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RESEARCH ARTICLE

Transient gene expression in secondary somatic embryos from coffee tissues electroporated with the genes *gus* and *bar*

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Keywords: *bar*, *Coffea arabica,* electroporation, genetic transformation, *gus*, secondary somatic embryos, somatic embryos.

Different electroporation conditions were evaluated, toward the goal of transformation of *Coffea arabica* cv. Catimor. The tissues assayed were: embryogenic calli, leaf sections from *in vitro* plants, and somatic embryos in globular and torpedo stage obtained from cell suspensions. The effect of 1 or 24-hour pretreatment with an enzymatic solution (2% cellulase, 1% macerozyme) and electric field strength (375, 625, 875 V/cm) was evaluated. In all the experiments the tissues were incubated in ASP buffer (potassium aspartate) during three hours, and then one hour with plasmid DNA (pCambia3201, containing *gus* and *bar* genes) at room temperature. The electroporation was performed at a capacitance of 900 µF. The effect of the parameters evaluated was determined by the transient expression of the *gus* gene. The optimal conditions for electroporation were one hour of enzymatic pretreatment of torpedo shape embryos, electroporation at 375 V and 900 µF. The culture of electroporated tissues in liquid media with 8 mg/l benzyladenine conducted to maximal regeneration through secondary somatic embryogenesis. The secondary somatic embryos were formed directly in the hypocotyl surface of the electroporated torpedo shape primary somatic embryos, the production of secondary somatic embryos was significantly greater than the production of primary embryos, therefore, this is an excellent method to propagate the products of genetic transformation. The secondary somatic embryos regenerated from electroporated torpedo shape somatic embryos were positive for *gus* expression, and also in the PCR analysis for the genes *gus* and *bar*.

Coffee is a very important crop in Latin America. As all the crops, the coffee is susceptible to different kinds of biotic stresses that affect yield significantly. In the cases where coffee does not have natural resistance for pests as leaf miner (*Leucoptera coffeella*) or the coffee berry borer (*Hypothenemus hampei*), it would be very useful to have a system for the genetic transformation permitting the incorporation of resistance to those insects. To establish a genetic transformation system it is necessary to have an explant competent for the transformation process, and an *in vitro* culture system, which permits a high frequency of regeneration. Furthermore it is necessary to have a system of genic transference that is simple, cheap, reproducible and independent of the genotype, that inserts the genetic sequence in a stable form (Birch, 1997). There are efficient regeneration systems for coffee through somatic embryogenesis from several tissues (Staritsky, 1970; Söndahl and Sharp, 1977; Dublin, 1984; García and Menéndez, 1987; Zamarripa, 1991). Spiral et al. 1993 transformed *Coffea canephora* (Robusta) with *Agrobacterium tumefaciens* and Leroy et al. 2000 obtained coffee plants of *Coffea arabica* and *Coffea canephora*

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resistant to the leaf miner also using the vector *Agrobacterium tumefaciens*. Hatanaka et al. 1999 regenerated transgenic plants of *Coffea canephora* by the incorporation of the genes *gus*, *nptII* and *htp* through *Agrobacterium tumefaciens*. Van Boxtel et al. 1995 transformed different coffee genotypes by bombardment, assaying different plasmids and promoter sequences. The main objective of our work is to develop a method for coffee genetic transformation by electroporation. The results of transitory expression of the *gus* gene and the PCR detection of the genes *gus* and *bar* in secondary somatic embryos obtained from electroporated coffee (*Coffea arabica* cv. Catimor) tissues are analyzed.

Materials and Methods

Electroporation. Electroporation assays were carried out on different tissues of *Coffea arabica* cv. Catimor cultivated *in vitro* following the procedure of embryogenic cell suspensions of Hermoso-Gallardo and Menéndez-Yuffá, 2000, leaf explants were used to originate the calli to obtain the embryogenic cell suspension cultures. Tissues were incubated in potassium aspartate (ASP) pH 5.8 buffer containing 70 mM aspartic acid, 5 mM calcium gluconate, 5 mM MES and 0.4 mM mannitol. Then, the tissues were incubated one hour at room temperature (28 $\pm$ 2ºC) in sterile cuvettes with buffer ASP and 100 µg/ml DNA of the plasmid pCambia3201 (isolated by the Promega kit Wizard Plus Midipreps). A pulse generator BIO-RAD (Gene Pulser II System) was used for the electroporation of the tissues.

