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Optimizing the Genetic Transformation of *Coffea arabica* Using *Agrobacterium tumefaciens*

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Abstract: The genetic transformation of *Coffea arabica* L. is an alternative strategy for obtaining plants with agronomic traits of interest that is less time-consuming than conventional breeding methods. Given the importance of coffee cultivation in Colombia, this study evaluated the main factors interfering with the genetic transformation of *C. arabica* using *Agrobacterium tumefaciens*. An efficient and reproducible method was accordingly developed that involved propagating "early" embryogenic calli in a liquid proliferation medium supplemented with 3 mg L^{-1} BAP for eight months, followed by sonication for 300 s in a suspension of LBA4404 OD_{600} of 0.5, harboring pCambia1301, and then incubation in this same suspension for 1 h. The vector pCambia1301 contained the *uidA* gene under control of the 35S promoter. A micropipette was used to remove the *Agrobacterium* suspension from the embryogenic callus. The remaining *Agrobacterium* suspension was blotted off by placing the embryogenic callus on filter paper. The embryogenic callus was then co-cultured for four days in a solid differentiation medium supplemented with 100 µM acetosyringone on filter paper. Subsequently, the embryogenic callus was post-cultured for four days in liquid differentiation medium under constant shaking at 100 rpm with 300 mg L^{-1} Cefotaxime, followed by selection with 50 mg L^{-1} hygromycin at 26 °C in the dark, with subcultures at 20-day intervals until somatic embryos were formed for subsequent culturing in germination medium. Molecular analysis confirmed the presence of the *uidA* gene in coffee seedlings transformed with strains LBA4404 and EHA105 and vectors pCambia1301 and pCambia2301 by polymerase chain reaction (PCR) analysis. This method successfully enables the stable integration of genes of interest in the coffee plant genome.

Keywords: *Agrobacterium tumefaciens*; *Coffea arabica*; coffee; genetic transformation; somatic embryogenesis

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1. Introduction

Coffee crops cover approximately 12 million hectares (ha) in 82 countries in the tropics and subtropics, mainly in Brazil and Colombia (Latin America), Vietnam and Indonesia (Asia), and Ethiopia and Ivory Coast (Africa). Approximately 10 million tons of green coffee beans are produced globally, making it one of the most traded commodities worldwide [1]. The International Coffee Organization (ICO) estimated that in 2022, between 12.5 and 25 million agricultural households worldwide depended on coffee production for a living [2]. *Coffea* belongs to the Rubiaceae family. To date, there are 130 known coffee plant species [3], of which *Coffea arabica* L. accounts for 60% of global production, followed by *Coffea canephora* Pierre ex A.Froehner, commonly known as Robusta, with 40% [4], and *Coffea liberica* Bull ex Hiern with less than 1%. *Coffea arabica* (2n = 4x = 44) is the only tetraploid and autogamous species of the genus. The low diversity of cultivated varieties of this species can be attributed to its allotetraploid origin, reproductive biology (self-compatible), and recent evolution [5]. In Colombia, *C. arabica* occupies the largest planted area in the country (842,399 ha), with a harvest value of COP 11,143,095 in 2023 [6]. Hence, it is the leading agricultural export product, with some 548,546 coffee growers

deriving their livelihoods from this crop. In other words, in Colombia, approximately two million people currently depend on coffee for subsistence [7].

When conventional breeding methods are used, it can take between 25 and 30 years to produce a new variety of *C. arabica* with attributes of interest, such as resistance to *Hypothenemus hampei*, Ferrari (Coleoptera, Curculionidae, Scolytinae), commonly known as the coffee berry borer (CBB). This beetle depends on coffee berries for its survival, infests all coffee species in different proportions, and causes the greatest economic losses to coffee crops not only in Colombia but worldwide [8]. Genetic transformation, however, presents an alternative for developing pest-resistant *C. arabica* plants in less time than that required by conventional breeding, as it allows gene transfer between different plant species and the subsequent expansion of their genetic pool. However, an in vitro plant regeneration system is necessary to produce transgenic coffee plants. Because the multiplication potential of somatic embryogenesis (SE) is significantly higher than that of other regeneration methods [9–11], SE has been the method of choice not only for developing transgenic coffee plants [12–16] but also for the multiplication of F1 hybrids [9,17] and the propagation and conservation of elite genotypes with high genetic and epigenetic stability [9]. SE is traditionally defined as a process by which plant somatic cells can be dedifferentiated into totipotent embryogenic stem cells and redifferentiated into a somatic embryo capable of regenerating plants under appropriate culturing conditions [18]. One study conducted by Campos et al. [19] suggests that cells capable of differentiating into somatic embryos do not undergo dedifferentiation. Hence, these meristematic cells retain totipotency and with the appropriate stimulus, they undergo multiplication and differentiation, forming new viable embryos.

Although somatic embryos have been obtained from coffee plant cells since 1970 [20], with numerous studies documenting the regeneration of somatic embryos produced from leaf explants over the last 50 years [11,17,21–24], the embryogenic capacity of coffee plant cells depends mainly on the genotype, which leads to the almost empirical development of specific protocols for each variety or clone [11,19,22]. Furthermore, other factors also influence the embryogenic response such as culture medium, growth regulators, and gelling agents [25]. Embryos can be obtained directly and indirectly by SE. In direct SE (DSE), between 1–10 somatic embryos are regenerated directly on the explant without forming a non-embryogenic callus [22]. The process is called low-frequency SE due to the low number of embryos produced [21]. In contrast, indirect SE (ISE) is characterized by the formation of several clusters of friable embryogenic calli (EC) that originate between 50 and 100 or more somatic embryos, which are formed from the proembryogenic masses [11]. ISE can be considered as a type of high-frequency SE because it produces many embryos [21].

Agrobacterium tumefaciens has been the preferred method for the genetic transformation of different plant species, including perennial species such as coffee [26], because it allows a low stable copy number of the gene of interest to be efficiently inserted in the plant genome. Although T-DNA does not present preferential integration in specific chromosomal sequences or regions of gene expression [27], a common characteristic is a slight local A + T motif enrichment at the pre-integration site and microhomology between the T-DNA border sequences and the pre-integration site [28]. Because of the low efficiency of the genetic transformation of *C. arabica* and *C. canephora* [12,26,29], very few genes of interest, such as Cry1Ac from *Bacillus thuringiensis* that confers resistance to the coffee leaf miner *Perileucoptera* spp. [12], have been incorporated into the coffee plant genome. In the field, transgenic plants of *C. canephora* expressing the Cry1Ac protein presented fewer lesions than the susceptible controls and exhibited stable resistance [13]. In addition, transgenic plants of *C. arabica* expressing a gene encoding the α-amylase inhibitor (*α-AI1*) of *Phaseolus vulgaris* L., an active inhibitor of the digestive α-amylases of the CBB, have been produced [14]. Similarly, the *Cry10Aa* gene from *B. thuringiensis* has been transferred to the coffee plant genome, conferring resistance to the CBB [15]. The berries that express this Cry10Aa protein cause mortality in first-instar larvae, with less than 9% seed damage as compared with 100% damage in untransformed berries [16]. Moreover, transgenic coffee

plants that suppressed caffeine synthesis have been produced using RNA interference technology by inhibiting the theobromine synthase gene *CaMXMT1* [30,31]. However, no information is available on the offspring of the transformed plants or commercial transgenic coffee varieties.

