# Genetic transformation of *Coffea canephora* by particle bombardment

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## Abstract

Stable transformation of *Coffea canephora* P. was obtained by particle bombardment of embryogenic tissue. Leaf explants were cultured on medium supplemented with 5 μM isopentenyl-adenosine to induce direct embryogenesis. Explants with somatic embryos were transferred to half strength MS medium with 9 μM 2,4 dichlorophenoxyacetic acid. After 2 weeks, the explants with somatic embryos and embryogenic tissue were bombarded with tungsten particles (M-25) carrying the plasmid pCambia3301 (containing the *bar* and *uidA* genes) using a high pressure helium microprojectile device. The bombarded explants were submitted to selection on medium containing 5 μM ammonium glufosinate herbicide as selective agent. After 6 months, putative transgenic embryos were transferred to a growth regulator-free medium for germination. The regenerated plantlets were β-glucuronidase (GUS) positive whereas no GUS activity was observed in non-transgenic controls. Incorporation of the *bar* gene into the genome was confirmed by PCR and Southern blot analysis of the regenerated transformed plants. Greenhouse grown transgenic coffee plants were found to withstand the recommended level of the herbicide *Finale*™ for weed control.

*Additional key words*: biolistic, transgenic coffee, ammonium glufosinate.

## Introduction

A traditional coffee breeding program may take more than 30 years (Carneiro 1999). The use of the genetic engineering to introduce new traits in genotypes of coffee can reduce the necessary time for obtaining new cultivars with desirable traits such as disease resistance and improved quality (Spiral *et al.* 1999). Pioneer experiments on genetic transformation of coffee used *Agrobacterium rhizogenes* for *Coffea canephora* (Spiral *et al.* 1993) and *C. arabica* (Sugiyama *et al.* 1995). Subsequently, *A. tumefaciens*-mediated transformation of *C. canephora* (Hatanaka *et al.* 1999, Spiral *et al.* 1999) and *C. arabica* (Spiral *et al.* 1999) were reported.

Direct gene transfer via particle bombardment offers some advantages in relation to transformation using *Agrobacterium* such as: the use of simpler constructions of vectors and less demanding transformation protocols, since the complex plant/bacterium interrelationship is eliminated (Gray and Finer 1993). In coffee, only transient expression of *uidA* gene has been reported using the biolistic method (Van Boxtel *et al.* 1995a, Rosillo *et al.* 2003). Selection of transgenic coffee plants has proven possible with use of chemical selection markers. The benefit of a chemical selection system is the given advantage of transgenic over non-transgenic tissue, facilitating the selection procedure. Chemical selection of transgenic coffee plants has mainly been conducted by means of antibiotics as hygromycin and kanamycin (Hatanaka *et al.* 1999). Genes that confer resistance to herbicides are an alternative to select transformed plants. Leroy *et al.* (2000) obtained transgenic coffee plants resistant to leaf miner using the *csr1-1* gene isolated from *Arabidopsis thaliana* conferring resistance to the herbicide chlorsulfuron. Besides their use as a selectable marker, genes for herbicide resistance offer the possibility to alter the herbicides spectrum used in crops (Pfister *et al.* 1981, Ha *et al.* 2003/04) Ammonium glufosinate is widely used as a non-selective herbicide. The L-isomer of glufosinate is a structural analogue of

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*Abbreviations*: 2iP - $N^6$-(2-isopentenyl)-adenosine; 2,4-D - 2,4 dichlorophenoxyacetic acid; CTAB - cethyltrimethylamonium; GS - glutamine synthetase; GUS - β-glucuronidase; PAT - phosphinothricin acetyltransferase; PVP - polyvinylpyrrolidone; SDS - sodium dodecyl sulfate, SSC - sodium citrate.
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glutamate and, therefore, is a competitive inhibitor of the enzyme glutamine synthetase (GS) of bacteria and plants (Bayer *et al*. 1972). Due to the inhibition of GS, non-tolerant plant cells accumulate large amounts of toxic ammonia produced by nitrate assimilation and photorespiration. In genetically modified glufosinate-tolerant plants, glufosinate is metabolized by the action of the enzyme phosphinothricin acetyltransferase (PAT) into the non-phytotoxic metabolite N-acetyl-L-glufosinate. The ammonium glufosinate showed the best perspectives for the selection of transformed coffee tissue from five selective agents studied by Van Boxtel *et al*. (1995b). Several transgenic plants have been obtained by particle bombardment using the *bar* gene to generated herbicide resistance, such as dry beans (Aragão *et al*. 2002), pineapple (Sripaoraya *et al*. 2001), sugarcane (Falco *et al*. 2000), wheat (Rasco-Gaunt *et al*. 1999), papaya (Cabrera-Ponce *et al*. 1995), rice (Cao *et al*. 1992), among others but not in *Coffea*. In this report, we describe a simple procedure using particle bombardment to introduce the *bar* gene that confers resistance to ammonium glufosinate herbicide into embryogenic calli of *Coffea canephora* with subsequent recovery of transgenic coffee plants.