The plasmid pCambia3201 (Center for the Application of Molecular Biology to the International Agriculture of Canberra, Australia (CAMBIA); E-mail: vectors@cambia.org, contains the duplicated promoter of CAMV 35S, the genes *gus*, *nptII* and *bar*.

The effect of different factors related to the electroporation procedure, on the transient *gus* gene in coffee plants was evaluated:

1. Type of tissue. Material tested: embryogenic callus (4 months from the beginning of the culture), vitroplant leaves, globular (Eg) and torpedo shape (Et) somatic embryos from cell suspensions.

2. Pretreatment. Incubation of Et during 1 or 24 hours in enzymatic solution 2% cellulase R10 "Onozuka" and 1% Macerozyme R-10 "Yakult Pharmaceutical" in buffer 5 mM MES, 0.5 M mannitol and 25 mM $CaCl_2$ at pH 5.8.

3. Electrical field strength. Discharges of 375, 625, 875 V/cm were tested, at constant capacitance 900 µF.

4. Temperature. Temperatures of 0ºC, Room temperature (28 $\pm$ 2ºC), and 37ºC were tested during the incubation previous to electroporation.

***gus* detection.** The transient expression of the *gus* gene was detected using the method of Jefferson et al. 1987 with the modification of Kosugi et al. 1990 and Lacorte, 1998. The histochemical reaction was done 48 hours after the electroporation of the tissues. To improve the visualization of the color in the plant tissues, they were clarified with 70% ethanol. The number of explants with *gus* expression and the number of dots were counted. The positive control used for the *gus* reaction were tissues of transgenic potato (*Solanum tuberosum* cv. Desiree) vitroplants, donated by Dr. Eva de García from the laboratory of Biotecnología Vegetal at the Universidad Central de Venezuela.

Electroporated tissues. The explants used for electroporation were obtained from suspension cultures of *Coffea arabica* cv. Catimor according to the method of Hermoso-Gallardo and Menéndez-Yuffá, 2000.

Regeneration. The regeneration of the electroporated explants was obtained in a medium for the induction of secondary somatic embryogenesis, composed by 1/2 strength Murashige and Skoog, 1962 salts, 100 mg/l mio-inositol, 35 mg/l cysteine, 10 mg/l thiamine, 30 g/l sucrose and 8 mg/l benzyladenine, in solid media (8 g/l agar powder extrapure HI-MEDIA) or liquid. The liquid cultures were maintained in a gyratory shaker at 150 rpm, cultures were incubated in the dark at room temperature (28 $\pm$ 2ºC).

DNA isolation. Leaves from the *in vitro* plants were macerated in 2X CTAB (350 µl) and β-mercaptoethanol (1 µl), followed by incubation for 30 minutes at 65ºC, tubes were inverted every 5 minutes. Then the samples were centrifuged for 15 minutes at 14,000 rpm. The liquid phase was transferred to a clean eppendorf tube, then 350 µl chloroform-isoamyl alcohol (24:1) was added, followed by centrifugation for 15 minutes at 14,000 rpm. The aqueous phase was transferred to a clean eppendorf tube and was precipitated with 2.5 volumes of cold 100% ethanol, followed by incubation for 20 minutes at –20ºC. The supernatant was discarded and the pellet resuspended in distilled water.

PCR reaction. The primers used to detect the sequences of *gus* and *bar* genes were the following: GUS1 (*gus*): 5'-GGTGGGAAAGCGCGTTACAAG-3'; GUS2 (*gus*):5'-GTTTACGCGTTGCTTCCGCCA-3'; LK2B (*bar*):5'-CCAGAAAC-CCACGTGATGCC-3'; LK1F (*bar*) 5'-CAGGAACCGGCAGGAGTGGA-3'. The amplification conditions were: 50 ng genomic DNA, 10 µl total mix volume, 1 µl buffer 10X, 1 µl 2.5 mM dntp, 1 µl 25 mM $MgCl_2$, 0.5 µl of primer (100 µM), 0.5 U/µl Taq polymerase (PROMEGA).