Successful regeneration and transformation in *C. arabica* can be restricted not only by high genotypic dependence, need for exogenous hormones, the *Agrobacterium* strain, method of infection, and culture conditions pre- and post-inoculation with *Agrobacterium* but also by the use of complex and inefficient genetic transformation methods. The foregoing evidences the importance of addressing the factors influencing the genetic transformation of this important crop in Colombia. This study accordingly aimed to develop an efficient and reproducible method for the genetic transformation of *C. arabica* by evaluating the factors that affect the integration of the *uidA* gene, which encodes GUS, into its genome. This is the first study that addresses the optimal combination of the following factors: age of EC, pre-culturing time, *Agrobacterium* strain, transformation vector, coffee genotype, sonication time, co-culturing medium, and co-culturing time.

2. Materials and Methods

2.1. In Vitro Culture Conditions

To induce indirect SE, leaves were collected at the Naranjal Experiment Station (Chinchiná, department of Caldas, Colombia) from coffee trees of genotypes BK.620 and BI.625 produced by the crossing of *C. arabica* cv. Caturra and the Timor hybrid. Climatic conditions at this site are as follows: average annual temperature, 20.8 °C; average relative humidity, 78%; annual rainfall, 2686 mm; and solar brightness, 1817 h yr^{-1}. The EC was regenerated as described by Molina et al. [22]. Young leaves were first disinfected with commercial sodium hypochlorite supplemented with 1% Tween 80 for 10 min, followed by three washes with sterile distilled water, after which 1 cm^2 explants were placed in primary culture medium supplemented with Murashige and Skoog (MS) salts [32], 1 mg L^{-1} thiamine, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} pyridoxine, 100 mg L^{-1} myoinositol, 37 mg L^{-1} cysteine, 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 8 mg L^{-1} kinetin, 30 g L^{-1} sucrose, and 8 g L^{-1} Bacto Difco Agar. After 1 month of culturing, explants were subcultured to a secondary culture medium supplemented with half-strength MS salts (MS/2) containing 3 mg L^{-1} 6-benzylaminopurine (BAP) instead of kinetin and 2,4-D. Cultures were left in the dark at 25 ± 2 °C until the EC was formed. Within 4–6 months, the friable EC developed on explants on the secondary medium [22]. To initiate cell suspension, 1 g friable EC was transferred to 250 mL Erlenmeyer flasks containing 100 mL MS/2 liquid proliferation medium, 10 mg L^{-1} thiamine, 0.2 mg L^{-1} myoinositol, 0.04 mg L^{-1} cysteine, 3 mg L^{-1} 6-BAP, and 30 g L^{-1} sucrose. The cell suspensions were then propagated by constant shaking at 100 rpm in the dark at 25 ± 2 °C, subculturing at 20-day intervals. "Early" EC were produced by propagating cell suspensions in liquid proliferation medium for 6–8 months after beginning the culturing process. "Differentiated" EC were produced by propagating cell suspensions for 12–14 months after beginning the culturing process. During pre-culturing, co-culturing, and post-culturing, the EC was maintained in a differentiation medium with MS/2 salts, 10 mg L^{-1} thiamine, 0.1 mg L^{-1} myoinositol, 0.04 mg L^{-1} cysteine, 0.5 mg L^{-1} kinetin, and 30 g L^{-1} sucrose. The solid culture medium was solidified with 2.8 g L^{-1} Phytagel, and cultures were left in the dark at 25 ± 2 °C.

2.2. Agrobacterium Tumefaciens Strains and Vectors

Agrobacterium tumefaciens strains EHA105 and LBA4404 harboring the binary vectors pCambia1301 and pCambia2301 were used for transformation experiments. Both vectors carried the ß-glucoronidase gene (*uidA*) as a reporter marker. In addition, the vector pCambia1301 carried the selectable marker gene for hygromycin phosphotransferase (*hpt*), whereas the vector pCambia2301 carried the selectable marker gene for neomycin phosphotransferase (*nptII*). Both genes were controlled by the cauliflower mosaic virus promoter (CaMV35S). Vectors pCambia1301 and pCambia2301 were introduced into *A. tumefaciens*

strains LBA4404 and EHA105 by electroporation. DNA (1 µL; 100 ng/µL) from either vector pCambia1301 or pCambia2301 was added to 30 µL electrocompetent cells of *A. tumefaciens* strains EHA105 or LBA4404. The cells were carefully shaken and transferred to a previously chilled electroporation cuvette (0.2 cm gap), which was subsequently placed in the cuvette holder. Electroporation was performed in a Bio-Rad electroporator model Gene Pulser® II Apparatus under the following conditions: 25 µF, 2.5 volts, and 200 W. Immediately after the electrical pulse, 800 µL of super optimal broth with catabolite repression (SOC) medium was added, and the cells were transferred to a microcentrifuge tube and incubated at 28 °C with constant shaking at 225 rpm for 1 h. After this time, dilutions of 10^{-0}, 10^{-1}, 10^{-2}, and 10^{-3} were made in LB medium containing kanamycin (100 mg L^{-1}), followed by selection of individual colonies of each vector in LB medium containing kanamycin (100 mg L^{-1}) at 28 °C for 12 h. After confirming the introduction of the vectors into the *A. tumefaciens* strains by restriction enzyme digestion, competent *A. tumefaciens* cells containing vectors pCambia1301 and pCambia2301 were prepared in 10% glycerol.

