

Plant regeneration of sugarcane cv. RB931003 and RB98710 from somatic embryos and acclimatization

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ABSTRACT

The aim of this study was to establish a protocol for regeneration and acclimatization of sugarcane (RB931003 and RB98710) cultivars from somatic embryos. For the cultivar RB931003, the concentration of 0.25 mg L⁻¹ BAP was efficient and provided the shoot induction in 80% of embryogenic masses. For the cultivar RB98710, the highest percentage of shoot induction was observed in 0 and 0.25 mg L⁻¹ BAP, which promoted 70% and 75% of shoots from embryogenic masses, respectively. The MS medium without growth regulators proved to be appropriate for elongation and rooting of shoots and the use of substrate composed by vermiculite and MS salts was effective in the acclimatization of plants for both cultivars analyzed.

Key words: *Saccharum* spp., 6-benzylaminopurine, tissue culture, somatic embryogenesis

INTRODUCTION

The sugarcane crop stands out in the world mainly because it is a renewable energy source for producing ethanol and contributing to produce 65% of world sugar. Sugarcane is cultured in tropical and subtropical regions of more than 70 countries. In Brazil, the sugarcane cultivation is installed in an area of more than eight million hectares, producing over 624 million tons of sugarcane, according to the 2010/2011 crop forecasting. Brazil is the world leader in the use of sugarcane as a source of renewable energy and sugar exports (Conab, 2011). Due to the prominent position it occupies in the economy, there is interest in developing genetic breeding of this species. Currently, the cultivars of sugarcane are the result of interspecific crosses between *Saccharum officinarum*, *S. barbieri*, *S. sinense* and the wild species of *S. spontaneum* and *S. robustum*, classified as complex *Saccharum* spp. *In vitro* organogenesis is a fundamental step for obtaining genetically transformed plants. The tissue culture is also a powerful tool that can

accelerate the genetic breeding (Alves et al., 2011). In this sense, efficient protocols for *in vitro* regeneration should be established. As the morphogenic response is influenced by genotype, it is essential to adapt the protocols performed for each cultivar. *In vitro* cultivation of sugarcane has been described for various cultivars through callus culture, meristems and somatic embryos (Hendre et al., 1993; Srinivasan & Vasil, 1986). The somatic embryogenesis induction has been obtained from explants of sugarcane inoculated in medium supplemented with various auxin types (Ho & Vasil, 1983; Fitch & Moore, 1990; Brisibe et al., 1994). Taylor et al. (1992) described the induction of embryogenic callus in 18 cultivars, as well as to obtain cell cultures in suspension. Plant regeneration from embryogenic callus obtained from immature leaves was observed by Nieves et al. (2008), Los Blanco et al. (1997) and Vickers et al. (2005) using MS medium (Murashige and Skoog, 1962) supplemented with 2,4-D (2,4 dichlorophenoxyacetic acid) and Liu (1993), using different combinations of 2,4-D, KIN (kinetin),

BAP (benzylaminopurine) and calcium pantothenate.

The aim of this research was to establish appropriate conditions for obtaining plant regeneration from somatic embryos and acclimatization of plants of sugarcane (RB931003 and RB98710) cultivars.

MATERIAL AND METHODS

Culture conditions

All the cultures were maintained in a growth chamber under cold white fluorescent light, with a photon flux density of $60 \mu\text{mol m}^{-2} \text{s}^{-1}$, 12/12 light/dark regime and $25 \pm 2 \text{ }^\circ\text{C}$. The cultures were performed in Petri dishes (10 cm diameter and 2 cm height), containing 25 mL of culture medium and sealed with PVC film, or in glass flasks (6 cm diameter and 9 height) containing 40 mL of culture medium each and sealed with rigid polypropylene caps. All media had the pH adjusted to 5.8 and were autoclaved for 20 min at $120 \text{ }^\circ\text{C}$.

Embryogenic callus induction and proliferation of embryogenic masses

Young meristematic leaf-rolls of sugarcane cultivars (RB931003 and RB98710) were used as explants. The explants were collected from 2-month-old plants originated through vegetative propagation and cultured under greenhouse conditions. The explants were surface-sterilized by immersion in 70% alcohol (v/v) for 2 min and then in a 2 % sodium hypochlorite (v/v) for 30 min. They were subsequently rinsed three times in sterile distilled water and cut into sections of about 50 mm and immersed for 30 min in antioxidant solution composed of 250 mg L^{-1} of ascorbic acid, 25 mg L^{-1} citric acid and 1 g L^{-1} PVP (polyvinilpirrolidone). The explants were isolated in Petri dishes containing callus induction medium composed of MS medium supplemented with 30 g L^{-1} sucrose, 7.0 g L^{-1} agar, 500 mg L^{-1} PVP, 500 mg L^{-1} hydrolyzed casein and supplemented with 4.0 mg L^{-1} 2,4-D. The explants were incubated in the dark for thirty days.