Cycling conditions: 95ºC-1 min (1X), 94ºC-30 seconds, 65ºC-30 seconds, 72ºC-1 min (40X), 72ºC-10 min (1X). After amplification the samples were electrophoresed in 1.5% agarose gel.

Results and Discussion

The conditions for the transformation of coffee by electroporation were evaluated by the transient expression of the gene *gus*. *Gus* expression was observed in all the tissues and voltages assayed, except for the leaves without enzymatic pretreatment. *gus* expression in the tissues without enzymatic pretreatment (Figure 1a and Figure 1b) was observed as dark blue very small dots. The tissues with enzymatic treatment (Figure 1c, Figure 1d, Figure 1e and Figure 1f) showed more expression in the form of dots of indigo blue color.

Figure 2 shows that the best *gus* expression was observed in leaf tissues treated with enzymes during 24 hours and electroporated at 625 V/cm. However, the regeneration by secondary somatic embryogenesis was obtained only in the torpedo shape somatic embryos treated enzymatically for 1 hour or without enzymatic treatment, electroporated at 375 and 625 V/cm, and cultured in liquid media (Table 1). The tissues cultured in the liquid media after electroporation were less darkened than the ones cultured in solid media, which is better for their regeneration. When intact cells are electroporated, the level of gene expresión is lower when compared with protoplasts (Lin et al. 1997). This is likely due to the presence of the cell wall, considered as a barrier that limits the free movement of large molecules such as DNA. To improve the DNA incorporation to the cell, one alternative is the application of an enzymatic treatment to remove the pectin present in the cell wall. The pectin inhibits the DNA transference acting as a physical and chemical barrier (Lindsey and Jones, 1990). An improvement of gene expression have been observed when electroporated tissues and cells were treated with cell wall-digesting enzymes. This was the case with zygotic embryos (D'Halluin et al. 1992) and cell suspensions of maize (Laursen et al. 1994), somatic embryos of *Manihot esculenta* (Luong et al. 1995), embryogenic calli of *Ipomoea batatas* (Mitchell et al. 1998) and zygotic proembryos of *Nicotiana tabacum* (Shitao et al. 2000). The results of the present research showed that even though the somatic embryos are composed of tissues which are less compact than those from the leaves from vitroplants, the enzymatic treatment is necessary in both tissues in order to increase the number of points of *gus* expression. It is important to note that the enzymatic treatment stresses the tissues, influencing the liberation of phenols and nucleases (which turn the solution to a brown color), which is negative for the transformation because this can alter the DNA to be transferred. This problem was solved by succesive washes with buffer ASP, after enzymatic digestion of the tissues.

Gus expression in the embryos was higher than in the calli; Shitao et al. 2000 reported that the size, area or surface of the explant influence the efficiency of transformation. Southgate et al. 1998 found higher frequency of transformation (*gus* expression) electroporating friable calli of maize than when they used zygotic embryos. They suggested that embryos with regular surface are susceptible to great damage by the electrical impulse. In our experiments greater *gus* expression was obtained in the embryos than in the embryogenic calli. The callus used for electroporation was smooth, then it could have been severely damaged by the electrical impulse, and that could be the reason of less *gus* expression in the electroporated calli.

Regarding the type of somatic embryos, greater expression was found in the torpedo shape somatic embryos, indicating that this is the best tissue for coffee transformation, also because it has high capability for secondary somatic embryogenesis, favoring the regeneration of transgenic plants. Our results are similar to those obtained by Luong et al. 1995, who electroporated different somatic embryos of cassava, finding higher transient *gus* expression at the torpedo shaped stage.

