EMBRYOGENESIS

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High-efficiency somatic embryogenesis of a broad range of Brazilian *Saccharum* spp. hybrids (sugarcane) varieties using explants from previously established *in vitro* plants

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Abstract

The current study assessed the embryogenic potential of different Brazilian *Saccharum* spp. hybrid (sugarcane) varieties, using explants from previously established *in vitro* plants, and determined the morphogenic capacity of calluses to regenerate plants during consecutive subcultures. After examining various explant lengths (1 to 15 mm) for callus initiation, an optimal length of 12 mm was determined, and 14 sugarcane varieties were studied. Callus induction occurred on Murashige and Skoog medium with 3.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid, in the dark. After 40 d, calluses were divided according to their predominant type: the mucilaginous part remained in the dark on fresh induction medium, whereas the compact nodular callus fraction was transferred to a regeneration medium containing 1.86 mg L^{-1} 1-naphthaleneacetic acid and 0.09 mg L^{-1} 6-benzylaminopurine and was cultivated in light. This callus selection based on morphological type took place over three consecutive subcultures, spanning 22 wk. The embryogenic process was asynchronous and the formation of the first plants was registered at 6 wk of cultivation. The embryogenic callus regeneration rate was kept at over 60% until the third subculture in 13 of the 14 varieties examined. However, delayed regeneration was observed, likely due to the aging of calluses. The protocol reported here utilized the embryogenic potential of sugarcane varieties in combination with efficient and quick regeneration and should thus provide an attractive alternative source material for the production of transgenics and/or large-scale clonal multiplication of sugarcane.

Keywords *Saccharum* spp. callus culture · Selective subculture · Morphogenic capacity · Large-scale propagation · Regeneration

Introduction

Saccharum spp. (sugarcane) was brought to Brazil during colonial times and has since become one of the country's key economically important crops. Brazil is currently one of the world's leading producers of sugar and ethanol (Brasil 2016). For the 2017–2018 harvest, the cultivated area for sugar and ethanol production has been estimated at 8.73 million ha in the Brazilian producer states, with an average productivity of approximately 72,000 kg ha^{-1} (Conab 2018).

Greatly heterogeneous, sugarcane plants are normally propagated by cuttings of stalks with one or more buds (Tiwari *et al.* 2010; Singh *et al.* 2013), or, within a recently adopted modality, by implementing a system of planting pre-sprouted plantlets (Landell *et al.* 2012). Because of the demand for sugarcane propagation is coupled with limited availability of seeds, a variety of diseases affect sugarcane, and world pressure for increased sugarcane productivity, the need to exploit biotechnological tools to obtain first-class, large-scale, and high-yield material is evident (Tiwari *et al.* 2010).

Tissue culture includes several techniques likely to be important allies for the production of economically significant species, among which somatic embryogenesis should be highlighted. Somatic embryogenesis in sugarcane is a process that has been under study for a number of years by researchers worldwide. Nonetheless, established protocols currently available are not always clearly disclosed and/or do not apply to every sugarcane variety. Further, there is scant knowledge on the persistence of regeneration capacity by embryogenic calluses during *in vitro* cultivation. Most protocols involve

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immature leaves taken from stem tips, gathered from field plants (Taparia *et al.* 2012; Alcantara *et al.* 2014; Kaur and Kapoor 2016), which are usually not physiologically uniform among the specimens and/or at different seasons, impairing the method's reproducibility. Moreover, several inconveniences are extant, such as a high rate of contamination by endophytic microorganisms and the release of large amounts of phenolic compounds into the culture medium (Garcia *et al.* 2007). Evaluating the efficiency of various sterilizing agents and the exposure of explants to heat, Moutia and Dookun (1999) verified that none of the tested methods could completely eliminate endogenous microorganisms and indicated that other resources must be discovered to increase micropropagation success.

The current study defined several cultivating conditions that ensured the efficient and quick regeneration of 14 Brazilian sugarcane varieties from embryogenic callus. Somatic embryogenesis has a number of practical applications, particularly massive propagation. It may also be employed for the production of synthetic seeds, cryopreservation of germplasm, and genetic modification by transformation (von Arnold *et al.* 2002).

