

TRANSIENT GENE EXPRESSION IN ELECTROPORATED INTACT TISSUES OF *Stylosanthes guianensis* (AUBL.) SW.¹

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ABSTRACT: Genetic transformation through protoplast electroporation has been established for commercially important plant species. In this work, explant sources, electric field strengths, electroporation buffers, DNA forms and osmotic pretreatment were assayed in order to optimize transient reporter gene expression in electroporated tissues of *Stylosanthes guianensis*, a tropical forage legume. Higher transformation rates were obtained employing cotyledonary explants and an electric field strength of 250 V cm⁻¹. Linear plasmid DNA, chloride-free electroporation buffer and osmotic pretreatment with 1.6 mol L⁻¹ mannitol also improved transient transformation but non-significantly. Transgene specific PCR amplification was employed to prove the transformed status of the tissues.

Key words: GUS expression, direct DNA transfer, plant transformation, forage legume

EXPRESSÃO TRANSIENTE EM TECIDOS INTACTOS DE *Stylosanthes guianensis* (AUBL.) SW. VIA ELETROPORAÇÃO

RESUMO: A transformação genética através da eletroporação de protoplastos foi estabelecida para espécies vegetais comercialmente importantes. Neste trabalho, fontes de explante, intensidades de campo elétrico, soluções de eletroporação, configuração da molécula de DNA e pré-tratamentos osmóticos foram avaliados para otimizar a expressão transiente do gene repórter em tecidos eletroporados de *Stylosanthes guianensis*, uma leguminosa forrageira tropical. Taxas elevadas de transformação foram obtidas empregando-se explantes cotiledonares e 250 V cm⁻¹ de intensidade de campo elétrico. DNA plasmidial linear, solução de eletroporação livre de cloro e pré-tratamento osmótico com 1,6 mol L⁻¹ de manitol favorecerem a expressão transiente do gene repórter, porém não significativamente. A amplificação por PCR específica do transgene foi usada para demonstrar a ocorrência de transformação nos tecidos.

Palavras-chave: expressão de GUS, transferência direta de DNA, transformação de plantas, leguminosa forrageira

INTRODUCTION

Genetic transformation through protoplast electroporation has been established for commercially important plant species, such as *Brassica napus* (Guerche et al., 1987), maize (Rhodes et al., 1988) and rice (Shimamoto et al., 1989), but requires (i) an effective regeneration protocol from protoplast to fertile non-chimerical transgenic plants; (ii) development of genotype-specific regeneration protocols; (iii) long periods of *in vitro* cultivation which may induce genetic and epigenetic alterations; (iv) intense and expertise work.

The intact cell wall of bacteria and eukaryotic cells is known to be DNA-permeable to variable extents (Neumann et al., 1996; Lin et al., 1997; Shimogawara et al., 1998) thus, selective permeability lies on the cellular membrane. Electric pulses elevate the transmembrane potential promoting pore formation due to increased dipole moment of the hydrophilic lipid heads (Neumann et al., 1982; Kinosita & Tsong, 1977). The effects of an

electric field on cell walls are still unknown although transgene delivery and expression have been described for goat's rue embryos (Collen & Jarl, 1999), intact cells of maize (Songstad et al., 1993; Sabri et al., 1996), common bean, cowpea, and other grain legumes (Akella & Lurquin, 1993; Dillen et al., 1995), rice and wheat (Dekeyser et al., 1990; Xu & Li, 1994), alfalfa (Senaratna et al., 1991) and sugar beet (Lindsey & Jones, 1987).

DNA introduction into organized tissues is a promising means to avoid the forementioned constraints of protoplast electroporation and easily obtain wholly transformed plants (Lin et al., 1997).

Genetic transformation of *Stylosanthes guianensis* (Aubl.) Sw., a tropical forage legume, has been achieved with *Agrobacterium* infection, but at low efficiency (Wordragen & Dons, 1992; Sarria et al., 1994). Although being considered one of the less recalcitrant genus of legume to *in vitro* culture (Vieira et al., 1990), direct gene transfer has never been employed in *Stylosanthes*. Calli can be obtained from various explant

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sources, and they can further be induced to plant regeneration via organogenesis (Dornelas et al., 1992), thus suggesting that direct tissue electroporation could be effective to generate transformants.