For the proliferation, the embryogenic callus obtained were subdivided into 3.0 mm slices and transferred to the culture medium for development and proliferation of embryogenic masses, composed of MS culture medium, 30 g L^{-1} sucrose, 7.0 g L^{-1} agar, 500 mg L^{-1} PVP, 500 mg L^{-1} hydrolyzed casein supplemented with 2.0 mg L^{-1}

2,4-D. This procedure was performed every 20 days during three subcultures. The plant material was incubated in the dark and after each subculture.

Effect of BAP (6-benzylaminopurine) on shoot induction from somatic embryos of sugarcane

Embryogenic masses were obtained in the stage of development and multiplication of somatic embryos of sugarcane (RB931003 and RB98710) and were subdivided into 5.0 mm slices and transferred to MS medium supplemented with 30 g L^{-1} sucrose, 7.0 g L^{-1} agar and supplemented with 500 mg L^{-1} hydrolyzed casein and 500 mg L^{-1} PVP. The following concentrations of BAP: (0) control without BAP, (1) 0.25 mg L^{-1} , (2) 0.50 mg L^{-1} , (3) 1.00 mg L^{-1} were compared.

Plant material has been maintained in the dark for 15 days and then was transferred to light condition. After thirty days of culture, samples were evaluated according to the following variables: percentage of embryogenic masses producing shoots and percentage of embryogenic masses oxidized.

The experimental design was completely randomized in a 2x4 factorial scheme with ten replicates and four embryogenic masses per experimental unit. Treatments effect were analysed by ANOVA and means were compared by Tukey's multiple range test using Sisvar[®] software.

Plant development and acclimatization

Sugarcane shoots obtained in the previous experiments were transferred to MS medium, 30 g L^{-1} sucrose and 7.0 g L^{-1} agar, without plant growth regulators to promote the elongation and root development. In this stage it was used 100 shoots of each cultivar. The percentage of rooting plants, height of plants (cm) and leaf number per plant were evaluated after thirty days. Sugarcane plants were removed from the culture flasks, washed in water and transferred to flasks containing 25 mL of liquid MS medium without plant growth regulator and incubated in a growth chamber in light conditions for four days. For acclimatization, 50 plants of each cultivar were transferred to substrate composed of vermiculite and MS salts solution. Plantlets were distributed in polystyrene trays and kept in greenhouse under intermittent mist and covered with Sombrite[®] 50% of light reduction. Thirty days after the initial

culture period, a sample of ten plants of each sugarcane cultivars was evaluated for the percentage of surviving plants and fresh weight of shoots and roots (mg).

RESULTS AND DISCUSSION

Embryogenic callus induction and proliferation of embryogenic masses

The callus induction in the explants began after fifteen days after isolation and was originated from the central section of explants. The percentages of embryogenic callus induction in explants of sugarcane cultivars RB931003 and RB98710 after thirty days of initial period of culture were 35% and 27% respectively. In this stage, the callus presented itself with mucilaginous appearance and thirty days after the initial period of culture it was

possible to observe the embryogenic masses formation with multiple somatic embryos in globular stage (Figure 1A). Similar results were reported by Lamb et al. (2001) when studied the embryogenic callus induction of *Avena sativa* L. in the presence of 2.4-D. These authors indicated that callus initially was presented itself with translucent aspect and after two weeks of culture, somatic embryos began to develop from embryogenic callus, in accordance with the observations described here.

For the embryogenic masses proliferation on MS culture medium with 2.4-D, the reduction of 2.4-D concentration to 2.0 mg L⁻¹ successfully doubled the number of embryogenic masses in each subculture.

Table 1 - Effect of 6-benzylaminopurine (BAP) in the shoots induction from embryogenic masses and percentage of oxidation of sugarcane (RB931003 and RB98710) cultivars after 30 days of culture. São Cristóvão-SE, 2009.

BAP (mg L ⁻¹)	Shoot induction (%)		Oxidation (%)	
	RB 931003	RB 98710	RB 931003	RB 98710
0.00	25 bB	70 aA	75 Aa	30 bB
0.25	80 aA	75 aA	20 bA	25 bA
0.50	20 bA	17.5 bA	80 aA	82.5 aA
1.00	5 bA	15 bA	95 aA	85 aA
CV (%)	46.66		29.07	

Means followed by same lower case letter in the column and capital letter on the line do not differ by Tukey's test at 5%.