In fact, substantial efforts have been made over the last decade to develop an efficient genetic transformation system for sugarcane (Sengar *et al.* 2011). Different transformation techniques, such as electroporation (Seema *et al.* 2001), bombardment with particles (Taparia *et al.* 2012), and gene transfer mediated by *Agrobacterium tumefaciens* (Manickavasagam *et al.* 2004; Joyce *et al.* 2010), have been studied to employ genes of agronomic and industrial interest for the improvement of the genus *Saccharum*. However, before genetic transformation can be routinely successful, the type of explant employed and the morphogenic regeneration method used must necessarily be well defined. Under the totipotency theory, any living part of a plant may be used as material for *in vitro* growth (Krikorian and Berquam 1969). In general, embryogenic calluses have been a preferred tissue, chiefly due to their greater proportion of cells capable of clonal proliferation and subsequent regeneration under adequate cultivating conditions (Basnayake *et al.* 2011).

The current study improved the protocol and analyzed the embryogenic potential of 14 Brazilian sugarcane varieties through the use of *in vitro* plants as a source for explants. Moreover, the present study not only described the morphoanatomical stages involved in the process, but also assessed the morphogenic capacity and regeneration frequency of consecutive subcultures.

Materials and Methods

Plant material Sugarcane plants with 8-mo-old were used as source of stem cuttings. In the laboratory, stem tips were disinfected with 70% (*v/v*) alcohol for 1 min, with sodium hypochlorite (NaClO; 2.5% (*w/v*) active chlorine) for 20 min, and rinsed three times in autoclaved distilled water, for the preparation of *in vitro* explants. After surface sterilization, shoot tips with 2 or 3 leaf primordia and approximately 1–2 mm were extracted with the aid of a stereomicroscope, and pre-cultured for 72 h in the dark in 25 × 150 mm test tubes containing 10 mL of MS (Murashige and Skoog 1962) salts and vitamins, supplemented with 0.2 mg L^{-1} 6-benzylaminopurine (BAP), 0.1 mg L^{-1} kinetin (Kin), 20 g L^{-1} sucrose, and solidified with 2.3 g L^{-1} of Phytagel™, according Nogueira *et al.* (2013). After 40 d of cultivation at 25 ± 2°C with a 16-h photoperiod and irradiance of 30 μmol m^{-2} s^{-1} provided by Universal 40 W cool white fluorescent lamps (Osram, Alphaville Barueri, SP, Brazil), the regenerated 5-to 7-cm long shoots were transferred to 250-mL glass flasks containing the same culture medium used to establish the plants, where, after one 30-d subculture cycle, multiplied shoots were used as a source of explants for somatic embryogenesis experiments. The media pH was adjusted to 5.8 ± 0.1 with KOH prior to autoclaving at 121°C and 101.3 kPa for 20 min.

Determining explant size for induction of somatic embryogenesis To determine the influence of explant size on induction of somatic embryogenic callus, shoots from two sugarcane varieties (RB85-5453 and IAC86-2210) were used as explant sources. Shoots, roughly 3.0-cm long, were isolated in a laminar flow cabinet and cut transversely into 1.0-, 3.0-, 6.0-, 9.0-, 12.0-, or 15.0-mm segments. Regardless of length, one shoot was used for each explant. Explants were placed on an induction culture medium containing MS salts and vitamins, supplemented with 3.0 mg L^{-1} 2,4-diclorophenoxyacetic acid (2,4-D; Ho and Vasil 1983), 30 g L^{-1} sucrose, and solidified with 2.3 g L^{-1} of Phytagel™. The medium pH was adjusted to 5.8 ± 0.1 with KOH prior to autoclaving at 121°C and 101.3 kPa for 20 min. After transfer to induction medium in a laminar flow cabinet, the explants were kept in the dark at 25 ± 2°C. Average primary callus formation rate was assessed every wk over a total of 40 d of cultivation.

The experiment was conducted in an entirely randomized design. For replication, three 15 × 90 mm Petri dishes were employed, each with five shoot explants placed horizontally onto the medium for each length group and sugarcane variety.

