GENETIC TRANSFORMATION OF SUGARCANE BY Agrobacterium tumefaciens USING ANTIOXIDANT COMPOUNDS

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ABSTRACT

The effects of 3 antioxidant compounds -ascorbic acid, cysteine and silver nitrate- on the growth of the *Agrobacterium tumefaciens* strain At 2260 and its interaction with plant cells, as well as on the viability and callus formation of meristematic tissue, were evaluated using explants from *in vitro* and field-cultured sugarcane plants commercial variety Ja 60-5. We achieved the transfer of a binary vector bearing the *uid1* and *bar* genes to sugarcane meristematic explants, and obtained the generation of BASTA®-resistant and GUS-positive calli. The use of an antioxidant mix caused an 80 % cell death decrease in respect to controls, and the callus quality was not affected in any of the culture phases.

Key words: transgenic plants, plant-microorganism interaction, hypersensitivity, necrogenesis, BASTA®, GUS

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RESUMEN

Se evaluaron los efectos de tres compuestos antioxidantes - ácido ascórbico, cisteína y nitrato de plata - sobre el crecimiento de *Agrobacterium tumefaciens* cepa At 2260 y la interacción de éste con los explantes vegetales, así como sobre la viabilidad y la formación de callos en tejidos meristemáticos de caña de azúcar variedad comercial Ja 60-5, provenientes de plantas *in vitro* y de campo. Se logró la transferencia de un vector binario con los genes *uid1* y *bar* a explantes meristemáticos de caña de azúcar y se obtuvieron callos resistentes a BASTA® y GUS-positivos. El empleo de la mezcla de antioxidantes redujo en un 80 % la muerte celular con relación al control, y no alteró la calidad del callo en ninguna de las fases de cultivo.

Palabras claves: plantas transgénicas, interacción planta-microorganismo, hipersensibilidad, necrogénesis, BASTA®, GUS

Introduction

Sugarcane (*Saccharum officinarum* L.) is one of the most extended crops in tropical and subtropical zones. The sugar industry and the production of certain chemicals such as furfural, dextranes and alcohol depend on this plant. By-products derived directly from sugarcane and its industrial processing represent a valuable alternative source for animal feeding and the cellulose industry. The application of biotechnology to sugarcane studies has a significant impact on agricultural yields and industrial production. During recent years, plant tissue culture, molecular biology and plant transformation have been developed (1). Recently, the first transgenic sugarcane plants resistant to stemborer (*Diatraea saccharalis*) were reported; revealing the possibilities of genetic engineering of this crop and offering new breeding possibilities (2).

The DNA transfer to sugarcane cells has been a drawback in the genetic manipulation of these plant species. The plant transformation methodologies based on the naturally occurring *Agrobacterium tumefaciens* gene transfer system allowed many important crops to be genetically engineered. However, at first, they were mostly dicotyledonous plants due to the apparent stringency of the *A. tumefaciens* host range. Because of this, many important monocotyledoneous plants such as rice, wheat and sugarcane remained inaccessible to genetic engineering for a long time. For those cases, alternative direct transformation methods have been developed. In sugarcane, stable transformation by intact cell electroporation (3) and particle bombardment technology (4) were reported.

The development of efficient and reliable methods for the *Agrobacterium*-mediated gene transfer to monocotyledonous plants has represented the most important breakthrough of the last years in plant transformation technology. This approach has enabled the generation of transgenic plants in crops previously inaccessible for *Agrobacterium*-mediated transformation such as rice (5), maize (6) and banana (7).

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In this paper we report the evidences of *Agrobacterium*-mediated transfer of foreign DNA to sugarcane meristematic explants of the cv Ja 60-5. The conditions for precocultivation, cocultivation, callogenesis and selection of BASTA®-resistant calli are described. The effects of several antioxidants on the sugarcane-*Agrobacterium* interaction were evaluated. The presence of the heterologous DNA in the transformed sugarcane tissue was verified by polymerase chain reaction (PCR) and histochemical GUS assay.

Materials and Methods

Plant material

An aseptic culture from sugarcane cv Ja 60-5 was established according to Ho *et al.* (8) in an MS basal medium (9). The genetic transformation assays were performed with meristematic sections of sugarcane plant spindlers. The plant material was taken directly from the field and was sterilized in 1.5 % NaClO solution during 20 min. Table 1 shows the compositions of all the culture media used in this study.