We have evaluated the factors affecting plasmid DNA introduction and expression into organized tissue of *Stylosanthes guianensis* (Aubl.) Sw. and provided discussions about the effects of electric pulses on intact plant cell walls. To our knowledge, this is the first report of direct gene transference to this species.

MATERIAL AND METHODS

Plant material and tissue cultures

Seeds of *Stylosanthes guianensis* cv. Mineirão were *in vitro* germinated on half-strength MS medium (Murashige & Skoog, 1962) as described by Dornelas et al. (1992), at a density of 15 seeds per flask. Hypocotyls and fully expanded cotyledons were excised from 7 to 8 day old seedlings and completely developed leaves from 14 to 15 day old ones. Explants were cut into segments of approximately 2 mm².

After electroporation, the explants were thoroughly washed with autoclaved water, dried on sterile filter paper and placed on MS3 (MS salts, 3% sucrose, 0.18% Phytigel) during two days for the β -glucuronidase (GUS) test (Jefferson et al., 1987) or 14 days for polymerase chain reaction (PCR) analysis. The longer period of *in vitro* culture of the explants destined to PCR analysis was employed in order to rule out the possibility of amplification of non-transformed, surface contaminant DNA.

Cultures were maintained under 25°C and 16h light regime (65 $\mu\text{E m}^{-2} \text{s}^{-1}$) provided by cool white fluorescent tubes (Sylvania, 85 W).

Plasmid

Transformation vector pCambia 1301 (CAMBIA GPO Box 3200, Canberra ACT 2601, Australia) carrying β -glucuronidase (GUS) - *uidA*, driven by 35S promoter from cauliflower mosaic virus (CaMV) (Figure 1). The presence of a catalase intron into the coding sequence of *uidA* rules out the possibility of reporter gene expression in transformed endophytic microorganisms. The plasmid was transformed to *E. coli* DH5 α (Hanahan, 1983) and isolated by alkaline lysis (Sambrook et al., 1989). Supercoiled or plasmid DNA linearized through digestion with *Eco*RI (GIBCO BRL) was employed in transformation experiments.

Pre-pulse treatments

Hypocotyls (H), cotyledonary (C) and leaf (L) sections were presoaked in 5 mL of electroporation buffer supplemented with 0, 0.2, 0.8 or 1.6 mol L⁻¹ mannitol for 20 min at 0°C. Prior to pulsing, 10 μg of supercoiled or linear plasmid DNA were added to each 800 μL mixture of electroporation buffer and explants. The cuvettes were kept on ice for 10 min before pulsing.

Electroporation conditions

Commercial Bio Rad® Gene Pulser II with Capacitance Extender device was employed to deliver 50 to 250 V. cm⁻¹ electric field, by discharging a 900 μF capacitor to 0.4 cm electrode-gap cuvettes (800 μL volume) containing 10 explants in electroporation buffer I (Fromm et al., 1987), II (Tada et al., 1990) or III (Walbot, 1993).

Sample resistances were 20 Ω (buffer I and III) and 50 Ω (buffer II) as determined by the device measurements. The time, expressed as pulse decay constant, established by internal settings of the apparatus was $\tau = RC$.

Histochemical GUS assays

Explants were histochemically analyzed for β -glucuronidase activity as described by Jefferson et al. (1987). Electroporated tissues were longitudinally sliced and vacuum-infiltrated for 5 min with staining buffer containing the chromogenic substrate, 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) (McCabe et al., 1988) with addition of 20% methanol in order to suppress endogenous β -glucuronidase activity (Kosugi et al., 1990). After 24-hour incubation at 37°C, tissue pigments were removed by successive washes with 80% ethanol, and the explants were observed under the stereomicroscope.

PCR analysis

After 14 days of culture in MS3, genomic DNA was isolated as described by Edwards et al. (1991) from randomly chosen samples of electroporated explants and non-electroporated controls.