Gus transient expression was observed only when the tissues were incubated in buffer ASP during 3 hours, with hourly changes. Without this treatment the solution turned brown due to liberation and oxidation of phenols. Release of phenolics is a critical problem that affects genetic transfer in coffee, as was previously observed by Van Boxtel, 1994. Dekeyser et al. 1990 and Pesticelli and Sukhapinda, 1995 point out that incubation of the tissues in the buffer previous to electroporation, favors the diffusion of DNA and its entrance to the cell during the discharge of the electric pulse; in addition Luong et al. 1995 indicate that 2-3 changes of the buffer every hour, permits to eliminate phenols and nucleases that can degrade the DNA.

Incubation of different coffee tissues (with of without enzymatic pretreatment) in buffer with DNA at the temperature of 28ºC was optimal, because it produced greater number of explants and dots per explant with *gus* expression (Figure 2). These results confirm that the temperature of incubation is an important factor in electroporation, as was pointed out by (Salas et al. 2001), who affirms that temperature influences the permeability of the plasmatic membrane, and also the stable integration of the DNA into the genome.

The use of liquid medium with 8 mg/l benzyladenine induced the formation of secondary somatic embryos in the surface of the hypocotilar zone of primary somatic embryos electroporated. The production of secondary somatic embryos was significantly greater than that of primary embryos; this is an advantage for the propagation of products of genetic transformation. Besides, as the secondary somatic embryogenesis occurs directly, chances for genetic variation are lower in the progenies obtained with this method.

The secondary somatic embryos (Figure 3) that developed from the torpedo shaped somatic embryos electroporated were *gus* positive, indicating transient activity in the treated tissues under the previously mentioned conditions. Eight

secondary somatic embryos that were regenerated from the electroporated torpedo somatic embryos were evaluated for the presence of *gus* and *bar* genes by PCR analysis (Figure 4). All of them were *gus* positive and two were *bar* positive. These results indicate that the construction that harbors those genes is present in the secondary somatic embryos regenerated from the electroporated embryos, and the *gus* enzymatic assays gives evidence of its expression.

Concluding Remarks

Our results showed that electroporation is a promising technique for the transformation of intact tissues of coffee. Among the coffee plant material tested the somatic embryos in torpedo stage were the more appropriate material for the electroporation process, they showed the best *gus* transient expression and the best regeneration response through secondary somatic embryo formation. The optimal conditions previous to the electroporation of torpedo shape somatic embryos, were: one hour of enzymatic pretreatment, incubation or the tissues in buffer ASP during three hours, with changes every hour and incubation of the tissues with DNA in buffer ASP at 28ºC (room temperature) during one hour. Electroporation conducted at 900 µF, 375 V/cm, and culture of the electroporated tissues in liquid medium (with 8 mg/l benzyladenine) to induce secondary somatic embryogenesis. The tests showed that the secondary somatic embryos regenerated had *gus* expression and according to the PCR analysis the genes *gus* and *bar* were present in two of the eight embryos assayed.

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APPENDIX

Table

Table 1. Regenerative response of electroporated torpedo somatic embryos.

Media conditions for regeneration	Time of enzymatic treatment (hour)	Voltage (V/cm)	Dark brown %	Explants with white calli %	Explants with formation of secondary somatic embryos %
Solid	1	375	100.0	30.8	0
Solid	1	625	100.0	14.8	0
Solid	1	875	100.0	0	0
Solid	24	375	100.0	7.5	0
Solid	without	375	100.0	27.0	0
Liquid	1	375	77.3	0	43.2
Liquid	1	625	80.0	0	16.0
Liquid	1	875	100.0	0	0
Liquid	24	375	71.4	0	0
Liquid	without	375	100.0	0	30.8