Bacterial strains and plasmids

The *A. tumefaciens* C58C1Rifr: PGV2260 (At 2260) strain quoted by de la Riva *et al.* (10) was used for the establishment of the transformation methodology. The plasmid pGT GUSBAR containing the marker genes *uid*1 and *bar* under appropriate regulation signals for their expression in monocotyledoneous plants was constructed as follows: A 2.8 kb DNA fragment carrying the first non-coding exon-intron-exon sequence from the rice actin 1 gene, linked to the *bar* gene under the rice ubiquitin promoter regulation was obtained by *Eco*RI partial digestion of the plasmid pAHC-25 (11). This fragment was inserted in the *Eco*RI site of pBPFA5 (Coego A., personal communication). The resulting plasmid (pBPFA-GUSBAR), contained an expression cassette for the *uid*1 reporter gene (GUS activity), and the *bar* gene conferring resistance to the herbicide BASTA® (Hoechst AG, Germany) fused to the first exon-intron-exon sequence from rice Actin 1 gene downstream of the CaMV 35S promoter. The described 6.1 kb expression cassette was separated by *Hin*dIII digestion from pBPFA-GUSBAR and transferred to the binary vector pGTDNA, a pDE1001 derivative in which the neomycin-resistance cassette was deleted. The final construct, named pGT GUSBAR (Figure 1), was inserted in *A. tumefaciens* by direct transformation (12).

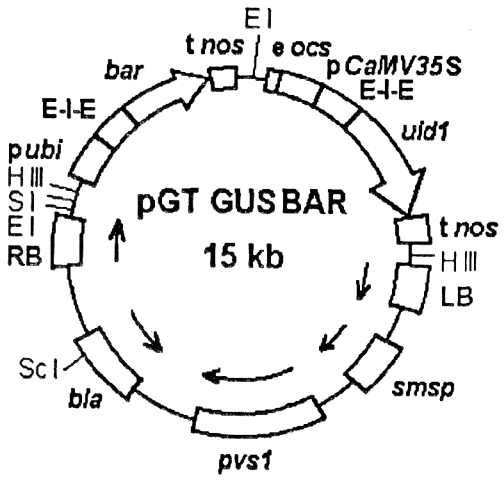

Figure 1. Schematic map of the plasmid pGT GUSBAR showing the reporter (*uid*1) and selection (*bar*) genes and the T -borders (RB: right border and LB: left border). *pubi*: promoter region of the maize ubiquitine gen; E-I-E: non coding exon-intron-exon regions; *tnos*: A. *tumefaciens* nopaline-synthase terminator; *eocs*: synthetic A. *tumefaciens* octopine-synthase enhancer; *pCaMV35S*: chimeric promoter derived from the promoter region of the gene encoding the 35S polyprotein of the Cauliflower Mosaic Virus; *bla*: ampicillin-resistance gene; *pvs*1: A. *tumefaciens* replication origin; *smsp*: streptomycin and spectinomycin resistance gene, and restriction sites: E I: *Eco*RI; H III: *Hin*dIII; S I: *Sac*I. and Sc I: *Sca*I.

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Table 1. Composition of the culture media for *Agrobacterium*-mediated genetic transformation of sugarcane cv Ja 60-5.

Culture medium	Composition
P+5	MS Salts, 1 mg/L nicotinic acid, 0.8 mg/L vitamin B_1, 0.5 mg/L vitamin B_6, 100 mg/L myo-inositol, 20 g/L sucrose, 500 mg/L casein hydrolysate and 5 mg/L 2,4 D.
P+5 AO	P+5 supplemented with 15 mg/L ascorbic acid, 40 mg/L cysteine and 2 mg/L silver nitrate.
P+5 AZ	P+5 supplemented with 60 g/L sucrose and 30 g/L glucose.
P+5 SEL	MS Salts, 1 mg/L nicotinic acid, 0.8 mg/L vitamin B_1, 0.5 mg/L vitamin B_6, 100 mg/L myo-inositol, 20 g/L sucrose, 5 mg/L 2.4 D, 500 mg/L claforan and 4 mg/L BASTA®.
P-	MS Salts, 1 mg/L nicotinic acid, 0.8 mg/L vitamin B_1, 0.5 mg/L vitamin B_6, 100 mg/L myo-inositol and 20 g/L sucrose.
P - AO	P- supplemented with 15 mg/L ascorbic acid, 40 mg/L cysteine and 2 mg/L silver nitrate.