## Materials and methods

Leaf explants of *Coffea canephora* P. were used to induce somatic embryogenesis. Completely expanded young coffee leaves (8 - 10 cm long) were collected from plants grown in the field. The leaves were pre-washed in running water and 70 % ethanol, and then surface-sterilized in 1 % sodium hypochlorite solution for 30 min followed by three rinses in with sterile distilled water. Explants (1 $cm^2$) were aseptically cut in a 250 mg $dm^{-3}$ cysteine solution. Then the explants were placed with adaxial side in contact with medium in 100 × 20 mm disposable Petri dishes containing 25 $cm^3$ of the embryogenesis medium (Hatanaka *et al*. 1991), supplemented with 30 g $dm^{-3}$ of sucrose, 100 mg $dm^{-3}$ cysteine, 8 g $dm^{-3}$ agar and 5 μM isopentenyl-adenosine (2iP). The pH was adjusted for 5.7 with KOH prior to autoclaving at 120 °C. After 40 d, the explants with somatic embryos were transferred to multiplication medium, consisting of half strength salts and organic constituents of the MS medium (Murashige and Skoog 1962) supplemented with 9 μM 2,4 dichlorophenoxy-acetic acid (2,4 D). During all cultivation period, the explants were maintained in the dark at 27 ± 2 °C. After two weeks, the explants containing somatic embryos and embryogenic tissue were transferred to direct embryogenesis medium containing 0.4 M mannitol. They remained in the medium for 4 h before being particle bombarded.

The plasmid pCambia3301 (Center for the Application of Molecular Biology to International Agriculture. Canberra, Australia) was used for the particle bombardment of embryogenic tissues of *Coffea canephora*. This plasmid contains *uidA* report gene encoding for β-glucuronidase (GUS) and the selectable *bar* gene encoding for the enzyme phosphinotricin acetyltransferase (PAT) in the T-DNA region, both under control of the CaMV35S constitutive promoter.

The tungsten particles - M-25 (*BioRad* Laboratories Inc, Hercules, CA, USA) were prepared by vortexing 5 mg with 0.5 $cm^3$ 70 % ethanol and incubated at room temperature for 15 min. The microparticles were spun in a centrifuge and the supernatant was removed. This proceedure was carried out twice. The microparticles were then re-suspended in glycerol (60 g $dm^{-3}$).

The DNA was precipitated onto the microparticles following the protocol described by Klein *et al.* (1987). The resulting suspension (0.006 $cm^3$) was then coated onto a carrier sheet for bombardment using the *Biolistic® PDS-1000/He Particle Delivery System* (BioRad Laboratories). The helium pressure was set at 1300 psi with a partial vacuum of 686 mm of Hg. Embryogenic tissues were positioned 60 mm from the microprojectile stopping plate. After the bombardment the explants were kept for a further 24 h on 0.4 M mannitol medium. The cultures were then incubated in the dark 27 ± 2 °C in direct embryogenic medium containing 5 μM ammonium glufosinate. Four months after particle bombardment, embryogenic tissues were submitted to histochemical analysis for GUS expression.

Somatic embryos regenerated on selective medium were transferred to growth regulator-free medium to allow germination. The putative transgenic plants developed *in vitro* were transferred to soil covered with a plastic bag, sealed with a rubber band and maintained in a greenhouse for acclimatization.

Transient expression of *uidA* gene was carried out 24 h after biolistic delivery. Explants were incubated in X-Gluc assay buffer (2 mM X-Gluc, 100 mM buffer phosphate (pH 8.0), 10 mM EDTA, 1 mM $K_4Fe(CN)_6$, 1 mM $K_3Fe(CN)_6$ and 20 % methanol, pH 8.0) for 24 h at 37 °C and fixed in a solution containing 10 % acetic acid, 50 % ethanol and 7.4 % formaldehyde. The explants were cleared in 70 % ethanol and observed under stereoscope microscope. Regenerated plants were also screened for *uidA* expression by sampling small segments of leaves from acclimatized greenhouse plants.

