



## Plant regeneration and histological study of the somatic embryogenesis of sugarcane (*Saccharum* spp.) cultivars RB855156 and RB72454

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**ABSTRACT.** The development of an efficient regeneration system is crucial for the genetic transformation of sugarcane. The aims of this study were to regenerate plants from somatic embryos and analyze the origin and division pattern of cells during the different embryonic developmental stages of the sugarcane cultivars RB855156 and RB72454. For both cultivars, the best results for embryogenic callus induction were obtained with explant cultures on culture medium containing 13.5  $\mu\text{M}$  2,4-dichlorophenoxyacetic acid (2,4-D) followed by callus culture on solid medium with 4.5  $\mu\text{M}$  2,4-D. A higher rate of shoot induction was observed in RB855156 with the addition of 8.9 and 17.8  $\mu\text{M}$  benzylaminopurine (BAP); this higher rate was also observed in RB72454 with the addition of 17.8  $\mu\text{M}$  BAP. Murashige and Skoog (1962) (MS) medium containing 2.5 and 5.0  $\mu\text{M}$  indolebutyric acid (IBA) for RB855156 and RB72454, respectively, was suitable for rooting. The plantlets were successfully acclimatized and the plant survival rate was 100% for both cultivars. Histological analysis revealed that the shoots in both cultivars originated from somatic embryogenesis.

**Keywords:** tissue culture, somatic embryos, anatomy, 2,4-D, Poaceae.

## Regeneração e análise histológica de embriogênese somática de cana-de-açúcar (*Saccharum* spp.) cultivares RB855156 e RB72454

**RESUMO.** O desenvolvimento de um protocolo eficiente em um sistema de regeneração é crucial para a transformação genética de cana-de-açúcar. Os objetivos deste estudo foram a regeneração de plantas a partir de embriões somáticos e a análise da origem e modelos de divisão celular durante os diferentes estágios dos embriões somáticos, das cultivares de cana-de-açúcar RB855156 e RB72454. Em ambas as cultivares, os melhores resultados para a indução de calos embriogênicos foram obtidos com o cultivo dos explantes em meio de cultura contendo 13,5  $\mu\text{M}$  de 2,4-ácido-diclorofenoxiacético (2,4-D), seguido do cultivo dos calos em meio de cultura sólido com 4,5  $\mu\text{M}$  de 2,4-D. As maiores taxas de indução de brotações foram encontradas com 8,9 de 17,8  $\mu\text{M}$  benzilaminopurina (BAP) para RB855156 e 17,8  $\mu\text{M}$  BAP para RB72454. O meio de Murashige and Skoog (1962) (MS) com 2,5  $\mu\text{M}$  de ácido indolbutírico (IBA) para RB855156 e 5,0  $\mu\text{M}$  IBA para RB72454 foram adequados para o enraizamento das brotações. As mudas foram aclimatizadas com sucesso e a taxa de sobrevivência foi de 100% para ambas as cultivares. As análises histológicas revelaram que a origem das brotações em ambas as cultivares ocorreu via embriogênese somática.

**Palavras-chave:** cultura de tecidos, embriões somáticos, anatomia, 2,4-D, Poaceae.

### Introduction

Sugarcane is a renewable energy source for the production of ethanol that is grown in the tropical and subtropical regions of more than 70 countries. Brazil is emerging as a world leader in the use of sugarcane as a source of renewable energy and sugar exports. There is a growing interest in developing breeding programs for this species. Genetic transformation is a potential alternative to sugarcane

breeding programs, and somatic embryogenesis is a fundamental method for the *in vitro* regeneration of plants.

The RB855156 and RB72454 sugarcane cultivars were developed by “Rede Interuniversitária para o Desenvolvimento do Setor Sucroalcooleiro” (RIDESA). The RB855156 cultivar exhibits early maturation and has the ability to sprout from stumps, while RB72454 has a high sucrose content,

exhibits late maturation and high agricultural productivity and has a long, useful period of industrialization (DAROS et al., 2010). Many of the stages of somatic embryogenesis in these cultivars have not yet been studied. Furthermore, some sugarcane cultivars are recalcitrant to plant regeneration; for example, the RB72454 cultivar does not respond to established protocols (LIMA et al., 2001).

The induction of somatic embryogenesis has been achieved in sugarcane explant cultures on medium supplemented with various types of auxins (BRISIBE et al., 1994; FITCH; MOORE, 1990; HO; VASIL, 1983). Plant regeneration from an embryogenic callus obtained from immature leaves was observed by Nieves et al. (2008), Blanco et al. (1997) and Vickers et al. (2005) using Murashige and Skoog (1962) (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D). Although various studies of callus induction in sugarcane had been reported, few studies have analyzed all stages of somatic embryogenesis.

This study is important because of the need to identify the cells associated with induction processes and the formation of structures capable of organized growth and eventual development into seedlings. For plant regeneration from somatic embryogenesis, histological analyses must be carried out to determine the route of regeneration and discover whether the plant originated from somatic embryogenesis or organogenesis. Additionally, it is important to determine whether somatic embryogenesis is a reliable technique for use in the genetic transformation of sugarcane.