2.3. Agrobacterium Transformation

Agrobacterium strains transformed with vectors pCambia1301 and pCambia2301 were cultured in 5 mL LB medium containing 50 µg mL^{-1} kanamycin and 5 g L^{-1} sucrose overnight at 28 °C, with constant shaking at 200 rpm. *Agrobacterium* cells were collected by centrifugation at 3000 rpm for 10 min at 25 °C, resuspended in 20 mL (A_{600} = 0.5) LB medium containing 50 µg mL^{-1} kanamycin, 5 g L^{-1} glucose, and 100 µM acetosyringone, and cultured overnight at 28 °C with constant shaking at 100 rpm. *Agrobacterium* cells were collected by centrifugation at 3000 rpm for 10 min at 25 °C and then resuspended in 40 mL (A_{600} = 0.5) of liquid differentiation medium. The EC was pre-cultured for 0–8 days in a solid differentiation medium containing 100 µM acetosyringone on sterile filter paper. Subsequently, 1.0 mL *A. tumefaciens* suspension containing the transformation vectors was added to 0.5 mL packed EC placed in 1.5 mL microcentrifuge tubes. These tubes were subsequently placed individually in the center of a sonicator bath (Ultrasonic Cleaner, Cole-Parmer) and sonicated four times (0, 60, 120 and 300 s) at 60 Hz. After 1 h incubation in the bacterial suspension, the *Agrobacterium* suspension was then removed from the EC using a fine-tip micropipette, after which the EC was placed on a sterile filter paper to blot off excess bacteria [33,34].

Embryogenic cells were subsequently co-cultured with 100 µM acetosyringone for four days in either 50 mL Erlenmeyer flasks with 25 mL liquid differentiation medium or Petri dishes with 25 mL solid differentiation medium on sterile filter paper. Co-culturing time on the solid medium with 100 µM acetosyringone on sterile filter paper was evaluated between days 0–8. Co-culturing was followed by four days of post-culturing, during which the EC were transferred to 50 mL Erlenmeyer flasks containing 25 mL differentiation medium with 300 mg L^{-1} Cefotaxime, after which selection began with 50 mg L^{-1} hygromycin for EC transformed with vector pCambia1301 and with 20 mg L^{-1} geneticin for EC transformed with vector pCambia2301. Cultures were maintained at 26 °C in the dark, with constant shaking at 100 rpm, sub-culturing at 20-day intervals.

The following tests were performed to evaluate the factors influencing the genetic transformation of coffee embryogenic cells:

1. Sonication time: Early EC of genotype BI.625 without pre-culturing was sonicated in a suspension of *A. tumefaciens* LBA4404 containing vector pCambia1301 for 0, 60, 120, and 300 s. After 1 h incubation in the suspension, the early EC was co-cultured for four days in both solid and liquid differentiation media.
2. Co-culturing time: Early EC of genotype BK.620 without pre-culturing was sonicated in a suspension of *A. tumefaciens* EHA105 containing vector pCambia1301 for 300 s. After 1 h incubation in the suspension, the early EC was co-cultured in solid differentiation medium with 100 µM acetosyringone for 0–8 days.
3. Pre-culturing time: Early EC of genotype BK.620 was pre-cultured in the dark in solid differentiation medium supplemented with 100 µM acetosyringone for 0–8 days

on sterile filter paper, followed by sonication of embryogenic cells for 300 s in a suspension of *A. tumefaciens* EHA105 containing vector pCambia2301. After 1 h incubation in the suspension, the early EC was co-cultured in a solid differentiation medium for four days.

4. Age of EC: Transformation efficiency of the *uidA* gene was evaluated using early and differentiated EC of genotype BI.625 without pre-culturing, followed by sonication for 300 s of embryogenic cells in a suspension of *A. tumefaciens* LBA4404 containing vector pCambia1301. After 1 h incubation in the suspension, the EC was co-cultured in a solid differentiation medium for four days.
5. Agrobacterium strain: *A. tumefaciens* strains LBA4404 and EHA105 were evaluated with the vector pCambia1301.
6. Transformation vector: Vectors pCambia1301 and pCambia2301, previously introduced into strain LBA4404, were compared.
7. Coffee genotype: The early ECs of genotypes BI.625 and BK.620 were co-cultured with *A. tumefaciens* strain LBA4404 containing the vector pCambia1301. *Agrobacterium* strains, transformation vectors, and coffee genotypes were evaluated using the same protocol used to evaluate the age of EC (described above, item 4).

2.4. Stable Transformation

2.4.1. Development of Transgenic Somatic Embryos

The transformed EC was subcultured at 20-day intervals in a liquid differentiation medium with MS/2 salts and depending on the vector used, was supplemented with either 50 mg L^{-1} hygromycin or 20 mg L^{-1} geneticin, as well as 300 mg L^{-1} Cefotaxime, until somatic embryos were formed. These embryos were then cultured in a germination medium with MS/2 salts, 1 mg L^{-1} thiamine, 1 mg L^{-1} nicotinic acid, 0.1 g L^{-1} myoinositol, 20 g L^{-1} sucrose, and 7 g L^{-1} agar (Plant TC, PhytoTechnology, Lenexa, KS, USA) to which either 50 mg L^{-1} hygromycin or 20 mg L^{-1} geneticin were added, depending on the vector used, as well as 300 mg L^{-1} Cefotaxime. The embryos remained in the dark until they produced the first pair of cotyledonary leaves. Plants were then submitted to a 12 h photoperiod (light intensity of 20 µmol·$m^{-2}s^{-1}$) at 26 °C. When seedlings had produced four pairs of leaves, they were transferred to plastic pots containing a soil substrate and kept in greenhouse conditions.

2.4.2. Histochemical GUS Assay

Transformation efficiency, defined as the total number of blue spots per gr dry weight co-cultured EC, was determined as reported previously [35]. The transformed EC was transferred to multi-well culture dishes into which 1 mL X-Gluc solution (2 mM X-Gluc, 100 mM sodium phosphate buffer pH 8.0, 10 mM EDTA, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, and 20% methanol) was added. Leaves and roots of transformed plants were also incubated for 24 h at 37 °C in X-Gluc solution, with the EC, leaves, and roots later being washed with 95% ethanol. A stereomicroscope was used to observe GUS activity in suspension cells, while its activity in leaves and roots was observed with the naked eye.