Assessing the embryogenic potential of Brazilian sugarcane varieties Fourteen Brazilian sugarcane varieties (RB83-160, RB99-395, RB86-3129, RB95-1541, CB45-3, SP85-4594, SP70-1143, SP13-3804, SP81-3250, SP85-1431, SP71-6949, VAT90-61, VAT90-212, and VAT90-186), retrieved as stem cuttings from the Germplasm Bank at the Embrapa Coastal Tablelands, Rio Largo, AL, Brazil, were

used to confirm sugarcane embryogenic potential. Simões Neto (2009) and Sindaçucar (2018) may be consulted to verify the varieties' agronomic characteristics and agronomic-industrial performance.

After the varieties were established *in vitro*, the stem segments were isolated and cut according to the results obtained in the preceding experiment and inoculated onto embryogenic callus induction medium.

Replication comprised of five 15 × 90 mm Petri dishes each with five explants placed horizontally on the culture medium for each variety. Cultures were kept in a growth chamber in the dark, at $25 \pm 2°C$, for a 40-d period. The formation of primary and embryogenic calluses was then assessed. The calluses were subsequently divided according to their morphological types: primary and embryogenic portions. Portions of primary callus were transferred to fresh induction medium and kept in the dark, whereas the embryogenic callus portions were inoculated onto a modified regeneration medium from Taparia *et al.* (2012), containing MS salts and vitamins, supplemented with 1.86 mg L^{-1} NAA and 0.09 mg L^{-1} BA, 30 g L^{-1} sucrose, and solidified with 2.3 g L^{-1} of Phytagel™, and transferred to light conditions in a growth chamber with a 16-h photoperiod and irradiance of 30 μmol m^{-2} s^{-1} provided by Universal 40 W cool white fluorescent lamps (Osram), at $25 \pm 2°C$. All chemicals above were obtained from Sigma-Aldrich®, St. Louis, MO.

The callus selection process, based on morphological types, was performed over three consecutive subcultures of 40 d each. After the end of each 40-d period, the primary callus portions were assessed with regard to the production of new calluses with the previously mentioned embryogenic features. On the other hand, the embryogenic calluses were assessed with regard to the regeneration percentage (percentage of green area containing somatic embryos at different development stages) and the number of plants (≥ 0.5-cm height) formed obtained by counting.

Morpho-histological analysis of somatic embryogenesis in sugarcane The histological analysis comprised the collection of primary and embryogenic callus samples and their fixation in formaldehyde, acetic acid, and 70% (*v/v*) ethanol at a 1:1:18 (*v/v*) ratio (FAA 70; Johansen 1940). The samples were subsequently dehydrated in an increasing ethanol series [70–100% (*v/v*)], infiltrated and embedded in Histo-Resin (Leica, Heidelberg, Germany), following manufacturer's recommendations. Crosswise and lengthwise serial sections (7–10-μm thick) were obtained by manual microtome (model RM 2125; Leica) and sections were then smoothed and placed on microscope slides on a hot plate heated to 40°C.

The samples were stained with toluidine blue (O'Brien *et al.* 1964), followed by permanent mounting with Entellan® (Merck, Darmstadt, Germany). Results were recorded under a light microscope (BA300; Motic, Xiamen,

PR China), coupled to a digital image capturing system (ImagePro Plus 4.5 software, Media Cybergetics, Rockville, MD).

Development and acclimatizing of regenerated plants Regenerated *in vitro* sugarcane plants were transferred to 250-mL glass flasks containing MS culture medium to which 30 g L^{-1} sucrose and 2.3 g L^{-1} of Phytagel™ were added to improve the development of both the shoot and root systems. After inoculation, the flasks were closed with plastic lids and sealed with transparent plastic film, and shoots were maintained in the growth chamber with a 16-h photoperiod and irradiance of 30 μmol m^{-2} s^{-1} provided by Universal 40 W cool white fluorescent lamps (Osram), at $25 \pm 2°C$, for 30 d.