The aims of this study were to regenerate plants via somatic embryogenesis and to analyze the origin and division pattern of cells during the various stages of somatic embryo development in the sugarcane cultivars RB72454 and RB855156.

## Material and methods

Plants of the sugarcane cultivars RB855156 and RB72454 at 6 to 8 months of cultivation were used for the experiments. Immature leaves (cylinders of approximately  $2 \times 10$  cm) were surface sterilized for 1 min. in 70% ethanol (v/v), rinsed three times with sterile distilled water, incubated for 20 min. in a 2.0% (v/v) sodium hypochlorite solution and rinsed again three times in sterile distilled water. Meristematic regions of transverse segments were removed, transversely cut into segments of 5-6 mm and used as explants.

The explants were cultured for 8 weeks on callus induction medium. Embryogenic calli were then

separated, transferred to callus multiplication medium and cultured for 4 weeks. Subsequently, embryogenic calli were transferred to regeneration medium (embryo conversion into plantlets), they were rooted, acclimatized and transferred to a greenhouse. The material obtained with the best treatment at each phase was selected to proceed to other phases.

## Induction of somatic embryogenesis

The explants were cultured in flasks (6 cm in diameter and 9 cm in height) with 30 mL of induction medium (MSC3), which was composed of MS medium supplemented with 50 mg L<sup>-1</sup> arginine, 50 mg L<sup>-1</sup> cysteine, 0.15 mg L<sup>-1</sup> citric acid, 500 mg L<sup>-1</sup> hydrolyzed casein, 20 g L<sup>-1</sup> sucrose and 0.6% agar (Vetec®).

Mixtures containing a standard concentration of 2,4-D and various concentrations of benzylaminopurine (BAP) or kinetin (KIN) (13.5 μM 2,4-D (control); 13.5 μM 2,4-D + 8.9 μM BAP; 13.5 μM 2,4-D + 17.8 μM BAP; 13.5 μM 2,4-D + 26.7 μM BAP; 13.5 μM 2,4-D + 9.3 μM KIN; 13.5 μM 2,4-D + 18.6 μM KIN; and 13.5 μM 2,4-D + 27.9 μM KIN) were compared for callus induction.

Additionally, various types and concentrations of auxins (6.8, 13.5 and 20.4 μM 2,4-D; 6.7, 13.4 and 20.1 μM trichlorophenoxyacetic acid; and 6.6, 13.2 and 19.8 μM picloran) were compared. The explants were maintained for a culture period of 8 weeks at 25 ± 2°C in the dark; following this, the percentage growth of embryogenic callus was evaluated.

## Growth of embryogenic callus

Embryogenic callus was grown in liquid and solid media composed of MSC3 supplemented with 30 g L<sup>-1</sup> sucrose (MSC1). Petri dishes were used for the solid medium and flasks were used for the liquid medium, and both contained 30 cm<sup>3</sup> of culture medium. Four concentrations of 2,4-D (0, 4.5, 9.0 and 13.5 μM) were compared in the liquid and solid media. Initially, 14 mg of callus was introduced for a subculture period of 15 days. The callus in the liquid medium was maintained under intermittent agitation at 120 rpm. The fresh weight of the callus (mg) was measured after 30 days of culture.

The cultures were maintained in a growth chamber at 25 ± 2°C under a 16/8h light/dark cycle under cold white fluorescent light with a photon flux density of 30 μmol m<sup>-2</sup> s for 30 days.

## Conversion of somatic embryos into plantlets

For the conversion of somatic embryos into plantlets, the treatments were composed of clusters of callus that formed embryogenic masses at the

same stage of development. The callus clusters were transferred into Petri dishes containing 30 cm<sup>3</sup> of MSC1 medium and subjected to the same *in vitro* conditions as previously mentioned. Four BAP concentrations (0, 8.9, 17.8, 26.7 and 35.6 μM) were compared. The percentage of shoot induction was evaluated after 30 days of culture.

#### Rooting and plant acclimatization

The rooting sugarcane shoots obtained in the previous experiment were transferred to MS/2 medium containing three concentrations (0, 2.5 and 5.0 μM) of indolebutyric acid (IBA). After 30 days of culture, the percentage of rooting plants, number of roots, root length (cm) and percentage of normal plant development were evaluated. The cultures were maintained in a growth chamber at 25 ± 2°C under cold white fluorescent light with a photon flux density of 30 μmol m<sup>-2</sup> s under a 16/8h light/dark cycle for 30 days.

For acclimatization, the rooted plants were transferred to plastic cups (7 cm in diameter and 10 cm in height) containing Plantmax<sup>®</sup> substrate. The percentage of surviving plants was evaluated after 30 days; these plants were then transferred to a greenhouse in pots (20 cm diameter and 17 cm height) containing Plantmax<sup>®</sup>.