Table 2. Effects of antioxidant treatments on the *A. tumefaciens* growth, the meristematic tissue viability and the quality of the obtained calli.

Treatments	Antioxidant compounds[1]	Bacterial OD_{620}[2]	% explant viability[3]	Callus quality[4]
AAS1	15 mg/L ascorbic acid	0.847	50	Type II
AAS2	30 mg/L ascorbic acid	0.803	50	Type III
CIS1	40 mg/L cysteine	0.570	60	Type II
CIS2	90 mg/L cysteine	0.598	80	Type III
Ag1	2 mg/L silver nitrate	0.490	70	Type II
Ag2	5 mg/L silver nitrate	0.010	60	Type III
P+5[5]	none	0.600	10	Type II
AO	15 mg/L ascorbic acid; 40 mg/L cysteine; 2 mg/L silver nitrate	0.512	90	Type II

[1] Antioxidant compounds supplementing the liquid P+5 medium.
[2] Average of 3 independent determinations of OD_{620} in YEB *A. tumefaciens* cultures grown at 28 °C for 20 h.
[3] Viability percentages represented as the averages from 10 independent stereoscopic determinations of the fraction of explant area remaining unstained after Evans Blue stain *vs.* total explant area.
[4] Callus qualities were assessed visually according to Ho *et al.* (8), and classified as follows:
Type II. Friable yellow calli, with high capacity of embryogenesis.
Type III. Soft, opaque, non-embryogenic white calli.
[5] P+5 medium without antioxidants used as negative control.

Evaluation of the effects of antioxidants on *Agrobacterium* growth

The effects of antioxidant (AO) compounds on the growth of *A. tumefaciens* At 2260 were assessed by measuring the optical densities of the bacterial cultures at 260 nm after 24 h of cultivation in a YEB medium (10) supplemented with these compounds (Table 2).

Evaluation of the effects of antioxidants on explant viability and callogenesis

The sugarcane meristematic explants (ca. 0.5 cm) were incubated in a P+5 medium (8), supplemented with the AO compounds for 60 h in the dark. The 8 treatments are described in Table 2. After exposure to the antioxidants, cell viability in the meristematic sections was evaluated using the Evans Blue method (13). Percentages of cell viability were determined as rates of unstained *vs* stained areas by direct stereoscopic observation of each treated ex-plant. Averages were calculated from 10 independent repetitions per treatment. The callogenic capacities of the antioxidant-treated explants were assessed in a solid P+5 medium and the generated calli were classified according to Ho *et al.* (8).

Genetic transformation

A. tumefaciens At 2260 containing the binary plasmid pGT GUSBAR, was grown in a liquid medium supplemented with ampicillin 100 mg/L, spectinomycin, streptomycin 100 mg/L, rifampicin 50 mg/L and carbenicillin 50 mg/L. When optical density (OD) at 620 raised to 0.6, the cells were collected by centrifugation and resuspended in the same volume P+5 medium supplemented with antioxidants (P+5-AO). The same operations were carried out in other sample series adding 20 mg/L of acetosiringone to the final concentration.

The effect of AO mixtures on the transfer of foreign genes from *A. tumefaciens* to sugarcane cells under 8 different treatments was evaluated (Table 3). The coculture was performed with explants from *in vitro* cultured plants and from field plants. After the explants were incubated in 10 mL of the P+5 or P+5-AO medium for 6-12 h in the dark, 1 mL of the *A. tumefaciens* culture was added and they were in-

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Table 3. Effects of antioxidant compounds on the *Agrobacterium*-sugarcane gene transfer.

Treatments[1]	Precoculture medium	Coculture medium	% GUS positive explants[2]	% BASTA®-resistant calli[3]	Analysis by PCR[4]
IN 1	P-AO	P+5 AO	100	15.5 (55)	+
IN 2	P-	P+5 AO	70	8.8 (55)	+
IN 3	P-AO	P+5	80	6.6 (55)	+
IN 4	P-	P+5	20	0 (55)	na
PC 1	P- AO	P+5 AO	100	26.5 (25)	+
PC 2	P-	P+5 AO	40	0 (25)	na
PC 3	P-AO	P+5	80	13.25 (25)	+
PC 4	P-	P+5	0	0 (25)	na

[1] IN: explants from *in vitro* cultured sugarcane plants; PC: explants field-grown sugarcane plants.
[2] GUS activity was tested 2 days after co-cultivation, using a standard histochemical procedure for 10 samples randomly selected from each treatment.
[3] Total number of *Agrobacterium* infected explants appears between parentheses.
[4] PCR analysis performed with genomic DNA extracted from one callus per treatment; na: samples not available.

cubated for 10 min with strong agitation. The explants were then placed on filter paper and for cocultivation on a solid P+5 or P+5-AO medium for 3 days.