PCR analysis was carried out to detect the *bar* gene. DNA was extracted from leaves of *gus*-positive plantlets and non transformed control using the following extraction buffer, 100 mM Tris HCl, 20 mM EDTA, 2 M NaCl, 50 mM CTAB, 2 M PVP, 196 mM spermidine, 285 mM β-mercaptoethanol, 105 mM sodium meta-bissulfite. The primers used for the gene *bar* amplification were: 5' - GTCTGCACCATGGTCAACC-3' and 5'-GAAGTCCAGCTGCCAGAAAC-3'. The PCR

reaction mix was incubated in a thermocycler (model *PTC-100*™, MJ Research, Inc., Watertown, USA) under the following conditions: 94 °C for 4 min, followed by 30 cycles at 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min with 5 min of final extension at 72 °C. The PCR product was analyzed by electrophoresis onto 1 % agarose gel stained with 100 g $dm^{-3}$ ethidium bromide and visualized under UV radiation.

For the Southern blot analysis, genomic DNA from coffee leaves was extracted using the extraction buffer described above. The leaves were frozen in liquid $N_2$ and ground to fine powder using a mortar and pestle. In order to determine the number gene copies integrated into the genome, 20 μg of DNA were digested with the restriction enzyme *Eco*RI in appropriate buffer and separated in 0.8 % agarose gel. After electrophoresis, the gel was treated once for 15 min in 0.2 M HCl, twice for 20 min (500 mM NaOH; 1.5 M NaCl) and twice for 20 min (500 mM Tris; 3 M NaCl). The DNA was transferred by capillarity to a nylon membrane (*Hybond*-N+, *Amersham*™ *Biosciences*, Sao Paulo, Brazil). The probe corresponding to the fragment of the *bar* gene was labeled with $^{32}$P-dCTP using the random primer method according to Sambrook *et al.* (1989). After hybridization overnight at 65 °C, the membranes were washed twice with different concentrations of sodium citrate (SSC) and sodium dodecyl sulfate (SDS) buffer (2X SSC, 0.1 % SDS at room temperature and 1X SSC, 0.1 % SDS at 65 °C) and exposed to *Kodak*™ X-ray film for 48 h.

Transformed and non-transformed plants were sprayed with 1 $cm^3$ $m^{-3}$ of commercial herbicide formulation, containing 2 g $dm^{-3}$ ammonium glufosinate (*Finale*™, *AgrEvo*, Wilmington, USA) at a field rate (60 mg ammonium glufosinate $m^{-2}$ or 0.3 $cm^3$ *Finale*™ $m^{-2}$). Plants were evaluated one week after herbicide spraying.

## Results and discussion

Leaf explants were bombarded approximately 8 weeks after culture, when they had formed callus along the edges of the leaves. Transient expression of *uidA* gene, assayed 24 h after microprojectile bombardment by histochemical assay, was observed in all the explants analyzed (Fig. 1*A*). The largest number of blue spots was observed when the distance traveled by the particles to the target was 60 mm. However, the number of blue spots observed was low (average of 35 per explant).

Selection was imposed immediately after bombardment. The explants were cultivated in direct embryogenesis medium with 5 μM of ammonium glufosinate in the dark in order to select transformed cells. The addition of ammonium glufosinate during regeneration has been essencial to select transformed rice tissue (Visarada and Sarma 2004). After four months on selective medium, putative transformed calli were visually identified on the surface of some bombarded explants. Such calli were observed in 12.5 % of the bombarded explants.

The putative transformed calli were subsequently transferred to direct embryogenesis medium without ammonium glufosinate and histochemically assayed. Small sections of the calli were used for GUS activity assay. The GUS-positive reaction was observed in all tested calli (Fig. 1*B*). Two months after the identification of the transformed calli, the somatic embryos were transferred to direct embryogenesis regulator-free medium for germination. Four somatic embryos from the same callus germinated and regenerated into plants on selective medium. As suggested by Van Boxtel *et al.* (1995b), ammonium glufosinate showed to be effective for the selection of transformed embryogenic tissue of *C. canephora*.