#### Histology

Samples of plant material at different stages of cell differentiation were collected at various points during callus and shoot induction. The samples were fixed in 50% formaldehyde/acetic acid/alcohol (FAA) (v/v) for 48h in accordance with the protocol of Johansen (1940). Next, they were transferred into 70% ethanol and dehydrated in an ethanol series. The samples were embedded in glycol methacrylate (GMA) following the procedure described by Feder and O'Brien (1968) and the manufacturer's instructions (JB4 - PolyScience<sup>®</sup>). Longitudinal sections were cut using a rotary microtome with a steel razor (type C) to a section thickness of 5 mm. The cuttings were placed onto slides and stained with 0.05% (v/v) toluidine blue for 10 min. (O'BRIEN et al., 1965). The permanent slides were mounted in synthetic resin (Entelan<sup>®</sup>). Images were captured with a Sony Cyber-shot<sup>®</sup> P200 digital camera attached to a Zeiss Axiolab<sup>®</sup> microscope. The scales were projected in the same optical conditions.

#### Experimental design

The experimental design was a completely randomized factorial scheme with five replications and ten explants per experimental unit. For each

phase, the factors included the treatments with regulators and the cultivars (RB855156 and RB72454). The regulators of somatic embryogenesis induction were 2,4-D, BAP and KIN. Growth of the embryogenic callus occurred in the presence of 2,4-D, conversion of somatic embryos into plantlets occurred in the presence of BAP and IBA was used for rooting and plant acclimatization.

The homogeneity of means was tested using Bartlett's test. For the callus induction and plant rooting experiments, the treatment effect was analyzed by ANOVA and the means were compared with Tukey's multiple range test ( $\alpha = 0.05$ ) using Sisvar<sup>®</sup> software. The results of callus growth and plantlet conversion experiments were subjected to a regression analysis using the MSTAT-C<sup>®</sup> program version 2.10 (Michigan State University, USA).

## Results and discussion

### Callus induction

The greatest percentage of embryogenic callus induction in both cultivars was achieved using 13.5 μM 2,4-D, with 70% induction observed for RB855156 and 65% observed for RB72454. No callus induction occurred with the treatments that lacked 2,4-D (Table 1). Lima et al. (2001) reported that embryogenic callus induction of the sugarcane cultivar RB72454 occurred on MS medium with 9.0 μM 2,4-D; however, this callus did not regenerate into plants.

**Table 1.** Percentage of embryogenic callus induction in two sugarcane cultivars cultured on different concentrations of BAP (benzylaminopurine) and KIN (kinetin) in the presence of 2,4-D.

Treatments	RB855156		RB72454	
Control	0.0	d A	0.0	d A
13.5 μM 2,4-D	70.0	a A	65.0	a B
13.5 μM 2,4-D + 8.9 μM BAP	20.0	b A	21.7	b A
13.5 μM 2,4-D + 17.8 μM BAP	10.0	c A	5.0	c B
13.5 μM 2,4-D + 26.7 μM BAP	0.0	d A	0.0	d A
13.5 μM 2,4-D + 9.3 μM KIN	0.0	d A	0.0	d A
13.5 μM 2,4-D + 18.6 μM KIN	0.0	d A	0.0	d A
13.5 μM 2,4-D + 27.9 μM KIN	0.0	d A	0.0	d A
CV (%)	11.9			

Means followed by same lowercase letter (vertically) and capital letter (horizontally) do not differ according to Tukey's multiple range test ( $p < 0.05$ ).

The induction of somatic embryogenesis in Poaceae was positively affected by the association between auxins and cytokinin (BHASKARAN; SMITH, 1990). However, the association of 2,4-D with BAP at concentrations ≤ 17.8 μM BAP led to a low percentage of embryogenic callus induction in this study. No embryogenic callus induction was observed with 26.7 μM BAP or treatments containing 2,4-D combined with KIN (Table 1).

The response of both cultivars to the treatments was similar, and significant differences were only observed for the treatments containing 13.5  $\mu\text{M}$  2,4-D and 13.5  $\mu\text{M}$  2,4-D with 17.8  $\mu\text{M}$  BAP. The RB855156 cultivar exhibited superior somatic embryogenesis induction under these treatments (Table 1). A similar result was observed for the Q165<sup>A</sup> sugarcane cultivar cultured on medium with auxin (LAKSHMANAN et al., 2006).

The highest percentage of embryogenic callus induction was observed with 13.5  $\mu\text{M}$  2,4-D. The use of trichlorophenoxyacetic acid and picloran resulted in a lower percentage of embryogenic callus induction in the RB855156 cultivar, whereas no embryogenic callus formation was observed in the RB72454 cultivar, with the exception of explants cultured on medium containing 13.2  $\mu\text{M}$  picloran. Comparing the two cultivars, RB855156 exhibited a higher percentage of embryogenic callus induction (Table 2). Similar results were observed by Taylor et al. (1992), who studied embryogenic callus induction in 14 sugarcane cultivars. The best results for all the cultivars were observed when the explants were cultured on MS medium containing 13.5  $\mu\text{M}$  2,4-D.

