffee transformation, it has resulted in low transformation efficiencies (<1%) [35]. However, the observed frequency was still insufficient for the efficient recovery of a large number of putative transformation events. Further optimization of factors influencing *Agrobacterium*-mediated gene delivery, together with efforts to shorten the transformation process, may improve the frequency of GUS-positive putative transgenic calli in future studies.

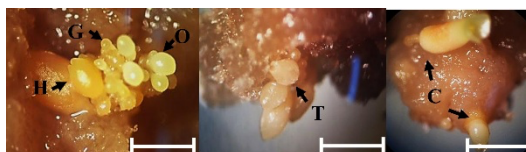


Fig. 3. Developmental stages of putative transgenic somatic embryos derived from coffee calli following *Agrobacterium*-mediated transformation. (G) globular stage, (O) oblong stage, (H) heart-shaped stage, (T) torpedo-shaped stage, and (C) cotyledonary stage (scale bar = 0.4 mm).

4. Conclusion

The optimization of *Agrobacterium*-mediated gene delivery in *Coffea arabica* cv. Catimor CIFC 7963 was achieved using friable calli co-cultivated with *Agrobacterium tumefaciens* strain EHA105 cultured for 20 hours and diluted at a ratio of 1:5 prior to inoculation. This treatment resulted in the highest recovery of hygromycin-resistant calli and enhanced transient GUS expression compared with the other conditions tested. A survival rate of 60.66% was obtained after three months of culturing on selective media. In addition, hygromycin-resistant calli were capable of progressing through multiple stages of somatic embryogenesis, producing 28 somatic embryos. These findings provide a useful foundation for the development of *Agrobacterium*-mediated gene delivery systems and future genetic improvement of *C. arabica* cv. Catimor CIFC 7963.

A limitation of this study is that stable transgene integration was not confirmed by molecular analyses. Therefore, the results should be interpreted as evidence of putative transformation based on callus survival under selection and transient GUS expression. Future studies should include molecular validation

and plant regeneration to confirm stable transformation, evaluate transgene inheritance, and assess the applicability of the optimized protocol for the introduction of agronomically important traits.

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