Callus formation and selection

After cocultivation, the explants were washed with sterile water, wipe dried and transferred to selective medium (P+5 SEL) where they remained for 5 weeks. The selective medium was replaced by a fresh one every 3 weeks.

Histochemical GUS assay

The BASTA®-resistant calli were analyzed by the histochemical GUS assay. The staining procedure was performed according to Jefferson (14) with the modification recommended by Hiei *et al.* (5). The 5-bromo-4-chloro-3-indolyl ß-D glucoronide (x-Gluc, Sigma, Chemical Co. USA) was used as the chromogenic agent.

PCR analysis

The presence of foreign DNA in the BASTA®-resistant calli was determined by PCR. The extraction of total DNA and the amplification reactions with the synthetic primers gus A +350 (5' GCC ATT TGA AGC CGA TGT CAC GCC 3') and gus A +1400 (5' GTA TCG GTG TGA GCG TCG CAG AAC 3') were done as reported previously (15).The PCR products were analyzed by Southern blot using a 1.8 Kb DNA fragment containing the gene *uid*1 as a probe (16).

Results

Effect of different antioxidant compounds on necrogenesis of sugarcane meristems

To obtain sugarcane meristematic explants with low rates of necrogenesis in the cut surface zones and highly competent to transformation by *A. tumefaciens*, the effects of 3 AO compounds (ascorbic acid, cysteine and silver nitrate) were assessed during the precocultivation stage (Table 2). The fraction (percentage) of meristematic section areas remaining unstained by the Evans Blue reagent was taken as a cell viability criterion. Each compound was individually tested at 2 different concentrations in the liquid P+5 media. In all cases, a significant decrease in necrogenesis (less than 50 % of the explant areas) was observed after 60 h of incubation (Table 2). Although the hypersensitivity in each assayed treatment decreased, the best results were obtained when an AO mix was used. In this case, the meristematic explant necrosis was inhibited up to 90 % during the AO treatment. This result shows that the synergistic effects on the hypersensitive response propagation through the meristematic tissue among the AO agents used may exist. However, more detailed studies are needed to characterize the phenomena observed.

After the pre-incubation step, the explants were placed in the dark for 5 weeks. The morpho-physiological features of the formed calli were different in each case. For treatments AAS1, Cis 1, Ag 1 and AO, yellow and friable calli with high regeneration capacity (type II) similar to those obtained from the control (P+5) explants, were formed (Table 2). In all cases when higher levels of AO compounds were used, the calli resulted opaque and soft, with low regeneration capacity (type III). The negative action of silver nitrate on callus formation in potato cv Bintje and Désirée when the quality of the callus and its regeneration capacity were affected at a concentration of 10 mg/L, was described (17). In all the experiments concerning the effects of AO compounds on sugarcane callogenesis, there were no differences between the calli obtained from *in vitro* and field-grown sugarcane explants. All the treatments affected bacterial growth when compared with the controls, with the exception of Ag 2 which showed a negative effect.

Selective agent

To our knowledge, there were no previous reports on chemical selection of genetically transformed cells from the sugarcane cv Ja 60-5. The use of selective agents in transformation procedures is essential to avoid chimeric plants. For this reason we decided to determine the effectiveness of the herbicide BASTA® -a compound reported to be cytotoxic in monocotyledonous plants- for the selection of transformed sugarcane calli from cv Ja 60-5. The meristems from *in vitro* cultured plants were maintained in the callus formation medium P+5, supplemented with BASTA® at concentrations of 0, 1, 2, 3, 4 and 5 mg/L. After 2 weeks, the fraction of proliferated calli was determined. We observed that the meristematic tissues were sensitive to concentrations of 4 mg/L and higher (data not shown).

Effect of antioxidants on plant tissue *Agrobacterium* interaction

The effect of AO compounds on the interaction of *A. tumefaciens* with sugarcane meristematic tissues was studied using the *A. tumefaciens* At 2260 strain transformed with the binary plasmid pGT GUSBAR. This plasmid contains the genes *uid1* and *bar* under the posttranscriptional regulation signals for monocotyledonous plants, which include introns. This fact made impossible the expression of marker genes in the bacterial host as was previously reported (18). We observed no blue staining when pGT GUSBAR-transformed *A. tumefaciens* samples were submitted to a histochemical assay similar to

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the one used to evaluate the GUS activity in sugarcane explants (data not shown).