The pre-acclimatization process started while the plants were still in the growth chamber. Initially, at the end of the 30 d, the plastic wrap used to seal the flasks was removed. After 72 h, the flask covers were also removed. After a 48-h period without any kind of cover had elapsed, the shoots were rinsed in running water to remove culture medium residues and taken to the greenhouse, where they were transplanted into 60-cell trays containing a 1:1 ratio (*v/v*) substrate (Bioplant, Nova Ponte, MG, Brazil) and washed sand mix. Survival percentage was assessed after 21 d of acclimatizing in the greenhouse. In the greenhouse, plantlets were irrigated manually every 24 h but not fertilized. Greenhouse conditions were $75 \pm 5\%$ relative humidity, $30 \pm 3°C$, with photosynthetic photon flux density of 450–500 μmol m^{-2} s^{-1} and a 12-h photoperiod.

Data analysis Data obtained in the current study were submitted to analysis of variance (ANOVA), while means were compared by Scott-Knott test at 5% probability ($P \leq 0.05$), with the Sisvar 4.4 (Ferreira 2011) statistical analysis program. Data expressed as percentage were transformed by arcsine square root $[(0.01x \div 100)^{1/2}]$.

Results and Discussion

Determining explant size for induction of somatic embryogenesis Results (Fig. 1) showed that the length of the initial stem segment explant had a close relationship with primary callus formation and proliferation. The formation of calluses in the two tested sugarcane varieties (RB85-5453 and IAC86-2210) was noted by the seventh day of cultivation in the more internal leaf layers, chiefly in the basal part close to the explants' meristem region. After 40 d, the formation of primary callus with a brittle aspect (Fig. 2A) was more pronounced and reflected a clear influence of explant length on responses. Specifically, explants exceeding 9 mm in length were the most responsive, averaging roughly 76% callus
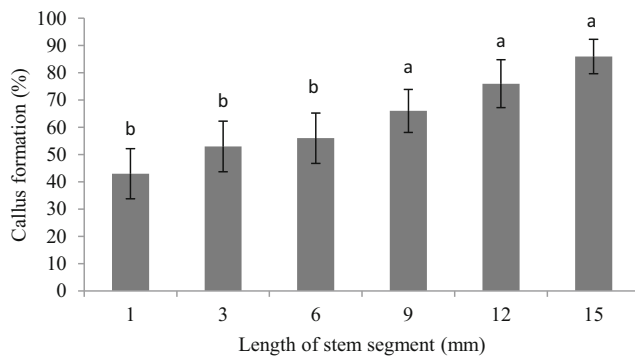
Figure 1. Influence of *Saccharum* spp. hybrids (sugarcane) stem explant length on primary callus formation rates on MS (Murashige and Skoog 1962) medium supplemented with 3.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid for 40 d. *Means* (± SE) with the *same letter* belong to the same group, by Scott-Knott test at $P \leq 0.05$.

formation (Fig. 1). Similar to the current findings, when Brisibe *et al.* (1994) used explants measuring 2 or 3 mm in length from leaf segments of *in vitro* plants for somatic embryogenesis in a sugarcane variety, an average of 48% of initial explants exhibited callus formation when inoculated on an induction medium. The lower callus induction rates of the shorter explants might reflect the strong phenolic oxidation found on the cut surface that might have damaged the development of callus-forming masses, as oxidation took place in larger explants on the most extreme portion and likely failed to interfere with the proliferation of callus at the opposite end. Owing to these results, the present investigation used explants with an initial length of 12.0 mm, measured from the basal part (meristem), excluding the first two most external leaves of the foliar segments, prior to inoculation.