2.4.3. PCR and Southern Blot Analysis of Transgenic Plants

The DNA of a randomly selected transformed plant was extracted from leaf tissue frozen at −80 °C and vacuum dehydrated in a lyophilizer (Labconco, FreeZone 6 L, Kansas City, MO, USA) using the CTAB method [36]. This involved incubating 80 mg macerated tissue in 500 µL 2× CTAB buffer (CTAB 2% w/v, Tris 0.1 M, EDTA 0.02 M, NaCl 8.2% *w*/*v*, polyvinylpyrrolidone 2% *w*/*v*, and ß-mercaptoethanol 1.5% *v*/*v*) and subsequent stirring at 65 °C for 30 min, after which a 500 µL chloroform/isoamyl alcohol (24:1) was added and centrifuged at 14,000 rpm for 15 min at 4 °C for DNA purification. The DNA was precipitated by adjusting the suspension to a concentration of 0.18 M sodium acetate, 0.7 M NaCl, and 500 µL isopropyl alcohol. Collected DNA was washed with

70% ethanol and resuspended by incubation for 30 min at 37 °C in 100 µL ultrapure water with 2.5 µg RNase-A. The primers 5′-GATCAGCGTTGGTGGAAAGCGCG-3′ and 5′-CACCGAAGTTCATGCCAGTCCAGCG-3′ were used to amplify the *uidA* gene. The reaction mixture for PCR was composed of sterile millipore water, 1× PCR buffer, 3.0 mM $MgCl_2$, 0.2 mM of each dinucleotide (dNTP), 0.8 µM primer, 2.5 U Taq polymerase (Gibco BRL), and 200 ng DNA for a final volume of 25 µL per tube. The mixture was incubated in a thermal cycler (PTC-200 MJ Research) under the following conditions: 1 cycle at 94 °C for 5 min; 40 cycles at 94 °C for 1 min, 60 °C for 1 min 30 s, and 72 °C for 3 min 30 s and an extension at 72 °C for 5 min. Eleven plants of each of the two transformation vectors, derived from independent transformation events, were selected among the plants that tested positive by PCR for molecular analysis via Southern blotting to determine the number of integration sites of the *uidA* gene in the coffee genome. The protocol reported by Noir et al. [37] was followed, and the DNA was digested with BglII and BsteII for the vector pCambia2301 and with Xho I for the vector pCambia1301. The DNA fragments were then separated on 0.8% agarose gels, transferred to nylon membranes, and hybridized with radioactive probes.

2.5. Experimental Design and Data Analysis

A series of seven experiments was conducted to evaluate the following factors affecting the genetic transformation of coffee: age of embryogenic cells, *Agrobacterium* strain, transformation vector, genotype, sonication times (0, 60, 120, and 300 s), co-culturing medium (solid and liquid), pre-culturing time, and co-culturing time. For all experiments, the experimental unit consisted of either a 50 mL Erlenmeyer flask with 25 mL liquid differentiation medium or a Petri dish with 25 mL solid differentiation medium, totaling three experimental units per treatment. Transformation efficiency, measured as the number of blue spots per gr dry weight EC, was considered as response variable. A completely randomized experiment design was used to evaluate the treatment effect in all experiments, except those of sonication time and co-culturing medium. In the latter, an analysis of variance for completely randomized designs was performed with a 4 × 2 factorial arrangement. Duncan's multiple comparison test was applied in the case of those experiments where the analysis of variance showed differences and the variation factors were qualitative. The regression or orthogonal polynomial test was used to identify differences between treatment groups for experiments with quantitative factors of variation.

3. Results

The detection of the GUS reporter gene by counting blue spots served to successfully monitor the transformation efficiency of all evaluated factors.

3.1. Age of EC

Coffee leaf explants were cultured in primary MS medium (Figure 1a) and after 1 month, were subcultured in secondary MS medium, producing non-embryogenic calli (Figure 1b). The yellow friable callus, developed on proembryogenic masses in the secondary medium between months 4 to 6 (Figure 1c), was transferred to liquid proliferation medium (Figure 1d). During the long-term maintenance of competent EC for use as explants in genetic transformation, most of the friable calli became dispersed and proliferated into suspension cells by subculturing at 20-day intervals on fresh liquid proliferation media. Two types of embryogenic clusters were produced, namely (1) early EC, composed of small, light-yellow friable clusters produced during EC propagation in the proliferation medium between months 6 and 8 by subculturing at 20-day intervals (Figure 1d) and (2) differentiated EC, composed not only of yellow clusters larger than those of early EC but also of small somatic embryos, produced during EC propagation in the proliferation medium during months 12 to 14, also subcultured at 20-day intervals (Figure 1e).

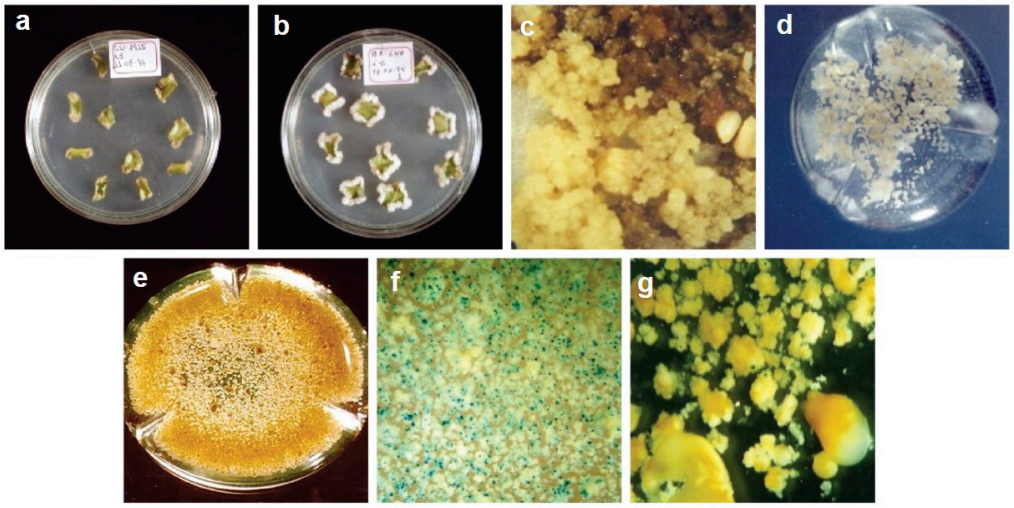

Figure 1. Proliferation of EC and evaluation of age of EC using GUS histochemical assay. (**a**) Explants of *Coffea arabica* on primary MS medium supplemented with 2,4-D and kinetin, (**b**) explants with non-embryogenic calli on secondary MS medium supplemented with BAP, (**c**) friable EC developed on secondary MS medium after six months of culturing as observed under the stereomicroscope, (**d**) early EC, (**e**) differentiated EC, (**f**) transformed early EC showing blue spots in GUS histochemical assay as observed under stereomicroscope, and (**g**) transformed differentiated EC showing blue spots in GUS histochemical assay as observed under stereomicroscope.

Results of the evaluation of transformation efficiency of the two types of EC obtained in the study indicated that transformation efficiency was significantly higher in early EC ($p \leq 0.0001$) than in differentiated EC (Table 1). This greater transformation efficiency in early EC clusters was characterized by a higher number of blue spots (Figure 1f) as compared with differentiated EC clusters, which showed very few blue spots (Figure 1g). GUS expression was very low in somatic embryos (Figure 1g).