Each 25 μL reaction mixture contained 20ng of genomic DNA, 10 mM TRIS-HCl (pH 8.4), 50 mM KCl, 2.0 mM MgCl₂, 160 μM of each dNTP, 1.0 U of *Taq* polymerase (GIBCO BRL) and 200nM of each *uidA* specific primer (*gus 1* – CCT GTA GAA ACC CCA CAA CG and *gus 2 c* – TGC AGC GCT ACC TAA GGC CG) (Figure 1), which provide an amplification product of 795 bp. The mixture was overlaid with sterile mineral oil and submitted to denaturation (2 min at 95°C) followed by 25 cycles of amplification (1 min at 94°C, 1 min at 45°C and 1.5 min at 72°C) and a final extension (7 min at 72°C) in thermal cycler (Perkin Elmer Cetus).

Statistical procedures and data analysis

A cuvette containing ten explants (approximately 2 mm²) was considered a replicate, and three replicates were evaluated for both GUS and PCR analysis in a randomized design experiment.

All tested variables, i.e. explant type, electric field strength, osmotic treatment, DNA form and buffer types, were independently assayed and compared to reference conditions. Reference conditions were: buffer I (Fromm et al., 1987), 10 μg of non-linearized plasmid DNA, no osmotic treatment previous to pulse and 100 V cm⁻¹

discharged by 900 μF capacitor (720 kJ) to cotyledon-derived explants.

GUS stained areas were visually evaluated and attributes were given from 0 to 5 according to their size, intensity and frequency per explant. Data are the mean number of three independent replicates (30 explants) \pm se (standard error). Mean comparisons were performed employing the Tukey test (1% and 5% probability).

RESULTS AND DISCUSSION

Effect of explant type on transgene expression

Cotyledons showed the highest levels of GUS expression (Figures 2A and 3A) independent of the buffer employed, DNA form, osmotic treatment and electric field strength, followed by hypocotyl-derived explants (Figures 2B and 3A). Leaves showed the lowest transformation rates (Figures 2C and 3A). Songstad et al. (1993) also reported low levels of expression in electroporated leaves of maize embryos. Qualitatively, as evaluated by the arbitrary expression scale, leaves also showed poor transformation rates (Figure 3B).

Pools of DNA from 20 randomly chosen explants of all transformed tissues showed PCR amplification of the transgene after 14 days of *in vitro* culture from two independent experiments (Figure 4), confirming their transformed status.

As reported for electroporated tissues of common bean (Dillen et al., 1995), maize (Songstad et al., 1993) cowpea (Akella & Lurquin, 1993) and rice (Dekeyser et al., 1990), GUS expression in *Stylosanthes* (Figures 2A to 2C) appeared as blue-stained areas, without clear definition of the cells which were actually expressing the reporter gene.

Optimizing field strength to foreign DNA deliver

Fixed discharge capacity was employed (C = 900 μF) in an RC circuit, thus the energy input was correlated to the applied voltage ($E = \frac{1}{2} CV^2$) and ranged from 180 kJ (at 50 V cm^{-1}) to 8820 kJ (at 350 V cm^{-1}). The strength of the electric field promotes pore formation in cellular membranes (Kinosita & Tsong 1977; Neumann et al., 1982), alters the viscosity of homogalacturan gel-like structure (Zablackis et al., 1995) and affects ionic interactions of positively-charged lysine and histidine residues from extensins to negatively-charged uronic acid from pectin (Showalter, 1993; Reiter, 1998) allowing macromolecular migration across these structures.

Highest transformation levels were observed employing electric fields ranging from 100 to 250 V cm^{-1} (Figure 5A). Discharges of 350 V cm^{-1} caused tissue injuries and reduced GUS expression levels, even when compared to low energy electric fields (50 V cm^{-1}) (Figure 5A).

Cotyledon and hypocotyl-derived tissues showed approximately the same patterns of GUS

expression, with the highest values ranging from 150 to 250 V cm^{-1} (Figure 5B), which are similar to those reported by Dillen et al. (1995) for bean embryonic axes. No GUS expression was observed with electric field intensities lower than 50 V cm^{-1} or higher than 350 V cm^{-1} .