Figures

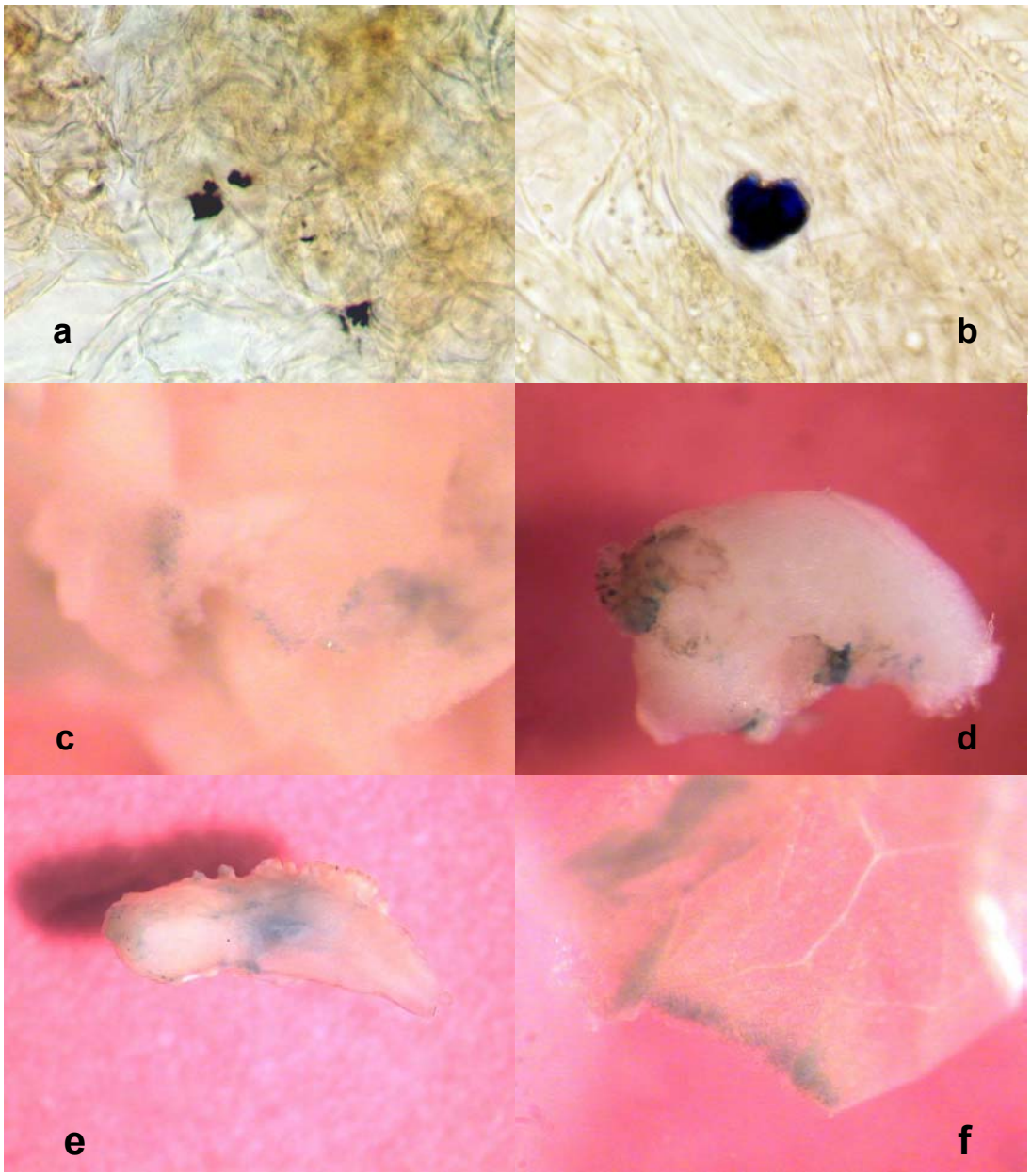
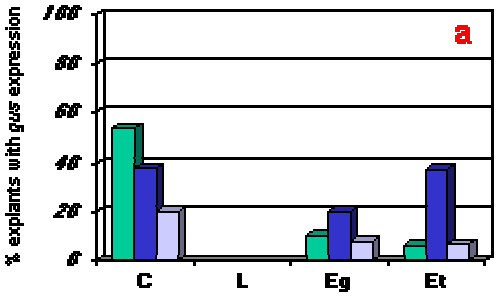