**Table 2.** The percentage of embryogenic callus induction of two sugarcane cultivars cultured on different concentrations of auxins, 2,4-dichlorophenoxyacetic acid, (2,4-D), trichlorophenoxyacetic acid and picloran.

Treatments	RB855156		RB72454	
6.8 $\mu\text{M}$ 2,4-D	24.0	b A	16.0	b B
13.5 $\mu\text{M}$ 2,4-D	68.0	a A	52.0	a B
20.4 $\mu\text{M}$ 2,4-D	8.0	cd A	10.0	c B
6.7 $\mu\text{M}$ trichlorophenoxyacetic	12.0	c A	0.0	c B
13.4 $\mu\text{M}$ trichlorophenoxyacetic	4.0	d A	0.0	c B
20.1 $\mu\text{M}$ trichlorophenoxyacetic	4.0	d A	0.0	c B
6.6 $\mu\text{M}$ picloran	12.0	c A	0.0	c B
13.2 $\mu\text{M}$ picloran	8.0	cd A	4.0	bc B
19.8 $\mu\text{M}$ picloran	15.0	b A	0.0	c B
CV (%)	34.8			

Means followed by same lowercase letter (vertically) and capital letter (horizontally) do not differ according to Tukey's multiple range test ( $p < 0.05$ ).

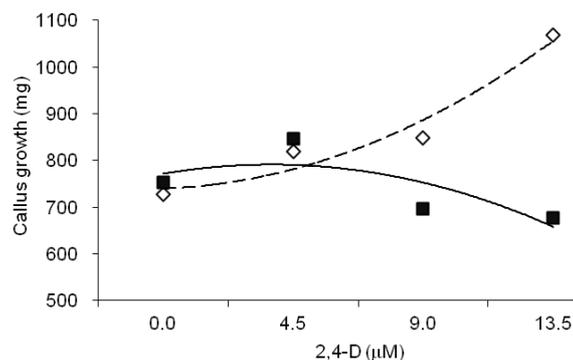
The use of 2,4-D for the induction of somatic embryogenesis in sugarcane has been reported in various studies (AHLOOWALIA; MARETZKI, 1983; CHEN et al., 1987; CHENGALRAYAN; GALLO-MEAGHER, 2001; FITCH; MOORE, 1990; GALLO-MEAGHER et al., 2000; HO; VASIL, 1983; LIU, 1993; OROPEZA; DE-GARCIA, 1996). However, the results observed here differ from those reported by Ho and Vasil (1983). These authors reported that low concentrations of 2,4-D were sufficient for embryogenic callus induction. In another study, the percentage of embryogenic callus induction in the sugarcane cultivar CP-841198 gradually decreased when the concentration of 2,4-D increased from 4.5 to 45.2  $\mu\text{M}$  after 12 weeks of

culture; the same results were observed when the explants were cultured on medium containing picloran (CHENGALRAYAN et al., 2005). In a study of the sugarcane cultivar CoJ88t, an increase in the concentration of 2,4-D from 18.1 to 36.2  $\mu\text{M}$  reduced callus induction from 86.4 to 76.0% (SINGH et al., 2008). In another study, dicamba (3,6-dichloro-2 hydroxybenzoic acid) was found to be more effective at inducing callus formation in the Nco310 sugarcane cultivar compared to 2,4-D, naphthaleneacetic acid (NAA) and 4-fluorophenoxyacetic acid (4-FPA) (BRISIBE et al., 1994). However, most studies showed that 2,4-D or 4-chlorophenoxyacetic acid (pCPA) were superior to other regulators in terms of the induction of somatic embryogenesis in sugarcane (LAKSHMANAN, 2006). Shoot regeneration from somatic embryogenesis of the sugarcane cultivar Q165<sup>A</sup> was found to occur in MS medium supplemented with NAA and pCPA (LAKSHMANAN et al., 2006).

### Callus growth

The greatest amount of callus growth in liquid medium was observed in the RB855156 cultivar in the presence of 13.5  $\mu\text{M}$  2,4-D; under these conditions, the callus weight was over 1,000 mg. Because the initial weight of each callus was approximately 14 mg, there was an average increase of 986 mg per flask. The optimal concentration of 2,4-D for callus induction in RB72454 was 4.5 mM.

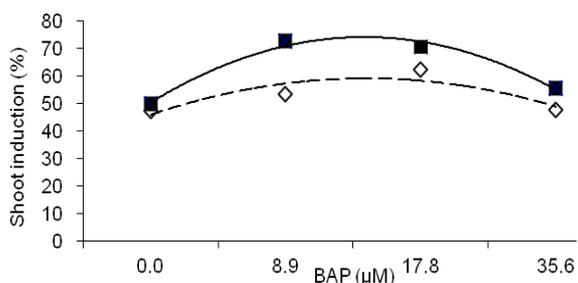
Callus growth occurred on solid medium at all concentrations of 2,4-D (4.5, 9.0 and 13.5  $\mu\text{M}$ ); however, no significant differences were observed between the treatments (Figure 1). The growth rate decreased in the absence of 2,4-D. The 4.5  $\mu\text{M}$  concentration of 2,4-D was chosen because it resulted in the same growth rate as the other concentrations. In another study, an increase in the concentration of 2,4-D was found to promote somaclonal variation in plants (SHOEMAKER et al., 1991).