Effect of electroporation buffer on DNA introduction

No statistical differences ($P < 0.05$) were observed concerning explants electroporated with buffer I, II and III. Tada et al. (1990) reported increased efficiency employing electroporation buffer lacking chloride in the transformation of rice protoplasts, but it is very likely that the effect of reactive oxygen derived from chloride reaction is not so drastic in intact cell walls. However, qualitative analysis of GUS expression showed that chloride-free buffers II and III had greater number of explants included in expression level 4 and 5 of the arbitrary scale in electroporated *Stylosanthes* cotyledons (Figure 6).

Influence of DNA configuration on gene expression

Linear DNA was most effectively introduced into whole tissues of *Stylosanthes* (Figure 7), as described for other Fabaceae (Akella & Lurquin, 1993; Dillen et al., 1995). Higher efficiency of transformation employing linearized plasmid DNA has been reported for bacteria (Rittich & Spanova, 1996), yeast and filamentous fungi (Kwon-Chung et al., 1998), plant protoplasts (Lin et al., 1997) and intact tissues (Lin et al., 1997; Sabri et al., 1996; Saunders et al., 1995; Dillen et al., 1995; Akella & Lurquin, 1993).

The transient nature of membrane pores and structural alterations of the cell wall induced by electric fields imposes conformational restrictions to the macromolecules to be introduced (Neumann et al., 1982).

Influence of the osmoticum pretreatment on transient GUS expression

No statistically significant enhancement ($P < 0.05$) was observed in the frequency of gene expression (Figure 8A). However, 0.8 mol L^{-1} and 1.6 mol L^{-1} mannitol increased the number and intensity of blue spots, as evaluated by the arbitrary scale (Figure 8B). There was positive correlation ($r^2 = 0.9129$) between the osmotic concentration (x) and the number of explants expressing GUS (y), described as $y = 8.82x + 37.35$.

Osmotic treatment of the target tissue has been reported to increase transformation rates from 7- to 10-fold in microorganisms (Armaleo et al., 1990; Shark et al., 1991) and plant cells (Russell et al., 1992; Vain et al., 1993). The increased distance between the cell wall and cytoplasm in plasmolyzed cells makes them less susceptible to organelle damage and cytoplasm loss when immersed in an electric field.

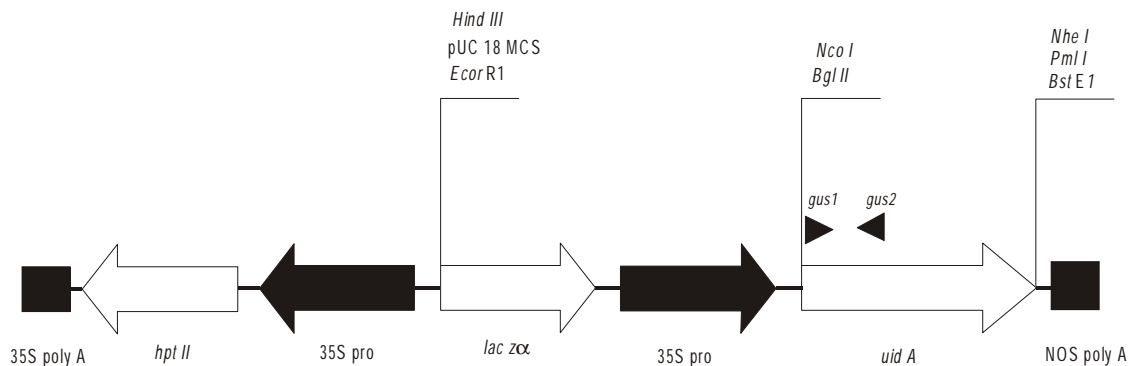


Figure 1 - Transformation vector pCambia 1301 expression cassette. PCR primers are indicated by (*gus1*) and (*gus2*). Arrows indicate cloning direction. Regulatory sequences (polyadenilation signals – poly A and promoters – pro) are shown as filled segments and coding sequences as unfilled ones. MCS = multicloning site, NOS = nopaline synthase, *lac*zα = α subunit of bacterial lactase.