Embryogenic potential of Brazilian sugarcane varieties
The 14 sugarcane varieties (Table 1) tested in the current study responded significantly to primary callus induction, with an average 94% formation rate (data not show). As in the preceding experiment, cell proliferation took place during the first weeks of cultivation from the explants' basal portion, which developed and gave rise to brittle calluses of yellowish color and mucilaginous aspect (Fig. 2A). However, a compact nodular callus of a whitish color was detected over time, especially in the first subculture, above or adjacent to the mucilaginous tissue (Fig. 2B). According to Ho and Vasil (1983), the mucilaginous callus features a brittle and brilliant aspect. It is not embryogenic and basically consists of slender and dissociated cells. On the other hand, the whitish compact calluses consist of small cells with a dense cytoplasm, which under ideal cultivating conditions will generate the embryoids of the species. Despite the efficiency in primary callus induction of the varieties in the present study, it became apparent that the evolution from the primary callus to the embryogenic stage was genotype-dependent and varied significantly in the course

of three consecutive subcultures (Table 1). In general, the monitoring of calluses over time revealed that primary calluses of a brittle and mucilaginous aspect gave rise to embryogenic nodular portions on their tissue during the first subculture in 13 of 14 tested varieties (Table 1), with SP85-1431 being the only exception. In most cases embryogenic callus production recurred in consecutive subcultures, i.e., the primary calluses preserved their morphogenic ability when kept on induction medium (Table 1). It should be recalled that during subcultures, the calluses were separated in portions according to their morphological features. Consequently, primary callus portions remained in the medium containing auxin to form new embryogenic calluses. The embryogenic callus portions were transferred to the regeneration medium. Among the sugarcane varieties studied that displayed a constant frequency in producing embryogenic calluses during the three subcultures under analysis, SP70-1143, VAT90-61, SP71-6949, and VAT90-212 maintained averages of 32.3, 33.3, 36.0, and 39.0%, respectively (Table 1).

When transferred to the regeneration medium, the nodular embryogenic calluses produced during the three subcultures multiplied and soon showed their ability to regenerate by means of an indicative appearance of globular stage embryos (*GE*; Fig. 2C). The embryos started differentiating from the border of the white compact nodular structures after approximately 10 d of cultivation, followed by coleoptile development and somatic embryo germination (Fig. 2D). During this stage, an asynchronous embryogenic process of the sugarcane varieties under study was indicated by the emergence of somatic embryos (*SE*) with scutellum and coleoptile, green regeneration areas containing somatic embryos at different stages of development, and regenerated plants (*RP*) on the same callus globular structures (Fig. 2C).

Garcia *et al.* (2007) confirmed the formation of globular embryos after 15 d, mature embryos after 25 d, and the start of plant conversion after 30 d from transferring calluses to the regeneration medium lacking growth regulators. Although these authors also employed *in vitro* explants as a source of explants, their regeneration process was more belated than that observed in the current study, since in the present study, it was possible to see the formation of the first sugarcane plants after 15 d on regeneration medium.

Indeed, the current protocol proved to be efficient: in addition to precocity in starting regeneration of the sugarcane varieties, the frequency reached 100% when embryogenic calluses from the first subculture were transferred to a regeneration medium and exposed to light conditions (Table 1; Fig. 2D). Nonetheless, the average number of plants formed per explant was significantly different among the varieties; for example, although VAT90-61, VAT90-212, SP70-1143, SP81-3250, SP81-3804, RB99-395, and SP85-4594 averaged 19 plants per embryogenic callus from the first subculture,