Leaf segments of transformed plantlets were also histochemically assayed to detect *uidA* gene expression. The deep blue colour on the cutting edges of leaves demonstrated stable expression of *uidA* gene whereas control plantlets (non transformed) did not show any GUS activity (Fig. 1*C*). Previous works suggest that endogenous GUS activity could be observed in coffee tissues (Spiral *et al*. 1999, Hatanaka *et al*. 1999, Van Boxtel *et al*. 1995a). Such activity was not observed in this work neither for the transient assay using calli nor for the stable assay using leaves. Probably, the use of methanol in X-Gluc solution for histochemical analysis avoided endogenous GUS activity (Kosugi *et al*. 1990).

The presence and the integration of the *bar* gene into the genome were confirmed by PCR and Southern blot analysis, respectively. The PCR analysis confirmed the presence of *bar* genes in coffee plants showing an amplified fragment of 450 bp. No amplification was detected on non-transformed plantlets (Fig. 2). In the Southern blot analysis the genomic DNA digested with enzyme *Eco*R I which, cuts pCambia3301 once, but not in the transgene, generated at least five fragments which hybridized to the *bar* probe. Several bands with different molecular mass were detected, indicating that either multiple independent insertions occurred or rearrangements had occurred in a concatemeric chains (Fig. 3). Transgenic plants produced by biolistic delivery commonly show concatemeric inserts (Cabrera-Ponce *et al*. 1995, Bower *et al*. 1996).

Functional expression of the *bar* gene was verified by localized application of herbicide on transgenic and control plants. Leaves of transgenic plants and non-transformed controls planted in a greenhouse were sprayed with 1 % commercial solution of herbicide (*Finale*™) and scored for herbicide damage. In control plants, herbicide application resulted in necrosis of the sprayed area after 48 h or in some cases death of the entire plant one week later. In contrast, transformed plants were completely resistant to herbicide spraying.

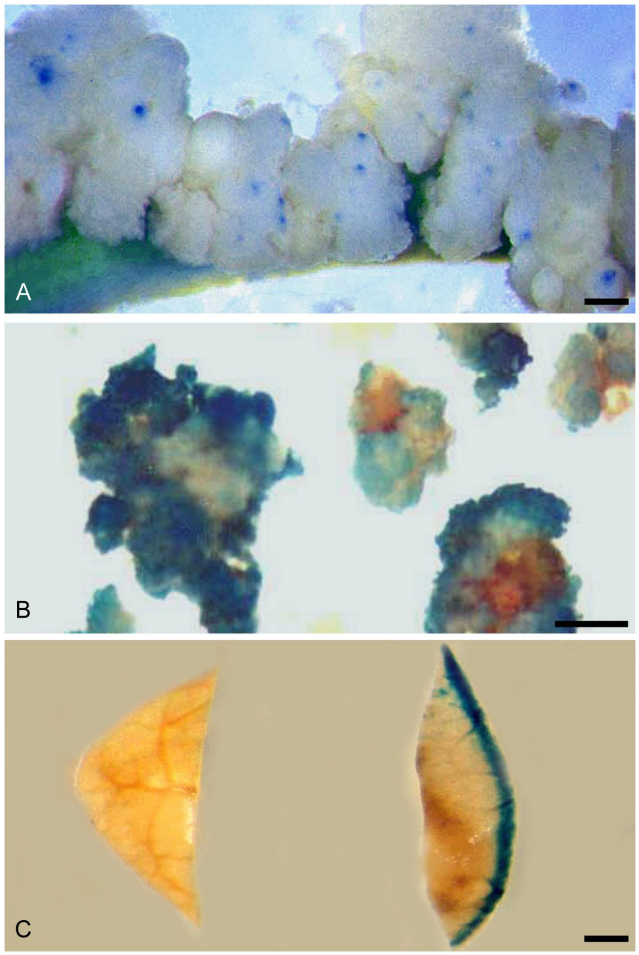


Fig. 1. Histochemical GUS assay on *Coffea canephora*. *A* - Transient expression of *uidA* gene on calli 24 h after biolistic delivery. *B* - Stable expression of *uidA* gene on calli after 5 months growing on selective medium with 5 μM ammonium glufosinate. *C* - Stable expression in leaf segments (right: transformed plant, left: non transformed plant). *Scale bar* on each picture represents 1 mm.