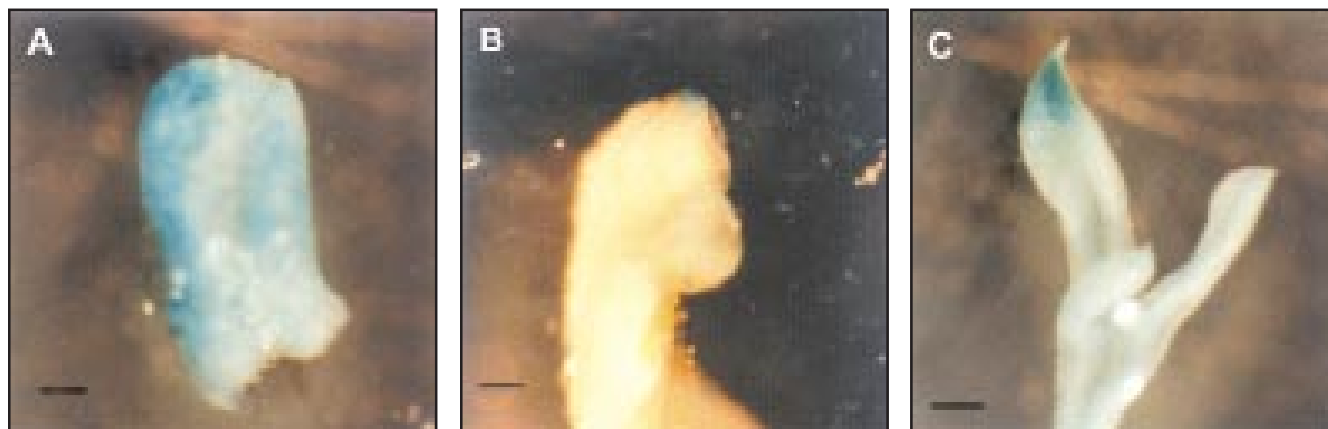


Figure 2 - GUS expression in electroporated *Stylosanthes guianensis* intact tissues. (A) cotyledon, (B) hypocotyl, (C) leaf. Bar=2 mm.

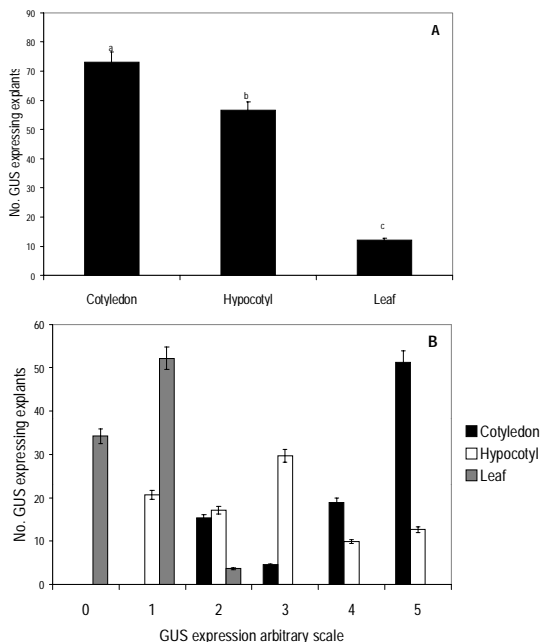


Figure 3 - Effect of the explant source on GUS expression in electroporated tissues of *S. guianensis*. (A) Number of explants expressing GUS. (B) Classification of GUS positive explants from different sources according to the arbitrary scale. Electroporation conditions: 900 mF, 100 V cm⁻¹, 20 W, buffer I, 10 mg of supercoiled DNA, absence of osmotic treatment. Bars indicate se and small cap letters, significant differences (P<0.05).