**Figure 1.** Callus growth of the sugarcane cultivars RB855156 and RB72454 in the presence of various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D).

### Conversion of somatic embryos into plantlets

A higher shoot induction rate was observed in the presence of 8.9 and 17.8  $\mu\text{M}$  BAP for the RB855156 cultivar and 17.8  $\mu\text{M}$  BAP for the RB72454 cultivar. Increasing the concentration of BAP to 35.5  $\mu\text{M}$  decreased the rate of shoot induction in both cultivars (Figure 2). However, when the Q165<sup>A</sup> sugarcane cultivar was cultured on medium containing KIN, BAP or NAA, no shoots were produced, although the authors observed some shoot induction when auxins and cytokinin were added to the MS medium at a minimum concentration of 4  $\mu\text{M}$ . Shoots were induced in nearly half of the explants cultured on MS medium with 4  $\mu\text{M}$  BAP and 10  $\mu\text{M}$  NAA after 8 weeks of culture. However, the proportion of explants that induced shoots was significantly lower, especially the proportion that produced more than 20 shoots per explant when BAP was combined with KIN (LAKSHMANAN et al., 2006).

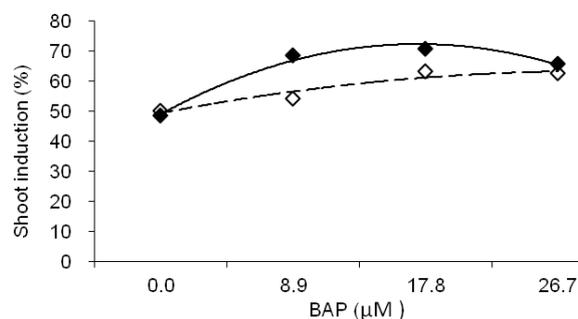


**Figure 2.** The effect of benzylaminopurine (BAP) on shoot induction in the sugarcane cultivars RB855156 and RB72454.

In the second experiment, the highest rates of shoot induction in the RB855156 cultivar were observed with 17.8  $\mu\text{M}$  and 26.7  $\mu\text{M}$  BAP. In the RB72454 cultivar, the addition of 26.7  $\mu\text{M}$  BAP resulted in the highest rate of shoot induction (Figure 3). The results presented here confirm the effect of cytokines on shoot induction as described by Ali et al. (2008). These authors studied the effect of a combination of BAP and KIN on shoot induction from sugarcane somatic embryos. The shoot induction rate was 60% in the presence of 1.11  $\mu\text{M}$  BAP and 1.16  $\mu\text{M}$  KIN. In another study, the addition of 4.44  $\mu\text{M}$  BAP to MS medium resulted in a shoot induction rate of 31.5% from embryogenic masses of sugarcane after 4 weeks of culture (ATHER et al., 2009).

Similar results were observed for sugarcane calli induced on medium containing 2,4-D. Shoot induction from somatic embryos occurred after the

calli were transferred to MS medium containing BAP and KIN (FALCO et al., 1996). In another study, shoot induction from embryogenic masses of sugarcane occurred in the presence of cytokines (FRANKLIN et al., 2006), confirming the results reported herein. Plant regeneration from an embryogenic callus obtained from immature inflorescence explants was observed on media containing 2,4-D (BLANCO et al., 1997) and various combinations of different concentrations of 2,4-D, KIN and BAP (LIU, 1993).



**Figure 3.** Effect of benzylaminopurine (BAP) concentration on shoot induction in the sugarcane cultivars RB855156 and RB72454.

### Rooting and plant acclimatization

Culture of the RB855156 cultivar on MS medium resulted in a higher percentage of rooting plants, greater number of roots, longer root length and greater percentage of normal shoot development compared to culture on MS/2 medium. Culture on MS medium containing 5.0  $\mu\text{M}$  IBA resulted in the highest percentage of rooted plants (98%) (Figure 4E); however, no statistically significant differences were observed between treatments (0; 2.5 and 5.0  $\mu\text{M}$  IBA) (Table 3).

**Table 3.** Effects of MS and MS/2 media and indolebutyric acid (IBA) concentration on rooting in the sugarcane cultivar RB855156.

IBA ( $\mu\text{M}$ )	Rooting plants (%)		Number of roots		Root length (cm)		Normal shoot development (%)	
	MS	MS/2	MS	MS/2	MS	MS/2	MS	MS/2
0	80.0 b A	70.0 a A	4.0 a A	3.0 a B	3.4 b A	2.0 a B	100.0 a A	30.2 a B
2.5	90.0 ab A	75.0 a B	4.5 a A	3.5 a B	4.8 a A	2.0 a B	98.0 a A	35.0 a B
5.0	98.0 a A	78.0 a B	4.2 a A	3.5 a B	5.0 a A	2.2 a B	100.0 a A	32.0 a B
CV (%)	10.5		9.6		6.7		9.9	

Means followed by same lowercase letter (vertically) and capital letter (horizontally) do not differ according to Tukey's multiple range test ( $p < 0.05$ ).