Figure 2. Depiction of typical somatic embryogenesis induced from stem segments of *Saccharum* spp. hybrids (sugarcane) varieties previously established *in vitro*. (*A*) Primary, brittle, mucilaginous callus formed after 40 d on MS (Murashige and Skoog 1962) induction medium supplemented with 3.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid; (*B*) whitish nodular compact embryogenic callus formed on mucilaginous tissue; (*C*) asynchronous embryogenic process following transfer to regeneration medium containing 1.86 mg L^{-1} 1-naphthaleneacetic acid and 0.09 mg L^{-1} 6-benzylaminopurine. (*D*) Plant formation on almost the entire callus surface. (*E*) Regenerated plantlets from somatic embryos. (*F*) Acclimatized plants in the greenhouse. *MC*, mucilaginous callus; *WC*, white compact nodular callus; *GE*, globular embryo structure; *SE*, somatic embryos; *RP*, regenerated plant. *Scale bars A, B, C, D,* and *E =* 0.5 cm; *scale bar F =* 1.0 cm.
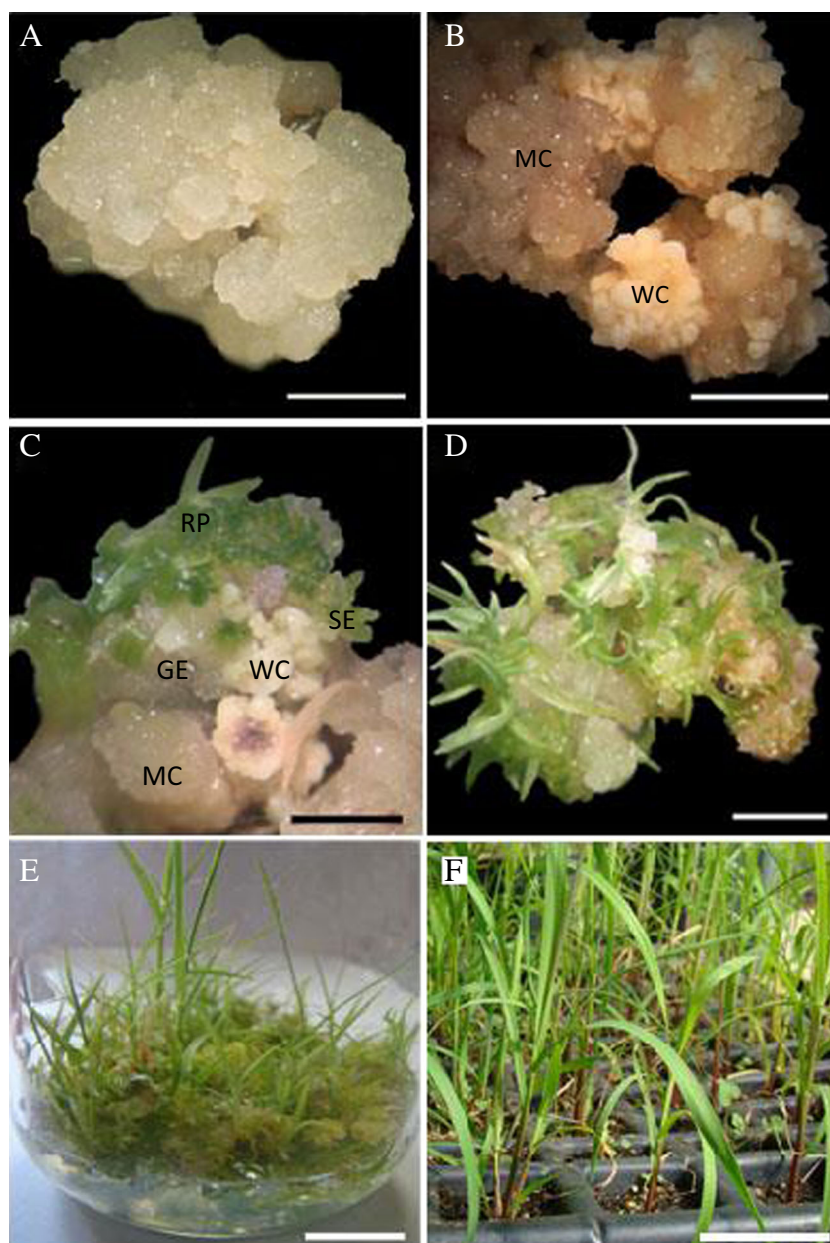
their individual values ranged from 15.3 to 23.3 shoots formed (Table 1).

The embryogenic callus regeneration rate stayed above 60% until the third subculture in 7 of the 14 sugarcane varieties assessed. Nonetheless, VAT90-186 and SP81-3250 lost their regeneration ability by the end of the experiment (Table 1). These data demonstrated the influence of time on somatic embryogenesis in sugarcane. Singh *et al.* (2008) confirmed maximum regeneration efficiency in 34.6% of calluses when inoculated on MS medium with the addition of 0.5 mg L^{-1} of BAP, whereas Dibax *et al.* (2011), studying two Brazilian sugarcane varieties (RB93-1003 and RB98-710), reported rates between 70 and 80% in inducing shoots based on embryogenic masses, also in the presence of BAP. In another study, Basnayake *et al.* (2011) obtained over 50% regeneration rates for embryogenic calluses with a nodular appearance inoculated on a basal MS medium without growth regulators.