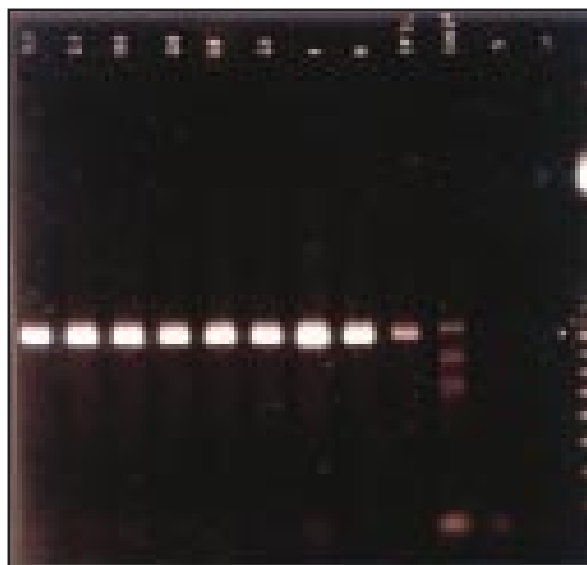


Figure 4 - Transgene PCR amplification using the genomic DNA of electroporated *Stylosanthes guianensis* tissues. Cotyledon (C), hypocotyl (H) and leaf (F) DNA from two independent experiments (1 and 2). Transgenic *S. guianensis* plants (1, 8 and 7/2.41, Quecini, 1999) and plasmid pCambia 1301 DNA were employed as positive controls; non-electroporated buffer-incubated tissue (S), and PCR mix without template DNA (C) were employed as negative controls. Molecular marker: 100 bp DNA ladder (Pharmacia).

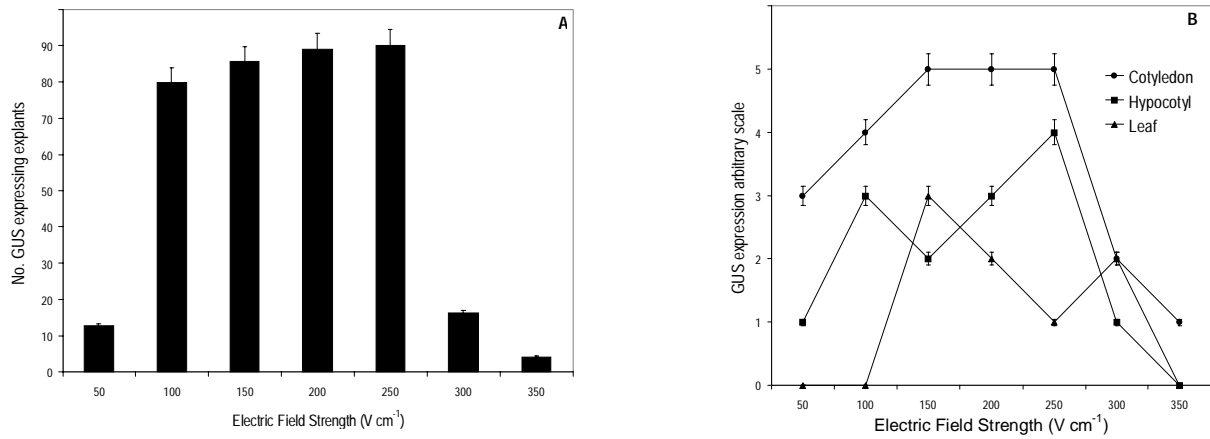


Figure 5 - Effect of electric field strength on GUS expression in electroporated tissues of *S. guianensis*. (A) Number of explants expressing GUS. (B) Classification of GUS positive explants electroporated with different field strengths according to the arbitrary scale. Electroporation conditions: cotyledons, 900 μF, 20 Ω, buffer I, 10 μg of supercoiled DNA, absence of osmotic treatment. Bars indicate se.

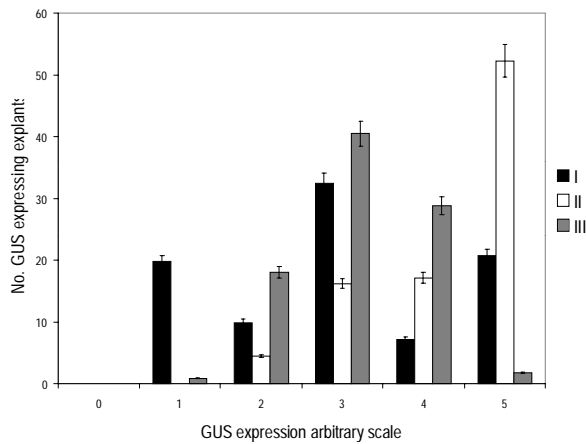


Figure 6 - Effect of electroploration buffer on GUS expression in electroporated tissues of *S. guianensis*. GUS positive explants electroporated with different buffers were classified according to the arbitrary scale. Electroporation conditions: cotyledons, 900 μF, 20 Ω (buffer I and III) and 50 Ω (buffer II), 100 V cm⁻¹, 10 mg of supercoiled DNA, absence of osmotic treatment. Bars indicate se.