When the RB72454 cultivar was treated with IBA, culture on MS medium resulted in a significantly higher rate of rooting compared to MS/2 medium. No significant differences were observed between the two culture media in terms of the number of roots and root length, although

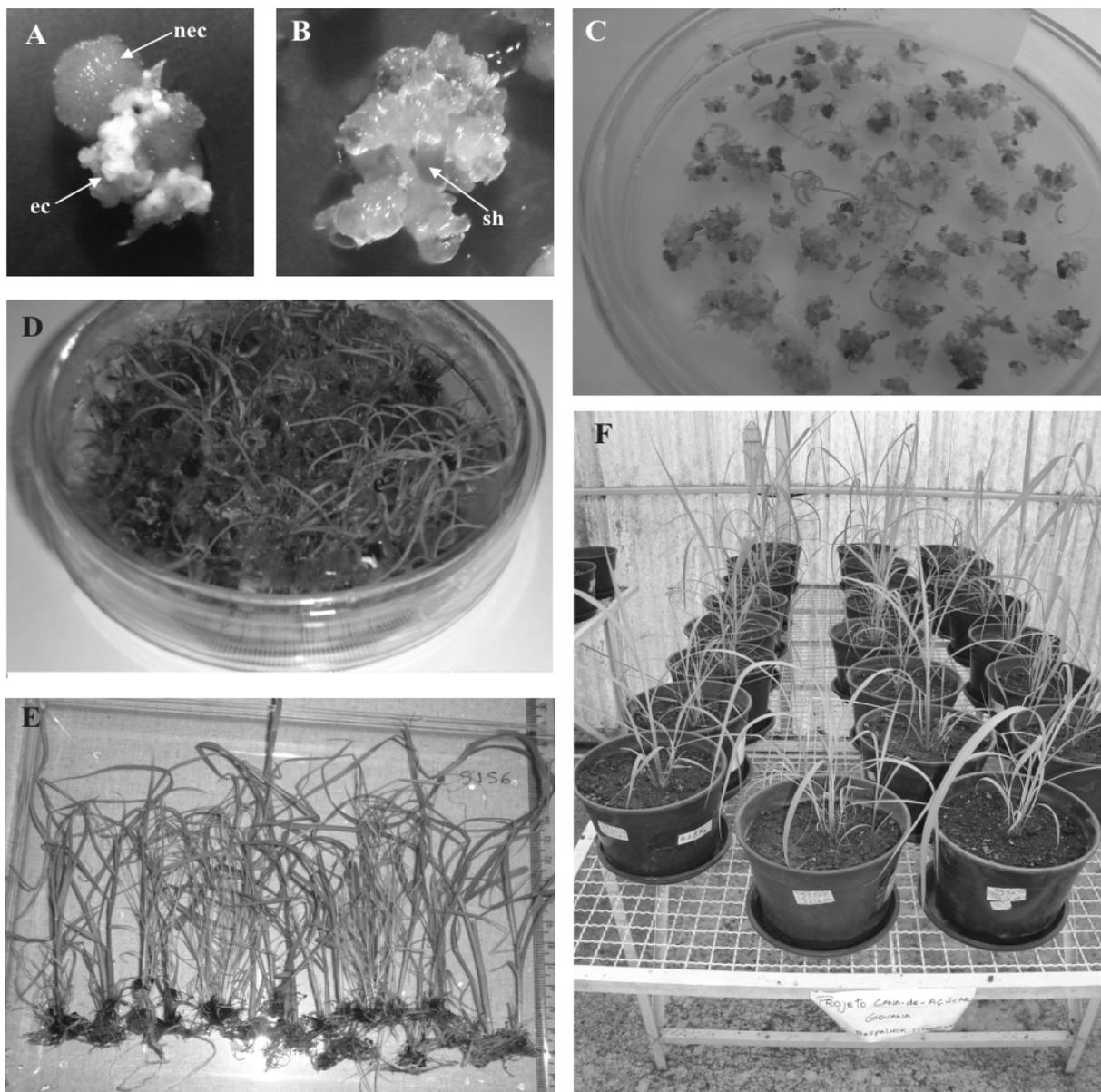
treatment with 5.0  $\mu\text{M}$  IBA and culture on MS medium resulted in longer root lengths. A significantly higher percentage of normal shoot development was observed with MS medium compared to the MS/2 medium. Regarding the IBA treatments, the longest root lengths were observed on MS medium containing 5.0  $\mu\text{M}$  IBA and MS/2 medium containing 2.5 and 5.0  $\mu\text{M}$  IBA (Table 4). Different results were observed for rooting in the sugarcane cultivars CP 77.400 and BL-4. In another study, the use of MS medium supplemented with 10.74  $\mu\text{M}$  NAA increased rooting by 90% after 6 days of culture (ALI et al., 2008).