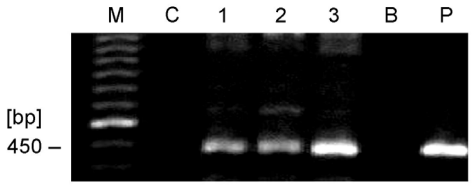


Fig. 2. PCR analysis of *Coffea canephora* plants transformed with the *bar* gene. *M* - Molecular marker 100 bp ladder; *C* - non-transformed plant; *1-3* - transformed plants; *B* - blank without DNA; *P* - pCambia3301 plasmid. *Arrow*: expected 450 bp fragment of the *bar* gene.

Reliable selectable marker is essential for the recovery of transgenic plants at the present time. A screenable selectable marker linked to an agronomic gene can make the identification of transformed plant efficient and fast. The data presented here indicate that the *bar* gene is suitable for this purpose and that herbicide screening can be used to successfully identify transgenic coffee plants.

For practical use in breeding programs, a reduced

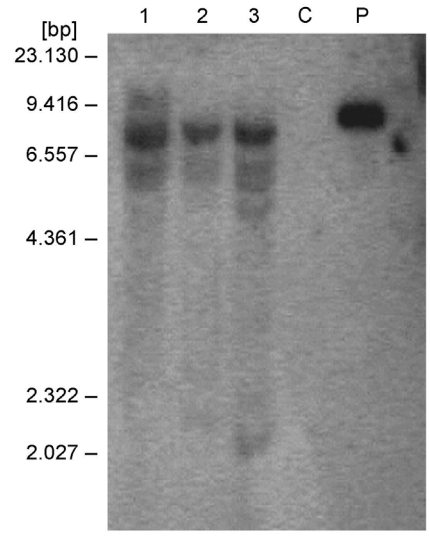


Fig. 3. Southern blot analysis of putatively transformed coffee plants. 20 μg DNA was hybridized with the *bar* probe. *Lane 1-3* - ammonium glufosinate-resistant plants. *Lane C* - control plant (non transformed). *Lane P* - pCambia3301 plasmid.

time for producing transgenic plants is required. The first transgenic coffee plants were obtained via *Agrobacterium*-mediated transformation selected on hygromycin and kanamycin (Hatanaka *et al*. 1999). The methodology described here is technically simple and time-saving. It avoids time consuming tissue culture protocols and yields transgenic plants in 40 weeks, with up to 12.5 % transformation efficiency. To our knowledge, this is the first work to successfully regenerate transgenic coffee plants using the biolistic transformation system, as well as to use the *bar* gene as selectable marker for coffee transformation.

## References


Aragão, F.J.L., Vianna, G.R., Albino, M.M.C., Rech, E.L.: Transgenic dry bean tolerant to the herbicide glufosinate ammonium. - Crop Sci. **43**: 1298-1302, 2002.

Bayer, E., Gugel, K.H., Haegele, K., Hagenmaier, H., Jessipow, S., Koenig, W.A., Zaehner, H.: Phosphinothricin and phosphinothricyl-alanyl-alanin. - Helv. chim. Acta. **55**: 224-239, 1972.

Bower, R., Elliot, A.R., Pottier, B.A.M., Birch, R.G.: High-efficiency, microprojectile-mediated co-transformation of sugarcane, using visible or selectable markers. - Mol. Breed. **2**: 239-249, 1996.

Cabrera-Ponce, J.L., Vegas-Garcia, A., Herrera-Estrella, L.: Herbicide resistant transgenic papaya plants produced by an efficient particle bombardment transformation method. - Plant Cell Rep. **15**: 1-7, 1995.

Cao, J., Duan, X., McElroy, D., Wu, R.: Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. - Plant Cell Rep. **11**: 586-591, 1992.

Carneiro, M.F.: Advances in coffee biotechnology. - AgBiotechNet **1**: 1-7, 1999.

Falco, M.C., Tulmann Neto, A., Ulian, E.C.: Transformation and expression of a gene for herbicide resistance in Brazilian sugarcane. - Plant Cell Rep. **19**: 1188-1194, 2000.

Gray, D.J., Finer, J.J.: Development and operation of five particle guns for introduction of DNA into plant cells. - Plant Cell Tissue Organ Cult. **33**: 219, 1993.