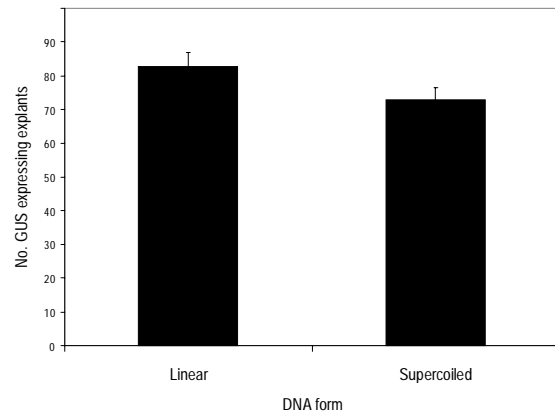


Figure 7 - Effect of the DNA form on GUS expression in electroporated tissues of *S. guianensis*. Electroporation conditions: cotyledons, 900 μF, 20 Ω, 100 V cm⁻¹, 10 μg of DNA, absence of osmotic treatment. Bars indicate se.

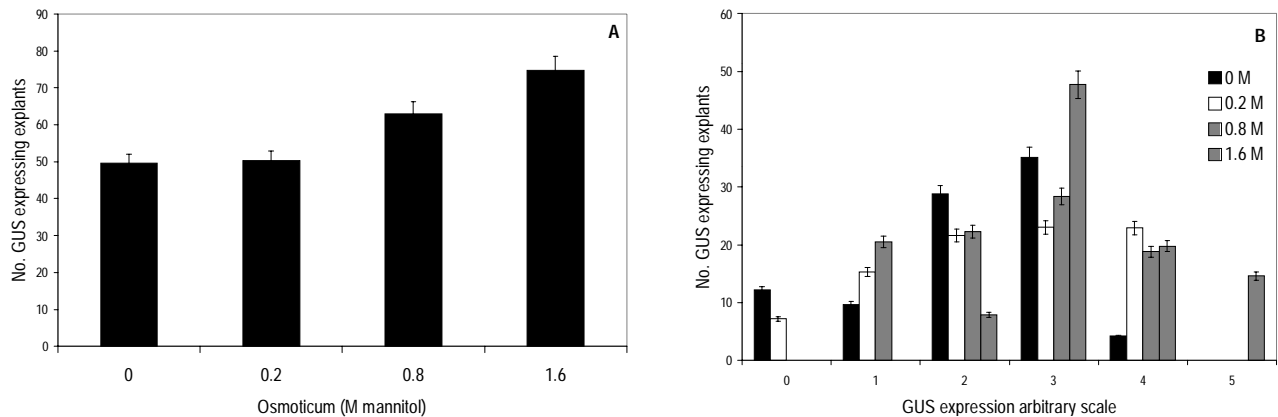


Figure 8 - Effect of osmotic pretreatment on GUS expression in electroporated tissues of *S. guianensis*. (A) Number of explants expressing GUS. (B) Classification of GUS positive electroporated explants pretreated with different concentration of mannitol according to the arbitrary scale. Electroporation conditions: cotyledons, 900 μF, 100 V cm⁻¹, 20 Ω, buffer I, 10 μg of supercoiled DNA. Bars indicate se.

CONCLUSIONS

Intact tissues of *Stylosanthes guianensis* (Aubl.) Sw. can be transformed with plasmid DNA after electroporation. Transient reporter gene expression is dependent upon biological and physical parameters. The explant source and energy input are the most important. Higher rates of transformation were obtained employing cotyledonary explants and electric fields ranging from 100 to 250 V cm⁻¹. Electroporation buffer, DNA form and osmotic pretreatment were not critical to transient transformation efficiency. To our knowledge, this is the first report on direct gene transfer in *Stylosanthes*, demonstrating the applicability of intact tissue electroporation to genetic manipulation of the genus.

Due to its importance for Latin American countries, the development of *Stylosanthes* biotechnological procedures must respond to increasing demands in terms of sustainable use of plant genetic resources as basic inputs for the future agriculture (see Izquierdo & de la Riva, 2000).

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