Table 1. Average transformation efficiency (number of blue spots per gr dry weight EC) according to the age of EC, *Agrobacterium tumefaciens* strain, co-culturing medium, transformation vector, and genotype.

Factor	Average Number of Blue Spots per gr Dry Weight EC
Age of EC	
Early	229,535 ± 9626 a
Differentiated	65,769 ± 5340 b
A. tumefaciens strain	
LBA4404	264,877 ± 12,176 a
EHA105	99,759 ± 4270 b
Co-culturing medium	
Solid	167,526 ± 80,923 a
Liquid	62,434 ± 54,765 b
Transformation vector	
pCambia1301	164,199 ± 8894 a
pCambia2301	146,344 ± 9150 b
Genotype	
BK.620	172,437 ± 87,017 a
BI.625	182,955 ± 87,810 a

Means with the same letter are not statistically different (Duncan = 0.05).

3.2. Pre-Culturing Time

The early EC was pre-cultured on solid differentiation medium with 100 µM acetosyringone for 1–8 days on sterile filter paper. Early EC propagated in proliferation medium was used in the no pre-culturing treatment. Results indicated early EC of coffee with no pre-culturing presented the highest transformation efficiency (82,086 blue spots per gr dry weight EC) as compared with coffee EC pre-cultured for 1–8 days in solid differentiation medium with acetosyringone, which yielded a lower number of blue spots, decreasing from 33,750 to 9927 with increasing pre-culturing time (Figure 2).

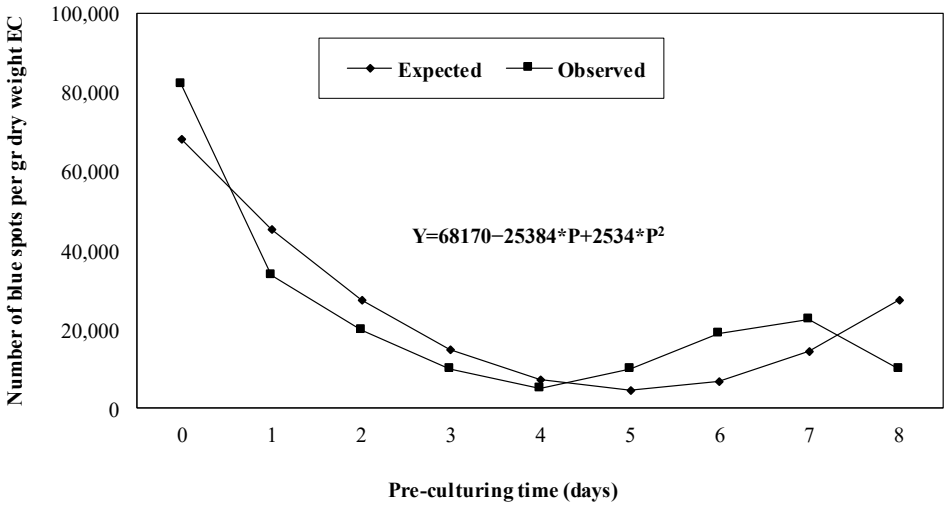

Figure 2. Regression of transformation efficiency (number of blue spots per gr dry weight EC) according to pre-culturing time.

3.3. Agrobacterium Strain, Transformation Vector, and Coffee Genotype

Different *Agrobacterium* strains containing a series of binary plant vectors have been used to obtain transgenic plants. Nonetheless, the ability of *Agrobacterium* to infect different genotypes differs among strains, affecting transformation efficiency [38]. In this study, strain LBA4404 proved to be more effective than strain EHA105 at infecting coffee EC because transformation efficiency was significantly higher with an increasing number of blue spots per gr dry weight EC (264,877) in comparison with strain EHA105, which produced 99,759 blue spots (Table 1). In addition, vector pCambia1301 showed a significantly higher number of blue spots than vector pCambia2301 (Table 1). Genotypes BK.620 and BI.625 did not differ in the number of blue spots per gr dry weight EC (Table 1).

3.4. Sonication Time and Co-Culturing Medium

To improve the transformation efficiency of coffee EC, the *Agrobacterium* suspension was cultured on LB medium with sucrose and glucose, as well as with 100 µM acetosyringone, prior to infection with EC. During the infection process, the *Agrobacterium* suspension was added to 0.5 mL of early EC packed. To increase T-DNA integration in coffee EC, four sonication times were tested, producing micro-wounds that facilitated the entry of bacteria into EC. Transformation efficiency was observed to increase when the EC in the *Agrobacterium* suspension was exposed to sonication between 60 and 300 s in both liquid and solid culture media (Figure 3). A significantly higher number of blue spots (278,992) was obtained with the longest sonication time tested (300 s) in solid culture media ($p \leq 0.0001$) as compared with the number of blue spots per gr dry weight in EC not exposed to sonication (68,819) or with the shortest sonication times evaluated (60 and 120 s). On the other hand, co-culturing in liquid culture medium showed a lower number of blue spots per gr dry weight at all sonication times tested (Figure 3).

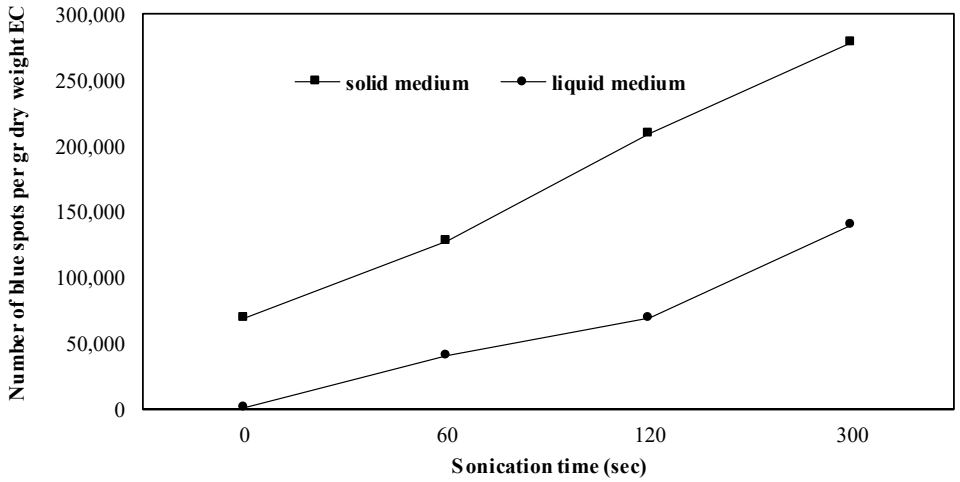

Figure 3. Transformation efficiency (number of blue spots per gr dry weight EC) according to sonication time in liquid and solid co-culturing media.