An aspect of the current study revealed that, despite preserving the regeneration ability of embryogenic calluses from a number of varieties, the number of shoots taller than 0.5 cm was significantly reduced over cultivation time. This was demonstrated by the observation that both shoot formation and growth slowed with aging of calluses. The ability to maintain a callus culture over extended periods of time is a unique feature of a species and a constant effort among researchers, as it allows such materials to be employed quickly in experimental processes (Pola *et al.*

Table 1. Influence of cultivating time on the frequency of embryogenic callus formation, regeneration rate, and average number of shoots formed, using stem segments from *in vitro* plants of Brazilian *Saccharum* spp. hybrids (sugarcane) varieties as the initial explant source

Variety	Subculture 1			Subculture 2			Subculture 3		
	Embryogenic callus (%)	Regeneration (%)	Number of shoots*	Embryogenic callus (%)	Regeneration (%)	Number of shoots*	Embryogenic callus (%)	Regeneration (%)	Number of shoots*
RB86-3129	28.0 ± 9.1 b	100.0 a	5.5 ± 0.5 b	30.0 ± 9.5 a	80.0 ± 13.3 a	8.6 ± 1.9 a	18.0 ± 7.8 b	40.0 ± 24.5 b	0.0 ± 0.0 a
SP85-1431	0.0 ± 0.0 b	0.0 b	0.0 ± 0.0 c	13.0 ± 9.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	13.0 ± 9.0 b	0.0 ± 0.0 b	0.0 ± 0.0 a
RB83-160	16.0 ± 7.5 b	100.0 a	9.7 ± 2.2 b	20.0 ± 8.1 b	100.0 ± 0.0 a	4.5 ± 1.8 b	17.0 ± 8.8 b	100.0 ± 0.0 a	6.3 ± 1.4 a
SP81-3804	56.0 ± 10.1 a	100.0 a	15.3 ± 2.5 a	32.0 ± 9.5 a	85.0 ± 15.4 a	10.0 ± 5.4 a	12.0 ± 6.9 b	100.0 ± 0.0 a	0.0 ± 0.0 a
VAT90-186	32.0 ± 9.5 a	100.0 a	10.4 ± 1.7 b	41.0 ± 10.1 a	50.0 ± 18.9 b	0.2 ± 0.0 b	20.0 ± 8.3 b	0.0 ± 0.0 b	0.0 ± 0.0 a
RB99-395	24.0 ± 8.7 b	100.0 a	16.2 ± 3.3 a	20.0 ± 8.2 b	62.0 ± 18.3 a	1.4 ± 0.7 b	12.0 ± 7.4 b	66.0 ± 33.0 a	0.0 ± 0.0 a
CB45-3	40.0 ± 10.0 a	90.0 a	11.5 ± 2.5 b	36.0 ± 9.8 a	80.0 ± 20.0 a	2.0 ± 1.0 b	0.0 ± 0.0 b	66.0 ± 33.3 a	1.33 ± 1.0 a
VAT90-61	24.0 ± 8.7 b	100.0 a	19.5 ± 2.2 a	40.0 ± 10.0 a	100.0 ± 0.0 a	2.8 ± 1.0 b	36.0 ± 9.8 a	77.0 ± 15.7 a	1.5 ± 0.2 a
VAT90-212	32.0 ± 9.5 a	100.0 a	20.5 ± 3.0 a	52.0 ± 10.2 a	85.0 ± 10.4 a	10.0 ± 2.5 a	33.0 ± 12.0 a	60.0 ± 24.5 a	0.0 ± 0.0 a
SP85-4594	16.0 ± 7.5 b	100.0 a	15.7 ± 3.5 a	36.0 ± 9.8 a	88.0 ± 11.1 a	7.0 ± 1.9 a	12.0 ± 6.6 b	100.0 ± 0.0 a	4.2 ± 2.2 a
SP70-1143	32.0 ± 9.5 a	100.0 a	23.3 ± 3.1 a	36.0 ± 9.8 a	75.0 ± 21.0 a	1.7 ± 1.1 b	29.0 ± 9.5 a	33.0 ± 16.4 b	0.0 ± 0.0 a
RB95-1541	16.0 ± 7.5 b	100.0 a	10.0 ± 3.8 b	8.0 ± 5.5 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 a
SP81-3250	24.0 ± 8.7 b	100.0 a	23.0 ± 3.2 a	16.0 ± 7.5 b	75.0 ± 16.4 a	2.6 ± 0.9 b	0.0 ± 0.0 b	0.0 ± 0.0 b	0.0 ± 0.0 a
SP71-6949	24.0 ± 8.7 b	100.0 a	14.0 ± 2.4 b	36.0 ± 9.8 a	71.0 ± 18.4 a	17.0 ± 4.5 a	48.0 ± 10.2 a	44.0 ± 17.5 b	0.6 ± 0.3 a