Figure 1. Expression of gen *gus* in electroporated tissues, without (a-b) and with (c-f) enzymatic treatment.

a-b: Cells of embryogenic calli.
c: Embryogenic calli.
d: Globular embryo.
e: Torpedo Shape embryo.
f: Leaf section from a vitroplant.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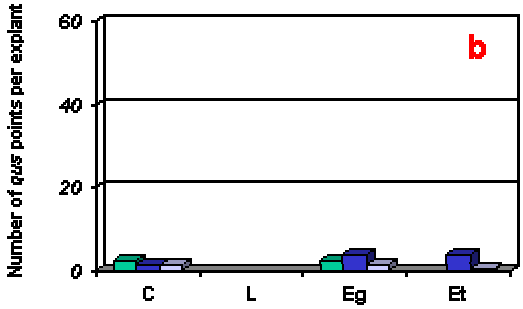
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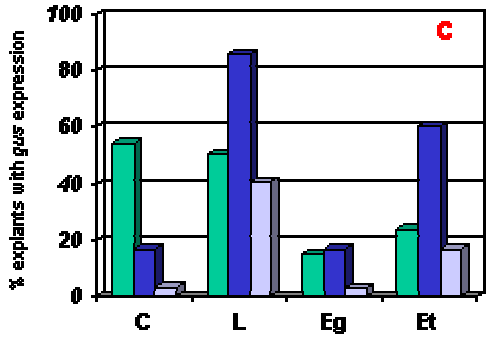
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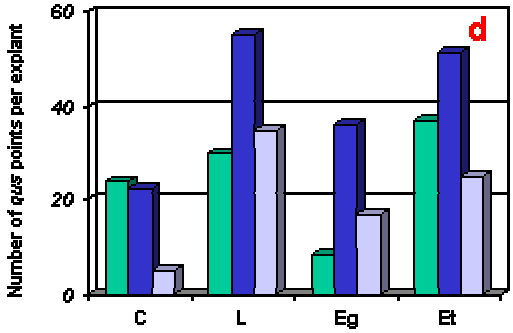
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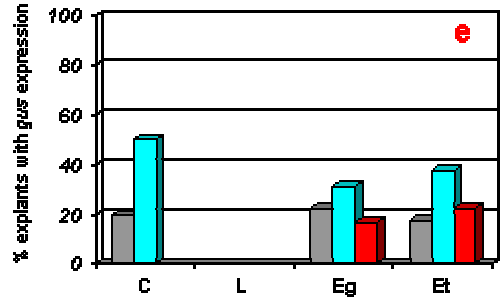
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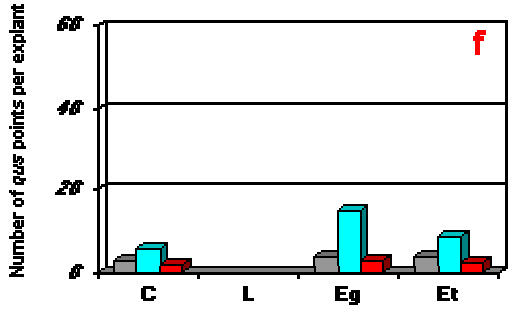
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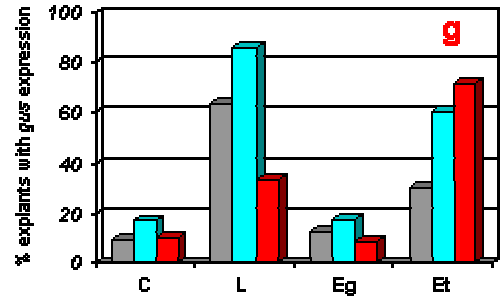
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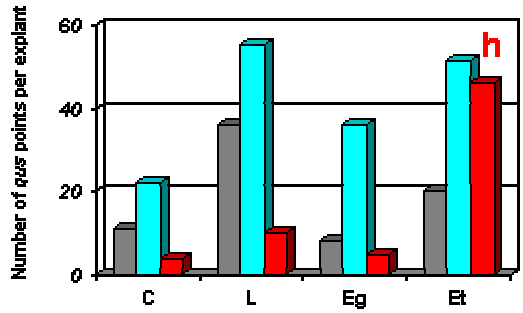
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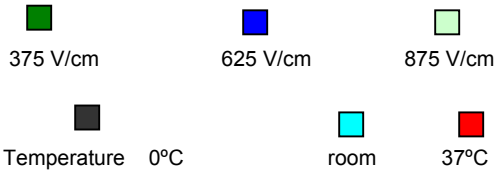
Figure 2.
a-b: Effect of voltage in the electroporation of coffee tissues without enzymatic treatment.
c-d: Effect of voltage in the electroporation of coffee tissues with 24 hours of enzymatic treatment.
e-f: Effect of temperature incubation with DNA, previously to the electroporation, without enzymatic treatment.
g-h: Effect of temperature incubation with DNA, previously to the electroporation, with 24 h enzymatic treatment.
c = calli; L = leaf; Et = torpedo shape somatic embryos; Eg = Globular shape somatic embryos.