3.5. Co-Culturing Time

In an effort to enhance the contact of *Agrobacterium* with coffee EC, as well as increase the insertion of the *uidA* gene, different co-culturing times of early EC were tested in solid culture medium containing 100 µM acetosyringone on sterile filter paper. Once the EC was infected by the *Agrobacterium* suspension with the help of sonication, the number of blue spots was observed to increase between days 1 to 4 of co-culturing (Figure 4), reaching their highest number at 4 days co-culturing in the dark ($p \leq 0.0001$). Prolonged periods (between 5 and 8 days) decreased the number of blue spots due to *Agrobacterium* overgrowth (Figure 4). The highest transformation efficiency occurred when all optimized factors were combined. Non-pre-cultured early embryogenic cells were sonicated for 300 s in a suspension of LBA4404 harboring pCambia1301, followed by co-culturing on solid medium with 100 µM acetosyringone on sterile filter paper for four days ($p \leq 0.0001$), obtaining 264,877 blue spots per gr dry weight EC (Table 1).

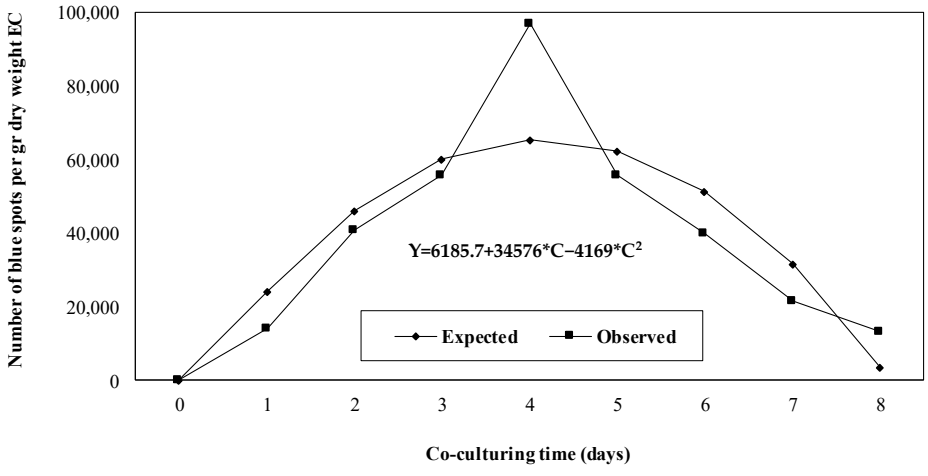

Figure 4. Transformation efficiency regression (number of blue spots per gr dry weight EC) according to the co-culturing time.

3.6. Regeneration of Transgenic Plants and Molecular Analysis

The EC co-cultured with *Agrobacterium* for four days on filter paper in solid differentiation medium was transferred during the four days of post-culturing to liquid differentiation medium supplemented with 300 mg L^{-1} Cefotaxime to initiate bacteria elimination. From this moment on, the EC was transferred to liquid differentiation medium containing 300 mg L^{-1} Cefotaxime with 50 mg L^{-1} hygromycin for vector pCambia1301

and 20 mg L^{-1} geneticin for vector pCambia2301. Untransformed EC showed a rapid browning, whereas transformed EC remained yellow in the liquid differentiation medium until somatic embryos began to develop. The inhibition of untransformed calli was higher in EC with 50 mg L^{-1} hygromycin (Figure 5a) than in those with 20 mg L^{-1} geneticin (Figure 5b). The GUS histochemical staining of stable calli showed an intense blue color (Figure 5a,b). In contrast, untransformed EC turned brown and died during selection with hygromycin and geneticin. Embryos were then cultured for three months in the dark in a germination medium without growth regulators. Selection was performed using hygromycin and geneticin, depending on the vector used, until plants produced the first pair of dicotyledonous leaves, after which these were incubated for four months under a 12 h photoperiod (Figure 5c,d). Leaves of hygromycin-selected transgenic plants (Figure 5e), as well as those selected with geneticin (Figure 5f), showed blue coloration. Leaves of untransformed plants did not show blue coloration (Figure 5g). Fifty transformed seedlings derived from independent transformation events with vectors pCambia1301 and pCambia2301, presenting four pairs of dicotyledonous leaves and good root development, were transferred to sterile soil under greenhouse conditions (Figure 5h,i). Agarose gel electrophoresis showed an amplification of a 1600 bp amplicon in the positive control vector PCR (Figure 6, L2 and L3). Hygromycin- and geneticin-resistant plants also showed the presence of a 1600 bp amplicon corresponding to the *uidA* gene (Figure 6, L4 to L7), whereas no bands were observed in the negative template PCR (Figure 6, L8). Hybridization by Southern blotting confirmed the presence of a band corresponding to the *uidA* gene in coffee seedlings transformed using vectors pCambia1301 and pCambia2301. The presence of the GUS gene-coding region fragment was confirmed in nine of the eleven plants transformed using vector pCambia2301 by digestion with enzymes Bgl II and Bste II (Figure S1). Similarly, a coding region fragment of the gene *hptII* was confirmed in eight of the eleven plants transformed using vector pCambia1301 and digested with the enzyme XhoI (Figure S2).