Table 2 - Production of fresh weight mass of shoots and roots and plant survival of sugarcane (RB931003 and RB98710) cultivars after acclimatization. São Cristóvão-SE, 2009.

Cultivar	Fresh weight mass of shoots (mg)	Fresh weight mass of roots (mg)	Plant survival (%)
RB931003	750 ± 26.89	215.8 ± 33.18	85
RB98710	1.980 ± 15.96	511.1 ± 50.36	100

Data represent means ± standard deviation of the mean.

Effect of BAP (6-benzylaminopurine) on shoot induction from somatic embryos of sugarcane

With regard to the percentage of shoots induction from somatic embryos and percentage of embryogenic masses oxidized, the variance analysis revealed significant interaction between cultivars and concentrations of BAP. According to the results observed with the percentage of shoot induction from somatic embryos, the best results has been obtained with RB931003 cultivar and the concentration of 0.25 mg L⁻¹ BAP, which promoted 80% of shoots induction after 30 days of culture (Figure 1B). For RB98710 cultivar, the

highest percentage of shoot induction from somatic embryos has been observed on the MS medium without BAP and at concentration of 0.25 mg L⁻¹ BAP, which promoted 70% and 75% of shoots induction from somatic embryos, respectively (Table 1 and Figure 1C). The present results confirmed the effect of cytokines described by Ali et al. (2008). These authors studied the effect of the combination between BAP and KIN on shoot regeneration from embryogenic masses of sugarcane and reported that the shoots regeneration was 60% with the use of 1.11 µM BAP and 1.16 µM KIN. In another study, Similar

results were observed by Falco et al. (1996) in their studies with callus of sugarcane induced on MS medium with 2.4-D. In this research, the shoot regeneration from somatic

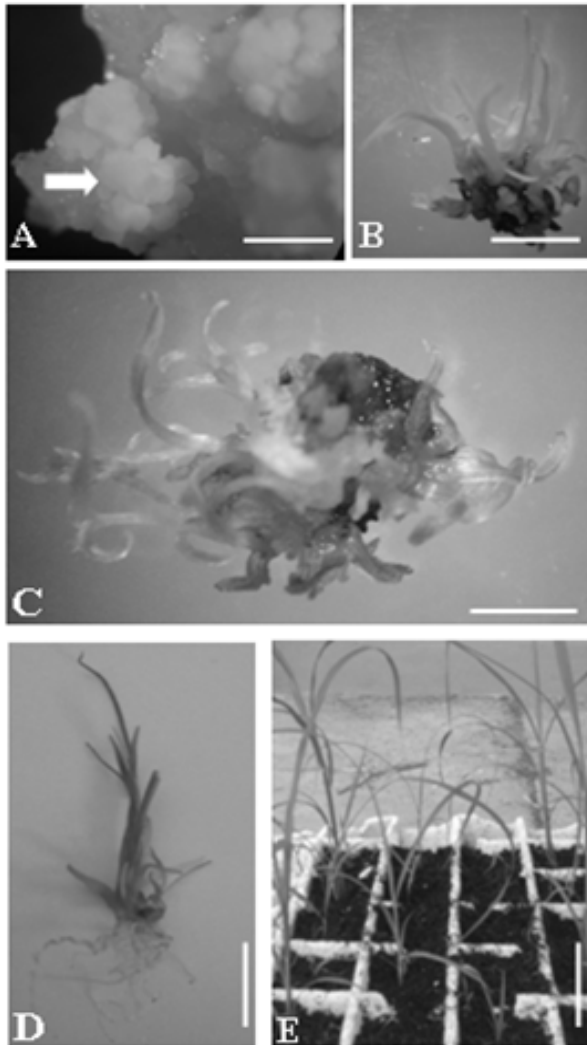


Figure 1 - *In vitro* plants regeneration of sugarcane (RB931003 and RB98710) cultivars from somatic embryos. (A) RB931003 embryogenic callus showing nodular structures induced after 30 days on MS medium containing 2.0 mg L^{-1} 2.4-D. (B) RB931003 shoots induction from embryogenic masses after 30 days of culture on MS medium containing 0.25 mg L^{-1} BAP. (C) RB98710 shoots induction after 30 days of culture on MS medium without growth regulators. (D) Detail of RB98710 plant elongated and rooted after 60 days of culture on MS medium without growth regulators. (E) Plants of RB931003 cultivar acclimatized after 30 days of culture on substrate composed of vermiculite and MS salts. Bars: (1 - 2 - 3 = 3 mm, 4 - 5 = 5 cm). Arrow = somatic embryos.