The AO treatment was selected to study the effect of AO compounds on gene transfer. The fractions of GUS positive and BASTA®-resistant calli were taken as criteria for transformation efficiency using different treatments, assayed with explants from *in vitro* culture (IN) and from the field (PC) (Table 3).

In all cases when the culture media were supplemented with antioxidants (IN1, PC1) during the pre and cocultivation, 100 % of infected explants became GUS positive (Figure 2A). In contrast, the infection efficiency was remarkably lower when antioxidants were not included. No GUS activity was found in infected controls.

The efficiency of callus formation in the infected explants cultivated on the P+5 selective medium AO was relatively high (5). The meristematic sections submitted to the IN1 and PC1 treatments showed a callus formation rate of 15 % and 25 %, respectively. In the rest of the variants the callus formation rate was much lower (0-13 %). Non-infected meristems did not proliferate at all in the selective medium. The presence of foreign DNA in BASTA®-resistant calli (Figure 2B) was confirmed by PCR (Figure 3) and histochemical GUS activity assay.

The addition of 20 mg/L of acetosiringone during bacterial growth provoked the early death of most meristematic tissues irrespective of the explant origin and the treatments applied. These results are in apparent contradiction with those reported by Hiei *et al.* (5), who supported that the use of acetosiringone and high sugar concentration promote a higher efficiency in the *A. tumefaciens*-mediated transformation of rice.

Discussion

The inclusion of AO compounds in the culture media decreases the cell death rate in the explants by inhibiting the hypersensitive reactions developed as a response to the damage generated during the manipulation of the tissue (19). In this study we demonstrated that 3 AO compounds -ascorbic acid, cysteine and silver nitrate- can decrease the hypersensitivity reaction on the cut zone in the sugarcane meristematic explants. The decrease of cell death