**Table 4.** Effect of MS and MS/2 media and indolebutyric acid (IBA) concentration on rooting of the sugarcane cultivar RB72454.

IBA ( $\mu\text{M}$ )	Rooting plants (%)		Number of roots		Root length (cm)		Normal shoot development (%)	
	MS	MS/2	MS	MS/2	MS	MS/2	MS	MS/2
0	78.0 b A	70.0 a A	3.0 a A	3.4 a A	1.8 b A	2.0 b A	99.0 a A	39.5 a B
2.5	85.0 ab A	74.0 a B	3.5 a A	3.2 a A	2.1 b A	2.3 ab A	100.0 a A	32.0 a B
5.0	91.0 a A	72.0 a B	3.2 a A	2.9 a A	4.7 a A	2.6 a B	100.0 a A	35.5 a B
CV (%)	10.5		9.8		9.7		8.8	

Means followed by same lowercase letter (vertically) and capital letter (horizontally) do not differ according to Tukey's multiple range test ( $p < 0.05$ ).

According to the plant acclimatization results, the survival rate of plants regenerated *in vitro* was 100%, and no phenotypic changes were observed (Figure 4F).



**Figure 4.** Plant regeneration of the sugarcane cultivar RB855156 via somatic embryogenesis. A) nec = non-embryogenic callus; ec = embryogenic callus. B, C and D) Somatic embryo development and formation of shoots; sh = shoots. E) Rooted plants cultured on MS medium containing 5.0  $\mu\text{M}$  IBA. F) Acclimatized plants.