embryogenesis occurred simultaneous after the callus transference to MS medium containing KIN

and BAP. In another study, Franklin et al. (2006) reported the shoot induction of sugarcane from globular somatic embryos in the presence of cytokines, confirming the results observed here. Regarding to the results observed in the literature, the genotype effect explains the organogenic diversity response to different sugarcane genotypes. The shoots induction from embryogenic masses started on the seventh day of culture and on the thirtieth day of culture it was intensified. The average number of shoots per embryogenic masses, regardless of the BAP concentration used, was 20 shoots for the RB931003 cultivar and 15 shoots for RB98710 cultivar.

With regarding to the percentage of embryogenic masses oxidized, the lowest rate of oxidation was found for the embryogenic masses of RB931003 cultivar on MS medium containing 0.25 mg L^{-1} BAP, which showed 20% of embryogenic masses oxidized after 30 days of cultivation. To RB98710 cultivar, the best results were observed in the absence of BAP and on presence 0.25 mg L^{-1} BAP, where it was found the lowest percentage of embryogenic masses oxidized (30% and 25% respectively). Among the two cultivars, the BAP concentrations above 0.25 mg L^{-1} showed oxidation induction of embryogenic masses greater than 80% and significantly compromising the shoot induction from embryogenic masses (Table 1). The rate of embryogenic masses oxidized probably occurred by cellular phytotoxicity presented in relation to higher concentrations of BAP.

Plant development and acclimatization

The MS culture medium without growth regulator promoted the plant rooting in 95% of shoots of RB931003 cultivar and 90% for RB98710 cultivar after thirty days of culture (Figure 1D). Divergent results were obtained by Ali et al. (2008) for seedlings rooting of sugarcane CP-77.400 and BL-4 cultivars. These authors related that MS medium without growth regulators promoted the rooting rate of 20% after fourteen days of culture while the MS medium containing $10.74 \text{ }\mu\text{M}$ NAA promoted the shoots rooting rate of 90% after six days of culture. Comparing the results observed by these authors with those obtained in the present study, the genotype effect explains the organogenic diversity response to different sugarcane genetic materials occur when cultured to similar

conditions. In the acclimatization stage, the use of the substrate composed by vermiculite and MS salts resulted in 85% survival plants rate of RB931003 cultivar and 100% for RB98710 cultivar (Table 2 and Figure 1E). According to the literature, the sugarcane acclimatization of plants regenerated *in vitro* can be achieved successfully if the plants are initially cultured in an environment with high humidity and use of substrates of simple composition. Similar strategies were described by Snymam et al. (2006) and Segar et al. (2009). These authors easily acclimatized seedlings of sugarcane using soil as substrate and initial plants culture in mist chamber and plant fertilization weekly. For fresh weight of shoots, they observed the rates 750 mg and 1980 mg for RB931003 and RB98710 cultivars, respectively (Table 2). With respect to fresh weight of roots, the values obtained were 511.1 mg and 215.8 mg for RB98710 and RB931003 cultivars, respectively (Table 2).

CONCLUSION

It was concluded in this research that the explants cultured under the conditions described here were responsive to embryogenic masses and shoots induction. The used procedures enabled the acclimatized plants in both sugarcane cultivars. The information described here may be used in the optimization of regeneration protocols for other cultivars of sugarcane and would be used in genetic breeding of sugarcane from genetic transformation.

RESUMO

O objetivo deste estudo foi o estabelecimento de um protocolo de regeneração e aclimatização de plantas de cana-de-açúcar de duas cultivares (RB931003 e RB98710) a partir de embriões somáticos. Para o cultivar RB931003, a concentração de 0,25 mg L⁻¹ de BAP, foi eficiente e proporcionou a indução de brotações em 80% das massas embriogênicas. Já para o cultivar RB98710, as maiores porcentagens de indução de brotações foram observadas na ausência de BAP e na concentração de 0,25 mg L⁻¹ de BAP, os quais promoveram 70% e 75% de brotações nas massas embriogênicas, respectivamente. O meio de cultura MS isento de regulador vegetal apresentou-se adequado para o alongamento e enraizamento das brotações e a utilização do substrato composto por vermiculita mais os sais do meio de cultura MS foi eficiente na aclimatização das plantas de ambos os cultivares analisados.

Palavras-chave: *Saccharum* spp., 6-benzilaminopurina, cultura de tecidos, embriogênese

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