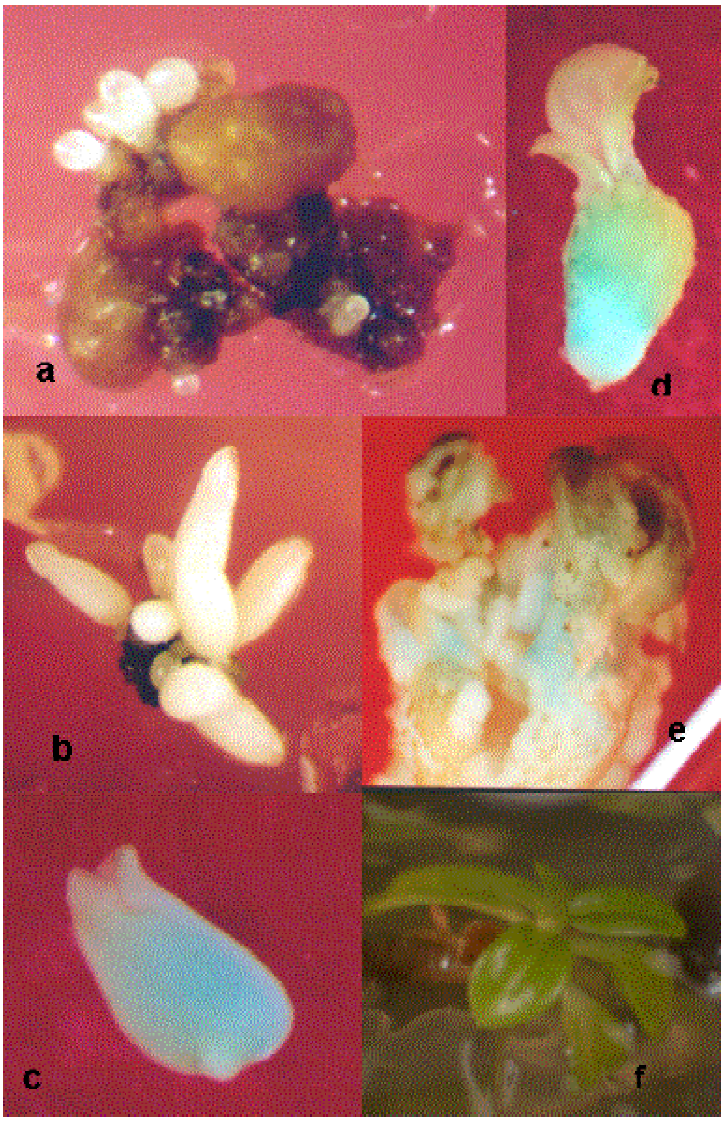


Figure 3. Regeneration by secondary somatic embryogenesis of electroporated torpedo shape embryos.

a: Globular embryos.
b: Group of embryos in different stages.
c-e: *gus* expression in a secondary torpedo shape embryos.
e: is a secondary embryo with tertiary somatic embryos on its surface.
f: Transformed plantlet.