Ha, S.B., Lee, S.B., Lee, D.E., Guh, J.O., Back, K.: Transgenic rice plants expressing *Bacillus subtilis* protoporphyrinogen oxidase gene show herbicide oxyfluorfen resistence. - Biol. Plant. **47**: 277-280, 2003/4.

Hatanaka, J., Arakawa, O., Yasuda, T., Ushida, N., Yamaguchi, I.: Effect of plant growth regulators on somatic embryogenesis in leaf cultures of *Coffea canephora*. - Plant Cell Rep. **10**: 179-182, 1991.

Hatanaka, T., Choi, Y.E., Kusano, T., Sano, H.: Transgenic plants of *Coffea canephora* from embryogenic callus via *Agrobacterium tumefaciens*-mediated transformation. - Plant Cell Rep. **19**: 106-110, 1999.

Klein, T.M., Wolf, E.D., Wu, R., Sanford, J.C.: High-velocity microprojectiles for delivering nucleic acids into living cells. - Nature **327**: 70-73, 1987.

Kosugi, S., Ohashi, Y., Nakajima, K., Arai, Y.: An improved assay for β-glucuronidase in transformed cells: methanol almost completely supresses a putative endogenous β-glucuronidase activity. - Plant Sci. **70**: 133-140, 1990.

Leroy, T., Henry, A. M., Royer, M., Altosar, I., Frutos, R., Duris, D., Philippe, R.: Genetically modified coffee plants expressing the *Bacillus thuringiensis cry*1Ac gene for resistance to leaf miner. - Plant Cell Rep. **19**: 382-389, 2000.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bio-assays with tobacco tissue culture. - Plant Physiol. **15**: 473-497, 1962.

Rasco-Gaunt, S., Riley, A., Lazzeri, P., Barcelo, P.: A facile method for screening for phosphinothricin (PPT)-resistant transgenic wheats. - Mol. Breed. **5**: 255-262, 1999.

Pfister, K., Steinback, K.E., Gardner, E., Arntzen, C.J.: Photoaffinity-labeling of an herbicide receptor protein in chloroplast membranes. - Proc. nat. Acad. Sci. USA. **78**: 981-985, 1981.

Rosillo, A. G., Acuna, J.R., Gaitan, A.L., Pena, M.: Optimised DNA delivery into *Coffea arabica* suspension culture cells by particle bombardment. - Plant Cell Tissue Organ Cult. **74**: 45-49, 2003.

Sambrook, J., Fritsch, E.F., Maniatis, T. (ed.): Molecular Cloning: a Laboratory Manual. - Cold Spring Harbour Laboratory Press, New York 1989.

Spiral, J., Leroy, T., Paillard, M., Petiard, V.: Transgenic coffee (*Coffea* sp.). - In: Bajaj, Y.P.S. (ed.): Transgenic Trees. (Biotechnology in Agriculture and Forestry. Vol. 44). Pp. 55-76. Spring-Verlag, Berlim - Heidelberg 1999.

Spiral, J., Thierry, C., Paillard, M., Petiard, V.: Obtention de plantules de *Coffea canephora* Pierre (Robusta) transformées par *Agrobacterium rhizogenes.* - Compt. rend. Acad. Sci. Paris **316**: 1-6. 1993.

Sripaoraya, S., Marchant, R., Power, J.B., Davey, M.R.: Herbicide-tolerant transgenic pineapple (*Ananas comosus*) produced by microprojectile bombardment. - Ann. Bot. **88**: 597-603, 2001.

Sugiyama, M., Matsuoka, C., Takagi, T.: Transformation of *Coffea* with *Agrobacterium rhizogenes*. - In: ASIC, 16ème Colloque Scientifique sur le Café. Pp. 853-859. Paris 1995.

Van Boxtel, J., Berthouly, M., Carasco, C., Dufuor, M., Eskes, A.: Transient expresssion of β-glucoronidase following biolistic delivery of foreign DNA into coffee tissue. - Plant Cell Rep. **14**: 748-752, 1995a.

Van Boxtel, J., Eskes, A., Berthouly, M.: Glufosinate as an efficient inhibitor of callus proliferation in coffee tissue. - *In Vitro* cell. dev. Biol. Plant **33**: 6-12, 1995b.

Visarada, K.B.R.S., Sarma, N.P.: Transformation of indica rice throught particle bombardment: factors influencing transient expression and selection. - Biol. Plant. **48**: 25-31, 2004.