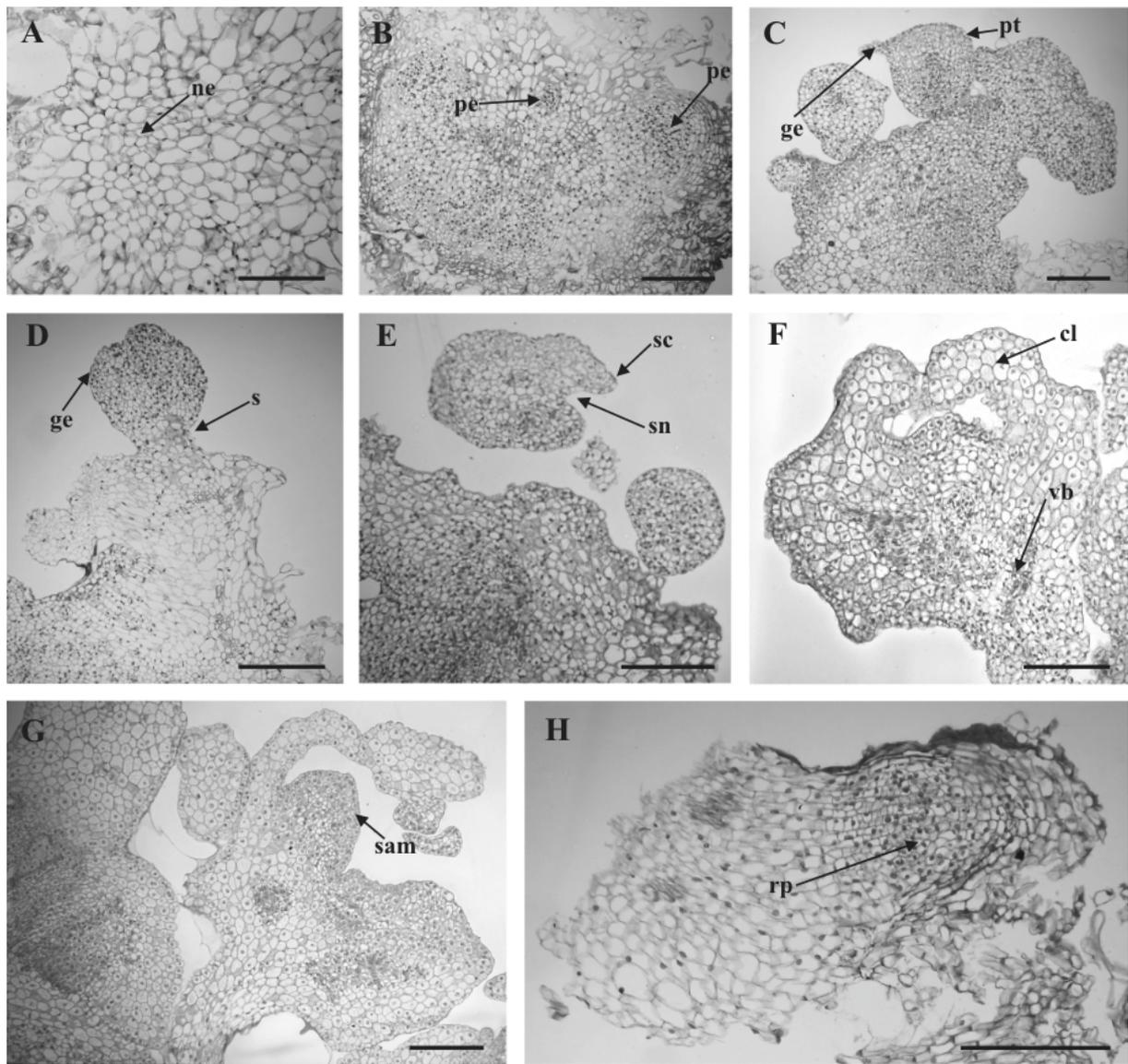
### Histological study

According to the results of the histological study, there were two types of callus induction: mucilaginous and nodular. The mucilaginous callus had a soft consistency, became necrotic, did not exhibit an embryogenic nature and did not regenerate into plants (Figure 4A). The nodular callus was whitish in color, composed of cells with a dense cytoplasm and a nucleus with an evident nucleolus and had a high nucleus/cytoplasm ratio, indicating the presence of meristematic zones that could lead to the development of somatic embryos (Figure 4A) and the formation of shoots (Figure 4B, C and D).

No significant differences were observed between the histological structures of RB72454 and

RB855156. The anatomy of both sugarcane cultivars was found to be similar to that previously reported for other monocotyledonous species (GEORGE, 1993) (Figure 5), including grasses (GUIDERDONI; DEMARLY, 1988; VASIL et al., 1984) such as *Saccharum* spp. (NADAR et al., 1976).

In some cases, non-embryogenic calli were found (Figure 5A), which had larger cells with generally obscured nuclei. However, various developmental stages of somatic embryogenesis were observed. Initially, we observed the formation of pro-embryos with six or more cells (Figure 5B). Generally, clusters of somatic embryos are formed by meristematic cells with a dense cytoplasm, large nucleus and many starch grains (GEORGE, 1993).



**Figure 5.** Histological study of somatic embryogenesis of the sugarcane cultivar RB72454. A) ne = non-embryogenic callus. B) pe = pro-embryos. C) ge = globular embryos, pt = protoderm. D) ge = globular embryos, s = suspensor. E) sn = node-scutellar, sc = scutellum. F) cl = coleoptile, vb = vascular bundle. G) sam = shoot apical meristema. H) rp = root pole. Bar = 200  $\mu$ m.

Globular embryos formed after cell division, and a protoderm was evident (Figure 5C). In some cases, globular embryos attached to the callus surface by a suspensor were observed (Figure 5D). However, in many cases, the suspensor was not clearly distinguishable or was not observed as a multicellular structure. When the suspensor was present, it ranged from a uniserial or biserial structure to a large multicellular structure, as previously observed by Ho and Vasil (1983).

The further development of globular somatic embryos was characterized by the formation of a terminal leaf node, called a scutellar node, which indicated the beginning of the formation of a scutellum (Figure 5E). The scutellum consists of cells that are richly cytoplasmic and irregularly shaped and contains tracheids in procambium that is bordered by typical epithelial cells; this was previously observed in sugarcane by Guiderdoni and Demarly (1988). An increased number of cell layers in the scutellum and other stages of embryonic development were observed when the coleoptile emerged (Figure 5F). The somatic embryos of monocotyledons initially go through a globular developmental stage; however, unlike dicotyledons, somatic embryos develop in the scutellum and coleoptile (GEORGE, 1993).

After several days of culture, an increased number of cell layers were observed in the scutellum and coleoptile; the apical shoot was also evident in the region of the scutellar node (Figure 5G). This showed continuity with the root vascular system (Figure 5F), as observed by Vasil et al. (1985). Later, a more advanced stage of somatic embryo development was observed (Figure 5G) with a shoot apical meristem, leaf primordia and a more developed coleoptile and scutellum aligned with the root meristems.

The cross-sections illustrate the differentiation of a somatic embryo with a bipolar organization formed by the shoot and root axis (Figure 5H), which was observed previously by other authors (HO; VASIL, 1983). According to the histological analysis of the two cultivars, plant regeneration in the present study occurred via somatic embryogenesis. The somatic embryos exhibited typical bipolar orientation of the apical and root poles and an absence of vascular connections between these structures and the underlying callus. The results described herein agree with the morphological characteristics of somatic embryos described by Reinert (1977) and Guiderdoni and Demarly (1988). These authors observed the same characteristics when they studied the somatic

embryogenesis of sugarcane. Both studies demonstrated the typical bipolar orientation and the absence of vascular connections with the original tissue of the explant.

## Conclusion

The sugarcane explants were responsive to shoot regeneration, and acclimatized plants were obtained for both cultivars. A histological investigation revealed that the adventitious shoots arose via somatic embryogenesis. All stages of development during somatic embryogenesis were observed in both cultivars. The information described herein could be used for regeneration protocol optimization for other sugarcane cultivars and for the production of plants through genetic transformation.

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