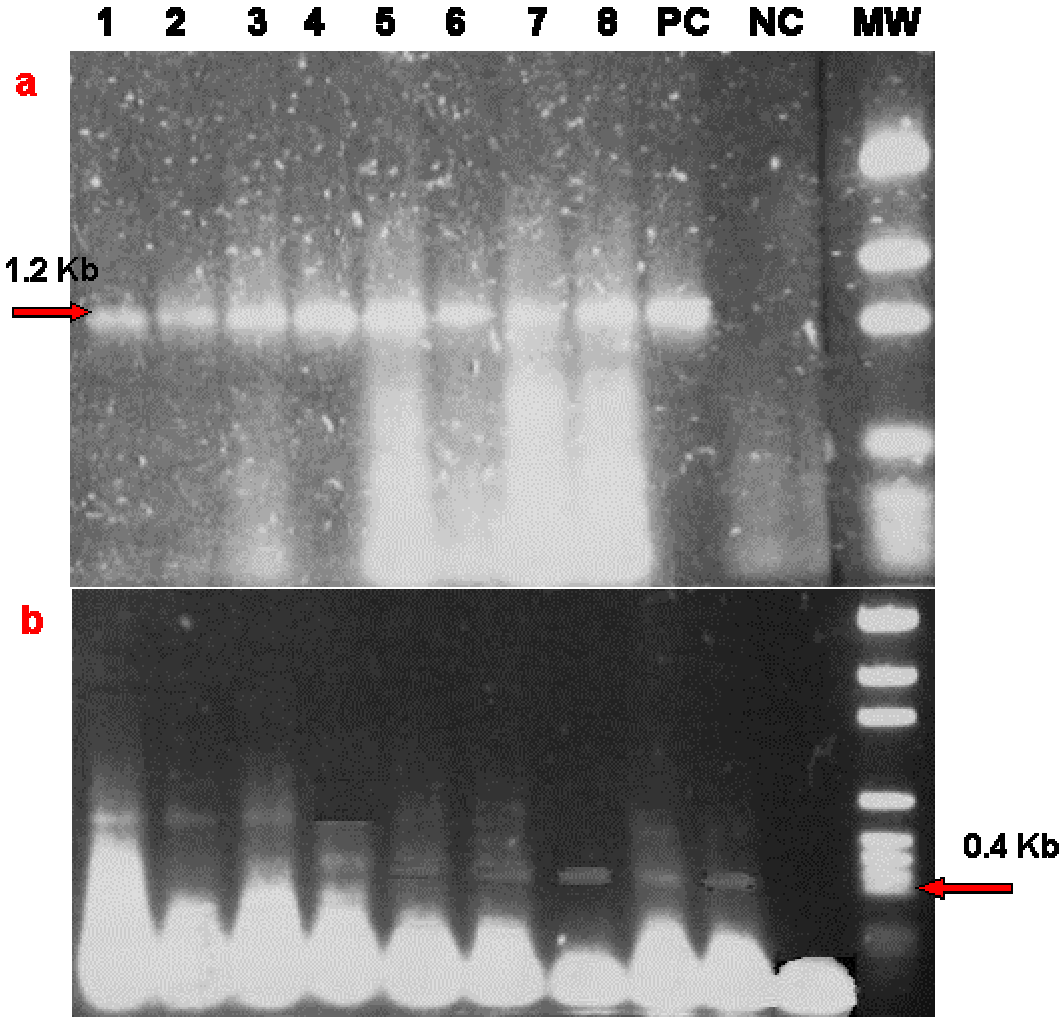


Figure 4. PCR products for the genes *gus* and *bar* in coffee plantlets regenerated by secondary somatic embryogenesis from electroporated torpedo shape embryos.

a: *gus* amplification.
b: *bar* amplification.
1-8 genomic DNA from regenerated coffee.
PC.= Positive Control (pCambia3201); NC = Negative Control (non-transformed coffee vitroplant); MW = Molecular weight standard PGEM.