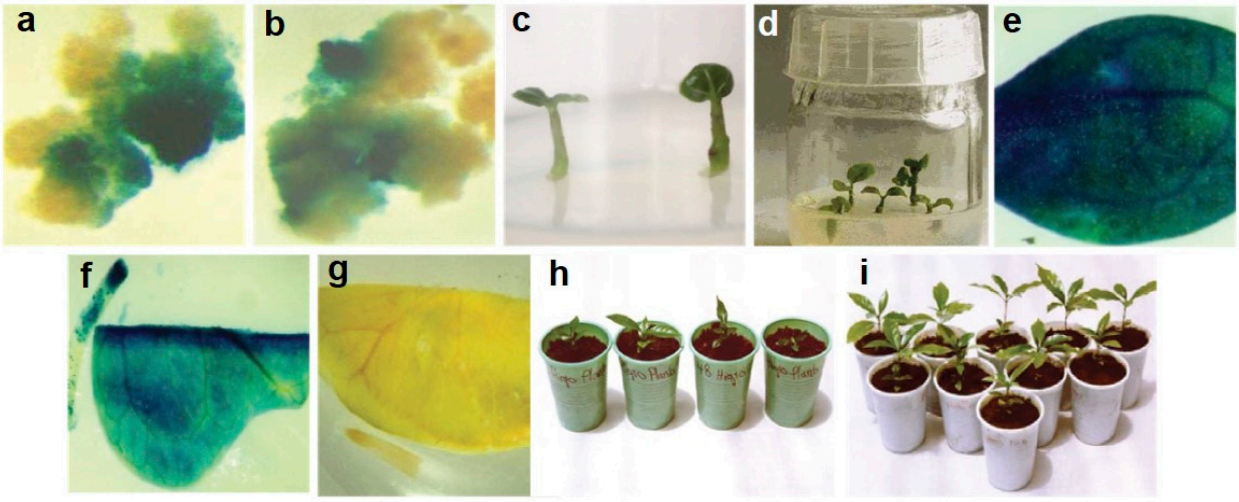

Figure 5. Proliferation of transformed suspension cultures and development of transformed plants of *C. arabica* using strains LBA4404 and EHA105 and vectors pCambia1301 and pCambia2301. (**a**) EC transformed using vector pCambia1301 and selected with 50 mg L^{-1} hygromycin as observed under stereomicroscope; (**b**) EC transformed using vector pCambia2301 and selected with 20 mg L^{-1} geneticin as observed under stereomicroscope; (**c**) somatic coffee embryos transformed using *A. tumefaciens* pCambia2301; (**d**) somatic coffee embryos transformed using *A. tumefaciens* pCambia1301; (**e**) leaf transformed using vector pCambia1301 and showing blue coloration in GUS histochemical assay; (**f**) leaf and root transformed using vector pCambia2301 and showing blue coloration in GUS histochemical assay; (**g**) leaf and root of untransformed control plants with no blue coloration; (**h**) transgenic coffee plants transformed using vector pCambia1301; (**i**) transgenic coffee plants transformed using vector pCambia2301.

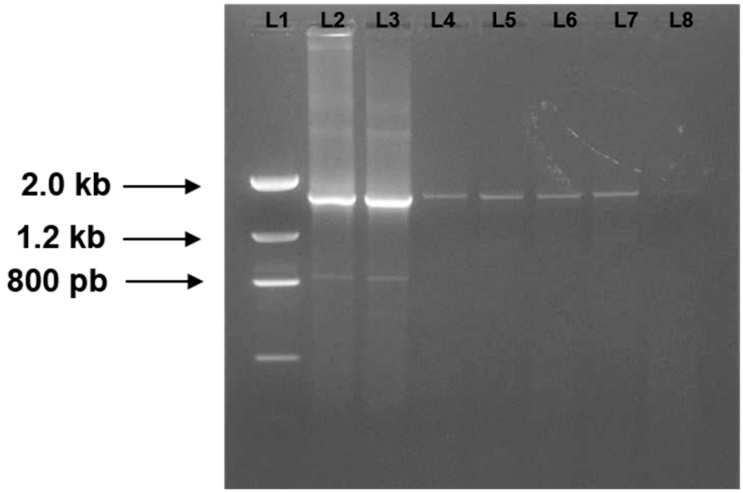

Figure 6. Molecular confirmation of transgenic *Coffea arabica* plant analysis for *uidA* gene presence in hygromycin-resistant plants and geneticin-resistant plants using *uidA* gene-specific primers. L1, molecular weight marker (Low DNA Mass Ladder) (ThermoFisher Scientific, Waltham, MA, USA); L2, positive control (vector pCambia1301 DNA); L3, positive control (vector pCambia2301 DNA); L4–L5, different transformed hygromycin-resistant plants using PCR; L6–L7, different transformed geneticin-resistant plants using PCR; L8, no template (negative control).

4. Discussion

The genetic transformation of *C. arabica* has been carried out using previously used methods that evaluated different factors affecting the stable integration of the gene of interest into the coffee genome [12,13,26,29,31,39,40]. However, the present study to improve the genetic transformation efficiency of *C. arabica* took into consideration most of the factors affecting the transfer of *Agrobacterium* T-DNA to plant cells, this being the first time that a method combined all these optimized factors.

In the case of coffee, the age of EC significantly affects genetic transformation efficiency. Early EC, produced during culturing for 6–8 months in liquid proliferation medium, presented higher transformation efficiency of the *uidA* gene ($p \leq 0.0001$) as compared with differentiated EC, produced during culturing for 12–14 months in the same culture medium (Table 1). This could be attributed to the fact that early EC are composed of actively dividing clusters (Figure 1d) and as a result, are susceptible to the formation of a higher number of wounds by sonication (Figure 1f) [34]. As a result, the expression of *vir* genes is activated, directing T-DNA cleavage and its subsequent integration into the coffee plant genome. On the other hand, fewer and more superficial wounds (Figure 1g) are produced by sonication in differentiated EC, which are composed of yellow clusters larger than those of early EC and somatic embryos (Figure 1e). Similarly, the yellow embryogenic callus of *C. arabica* variety Caturra propagated for seven months presented the highest transformation efficiency [26]. These results demonstrate that the age of EC is one of the most decisive factors for improving genetic transformation efficiency in coffee plants. To increase the transformation efficiency of *C. arabica*, early EC composed of a yellow friable callus, propagated for 6–8 months in liquid culture medium, should be selected.

Pre-culturing time refers to culturing EC in a differentiation medium with the addition of acetosyringone to promote cell division before co-culturing with *Agrobacterium*. The purpose of pre-culturing is to (1) ensure that the tissues are sufficiently competent to resist bacterial infection and (2) increase the expression of virulence genes (*vir*) once the *Agrobacterium* suspension enters in contact with the EC [38,41], thus facilitating the cleavage and insertion of the T-DNA into the embryogenic cells. In this study, however, coffee EC with no pre-culturing presented the highest transformation efficiency as compared with coffee EC pre-cultured for 1–8 days in a solid medium supplemented with 100 µM acetosyringone (Figure 2). These findings indicate that early EC of *C. arabica* undergoing

cell division, maintained for 6–8 months in liquid proliferation medium supplemented with BAP as the only growth hormone, are suitable for *Agrobacterium* infection and production of transgenic plants. This corroborates that the propagation of embryogenic cells in liquid medium increases their capacity to produce somatic embryos [9] and that cytokinins are essential for EC proliferation without requiring auxins after the induction of totipotent cells [42]. Similarly, the EC of *C. arabica* variety Caturra [26] and cotyledonary somatic embryos of *Hevea brasiliensis* Müll. Arg. [43] presented the highest transformation efficiency when not pre-cultured in a medium supplemented with 100 µM acetosyringone. In contrast, the transformation efficiency in *Cicer arietinum* L. was higher in explants pre-cultured between 3–5 days [44,45].