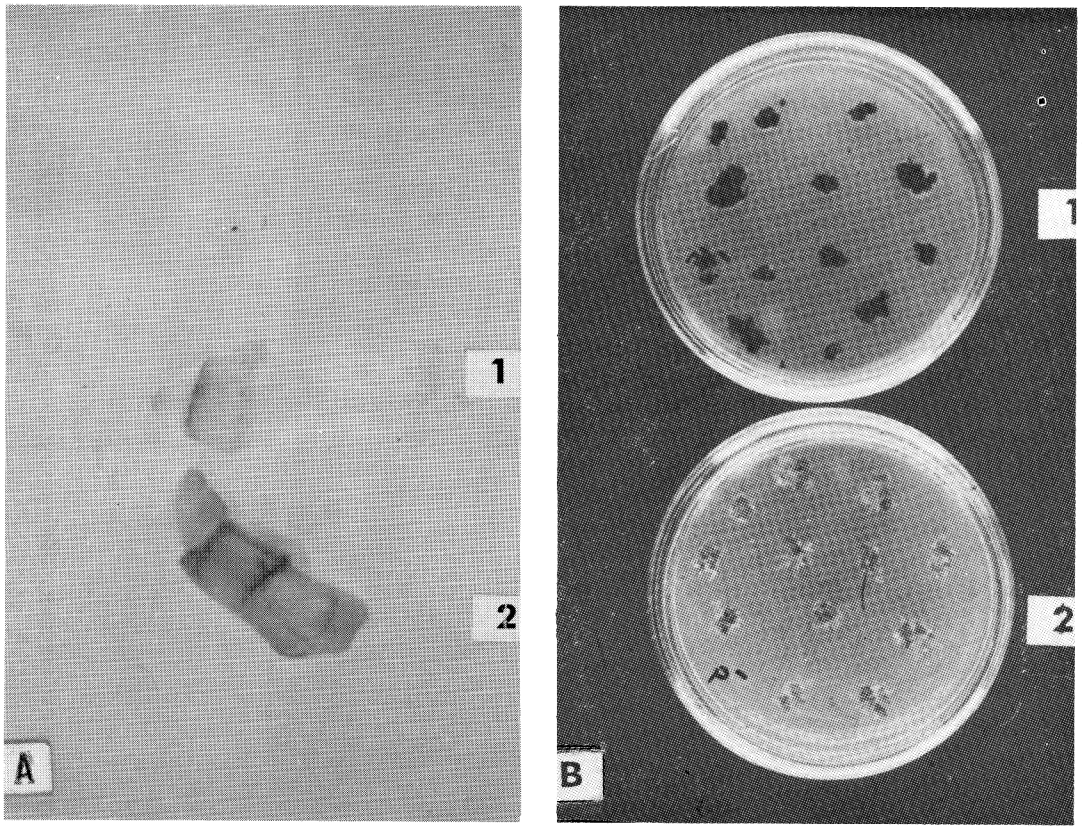

Figure 2. A) Meristematic tissues from *in vitro* sugarcane plants after 3 days of cocultivation with *Agrobacterium tumefaciens* At 2260 harboring the plasmid pGT GUSBAR. 1: Meristematic explants not cocultured with *A. tumefaciens* -- negative control. 2: Meristematic explants cocultured with *A. tumefaciens*, showing blue stain due to the presence of b glucuronidase encoded by the gene *uid*1. B) Sugarcane calli after 2 weeks of culture on the P- medium, supplemented with 4 mg/L BASTA®. 1: Non-transformed calli --negative control. 2: Transformed calli resistant to the herbicide BASTA®, showing small shoots (future publication). (See color plate on page 217).

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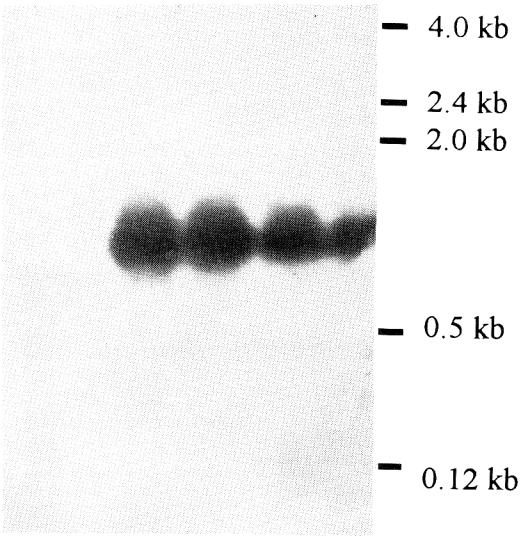

Figure 3. PCR-Southern blot analysis of the BASTA® resistant calli. Genomic DNA from sugarcane calli, cocultured by 4 treatments was the template for *uid*1 gene - specific amplification. The α-^{32}P ATP - labeled DNA probe (16) was also specific to the gene *uid*1. 1: DNA from non infested calli. 2-5: DNA from infested calli, treatments IN1, IN2, PC1 and PC3.

rates after cutting improved the competence of plant tissue to the *Agrobacterium*-mediated gene transfer. In the opposite cases, a fast hypersensitive response made the transformation impossible.

The results obtained are similar to those reported in grape (*Vitis vinifera*) (19) where a reduction of callus necrosis and an increase in the number of transformation events were observed when various AO compounds were added during explants *Agrobacterium* interaction. These studies showed that the target of the AO compounds mentioned should be the oxygen reactive species produced by the extracellular peroxidases and involved in the propagation of the local hypersensitivity response in the plant.

The host-spectrum of wild *A. tumefaciens* strains in natural conditions is limited mainly to dicotyledonous plants. Recently, the *Agrobacterium*-mediated transformation of several monocotyledonous plant species was successfully achieved under *in vitro* cell viability favoring conditions (5-7). We established those conditions for the transfer of foreign DNA to sugarcane meristematic explants. These explants were previously treated with AO compounds inhibiting cellular necrosis. The genetic transformation was performed and the GUS activity was histochemically detected in infected tissues as well as in regenerated calli. These calli also showed a remarkable resistance to BASTA®. The transgenic material was positive when tested by PCR.

The explants from field plants showed better callogenesis in the P+5 SEL medium compared to those obtained from plants cultured *in vitro*. This fact is possibly related with the capacity of dedifferentiation and the physiological conditions of the meristems.

The results obtained are an important step in the whole extension of genetic engineering to sugarcane by the development of efficient methodologies for stable transformation and the introduction of useful traits into the genome of this recalcitrant species. The application of an efficient regeneration technique (Figure 2B) recently allowed the production of *Agrobacterium*-transformed transgenic sugarcane plants, as confirmed by PCR, genomic Southern blot and field herbicide-resistance trials (manuscript in preparation).

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