This study revealed that micro-wounds of coffee EC exposed to 300 s of sonication were more numerous than those of calli not exposed to sonication, as well as those exposed to 60 and 120 s of sonication. These results are similar to those observed in *Glycine max* L. [34], *H. brasiliensis* [43], and *Passiflora cincinnata* Mast. [46], where micro-wounds became larger and more numerous with increasing sonication time, thus increasing transformation efficiency. Micro-wounds generated during sonication in coffee EC release compounds such as acetosyringone and a variety of monosaccharides, including glucose, galactose, and arabinose [41], as well as the sucrose, glucose, and acetosyringone added to the *Agrobacterium* growth culture media. All of these compounds stimulate the expression of *vir* genes, given that proteins VirA and VirG serve as members of a two-component sensory signal transduction genetic regulatory system [47]. VirA is sensitive to the presence of specific phenolic compounds induced in the wounds together with sugar molecules. Under this stimulus, VirA autophosporylates and, subsequently, transphosphorylates VirG protein. Upon phosphorylation, the VirG protein helps increase the level of transcription of *vir* genes [48], explaining why the incubation of EC with *Agrobacterium* suspension for 1 h post-sonication without shaking increased the excision and transfer of the *uidA* gene in the coffee genome. Sonicated coffee EC also showed normal growth up through the formation of transgenic plants, confirming the observations of Trick and Finer [34], who established that sonication could affect EC growth immediately after exposure. However, after two weeks, the EC proliferated under conditions similar to those of the control without sonication. These results demonstrated that sonication-assisted *Agrobacterium*-mediated transformation increases transformation efficiency in the EC of *C. arabica*.

Different *Agrobacterium* strains, such as LBA4404, C58, EHA101, and EHA105, are suitable for the transformation of coffee EC [26,40]. In this study, the number of blue spots was higher with *A. tumefaciens* strain LBA4404 as compared with strain EHA105 (Table 1). Moreover, strain LBA4404 was used to obtain transformed plants of *C. arabica* and *C. canephora* with the *cry*1Ac gene [12]. In contrast, leaf explants of *C. arabica* variety Catuaí agroinfiltrated with *A. tumefaciens* strain GV3101 showed a higher expression of the *uidA* gene than did leaves agroinfiltrated with strains LBA4404 and ATHV [49]. On the other hand, the present study did not find significant differences in transformation efficiency between the two coffee genotypes BK.620 and BI.625 used in the study (Table 1), probably because both are advanced lines of the Colombia variety, which was obtained by crossing the variety Caturra and the Timor Hybrid that share a common genetic origin [50].

This study found that transformation efficiency was highest when EC were co-cultured for four days on filter paper in solid medium with MS/2 salts and 100 µM acetosyringone, similar to what occurred in *C. arabica* variety Caturra [26] and *C. canephora* [51]. In *H. brasiliensis* [43], *C. arietinum* [44,45], *Zea mays* L. [52], and *Oriza sativa* L. [53], different co-culturing times have been used, ranging between 2 and 3.5 days. Co-culturing on filter paper benefits the transfer of T-DNA to embryogenic cells because *Agrobacterium* tends to increase the integration of T-DNA to these cells, attributable to the low availability of nutrients on filter paper. In *C. sativus*, co-culturing on filter paper in solid medium also suppressed explant necrosis, which led to a higher regeneration efficiency [54]. The constant shaking could explain the lower number of blue spots in EC co-cultured in liquid medium, as shaking restricts bacteria colonization to the areas between EC lobes. In

contrast, co-culturing in solid culture medium favors the growth of *Agrobacterium* over the entire cell suspension surface.

The EC transformed by the *uidA* gene was maintained in liquid differentiation medium into which Cefotaxime had been added. This antibiotic eliminated the bacteria and prevented overgrowth, allowing transformed plants to develop. This proved advantageous because *Agrobacterium* overgrowth adversely affects the regeneration of somatic embryos [55]. Cefotaxime has also been used to eliminate *Agrobacterium* during the development of transgenic coffee plants [12,26] because it has proven to be non-toxic for EC proliferation. Selective antibiotics hygromycin and geneticin allowed transformed EC to be selected and continue through plant development, with the stable integration of independent single copy inserts of the *uidA* gene in plants originated from the different transformation events. The 1.40% transformation efficiency achieved during this study was higher than that reported by Leroy [12], who reported a transformation efficiency less than 1% in *C. arabica*.

5. Conclusions

This study successfully demonstrated a reliable and stable genetic transformation of *C. arabica* using cell suspension cultures derived from leaf explants with *A. tumefaciens* strain LBA4404 harboring pCambia1301. This is the first report of successful transformation efficiency of EC of *C. arabica*, achieved by the optimal combination of factors such as the age of the EC, pre-culturing time, *Agrobacterium* strain, transformation vector, coffee genotype, sonication time, co-culturing medium, and co-culturing time in suspension cultures. Transformation efficiency was confirmed by the number of blue spots per gr dry weight EC due to its GUS reporter gene. This method of genetic transformation can be used to transfer CBB resistance genes to coffee.

One of these resistance genes is the *Lupinus bogotensis* aspartic protease inhibitor (LbAPI), which was found to be highly effective in inhibiting CBB aspartic proteases in vitro, with a mean inhibitory concentration (IC50) of 2.9 µg [56]. In vivo, the concentration of recombinant LbAPI required to cause 50% mortality in CBB larvae in artificial diets was 0.91% [57]. In addition, the α-amylase inhibitor from *P. vulgaris* caused an 88% inhibition of the α-amylase activity of the CBB, and transgenic plants expressing this inhibitor presented delayed borer development [14]. Although the development of transgenic plants and their subsequent planting in the field can be challenging, this is indeed an alternative for controlling CBB in coffee crops.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijpb15040086/s1, Figure S1: Southern blot analysis of coffee plant DNA transformed with vector *pC2301* and digested with enzymes Bste II and Bgl II. Lane 1, molecular weight marker λ Hind III; lanes 2, 3, 16, and 17, vector *pC2301*; lanes 4–14, 11 coffee plants transformed with *pC2301*; lane 15, negative control corresponding to untransformed coffee plant; Figure S2: Southern blot analysis of coffee plants genomic DNA transformed with vector *pC1301* and digested with enzyme Xho I. Lane 1, molecular weight marker λ Hind III; lanes 2, 3, 16, and 17, vector *pC1301*; lanes 4–14, 11 coffee plants transformed with vector *pC1301*; lane 15, negative control corresponding to untransformed coffee plant.

Author Contributions: Conceptualization, D.M. and R.A.; methodology, D.M. and R.A.; validation, D.M. and R.A.; formal analysis, D.M.; investigation, D.M. and R.A.; writing—original draft preparation, D.M.; writing—review and editing, D.M. and R.A.; project administration, D.M. and R.A. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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