Means (± SE) followed by the same *lowercase letter* in a *column* do not differ significantly by Scott-Knott test at $P \leq 0.05$.

*Number of shoots of $\geq$ 0.5-cm length

2009). However, this type of extended culture becomes feasible only when the ability to regenerate is also supported throughout the course of cultivation time. In general, the calluses of 13 sugarcane varieties in the present study maintained their regeneration abilities between the induction of embryogenic callus and the third subculture on regeneration medium for over 22 wk of cultivation (Fig. 3), with a notable exception of the cultivar SP85-1431 that in none of the subcultures presented plant regeneration (Table 1).

Apparently, no morphological variations were detected in the regenerated materials that could be related to somaclonal variation. In sugarcane, the detection of phenotypic variations due to the occurrence of somaclonal variation is relatively easy to observe *in vitro*, due to the reduced internodal length of the shoots, giving it a rosette appearance (Ramage and Williams 2004; Nogueira *et al.* 2015), or due the regeneration of albino shoots, which occurs due to lack of chloroplast (Gosal *et al.* 1998). Some shoots can have extremely narrow leaves, while others have wide, short, thick leaves (Ahloowalia and Maretzki 1983). Possibly, the fact that the number of induction subcultures was purposely limited to only three influenced this apparent lack of phenotypic variation, since it is quite common to find in the literature reports of somaclonal variation occurrence in sugarcane due to the excessive number of subcultures (Lee 1987; Sobhakumari 2012; Hsie *et al.* 2015; Nogueira *et al.* 2015; Martínez-Estrada *et al.* 2017).

Histological analysis Anatomically, the mucilaginous type of callus had a large portion of cells in an elongated format with large intercellular spaces (Fig. 4A). Nonetheless, meristematic cells containing dense cytoplasm and a voluminous nucleus could be detected in the aggregate (Fig. 4A, *arrow*). These meristem cells developed to form compact nodular masses, which consisted of small, juxtaposed cells undergoing intense cell division (Fig. 4B), as usually found during the initial formation of embryogenic calluses. These explant cell features of dedifferentiation and subsequent development of meristematic regions was similar for all varieties under study, and corroborated previous findings by such pioneers in the study of somatic embryogenesis of sugarcane as Ho and Vasil (1983) and Chen *et al.* (1988).

When transferred to a regeneration culture medium, compact nodular embryogenic calluses displayed pro-embryo formation in a linear stage with two to four cells (Fig. 4C), particularly in the peripheral region. Pro-embryos were apparently enveloped in a fine layer of cell wall (Fig. 4C). Intense cell divisions of the pro-embryonic apical cell gave rise to globular stage embryos (Fig. 4D). At this stage, a suspensor cell (*SP*; Fig. 4D) was seen in the globular pro-embryo's base, connecting the embryo to the compact nodular callus. The existence of the suspensor cell existence pointed to the embryos' single-cell origin, although multi-cell origin somatic embryos had also been detected (data not shown). According to Sané *et al.* (2006), the single-cell origin is a typical form of embryogenesis development where the competent cell
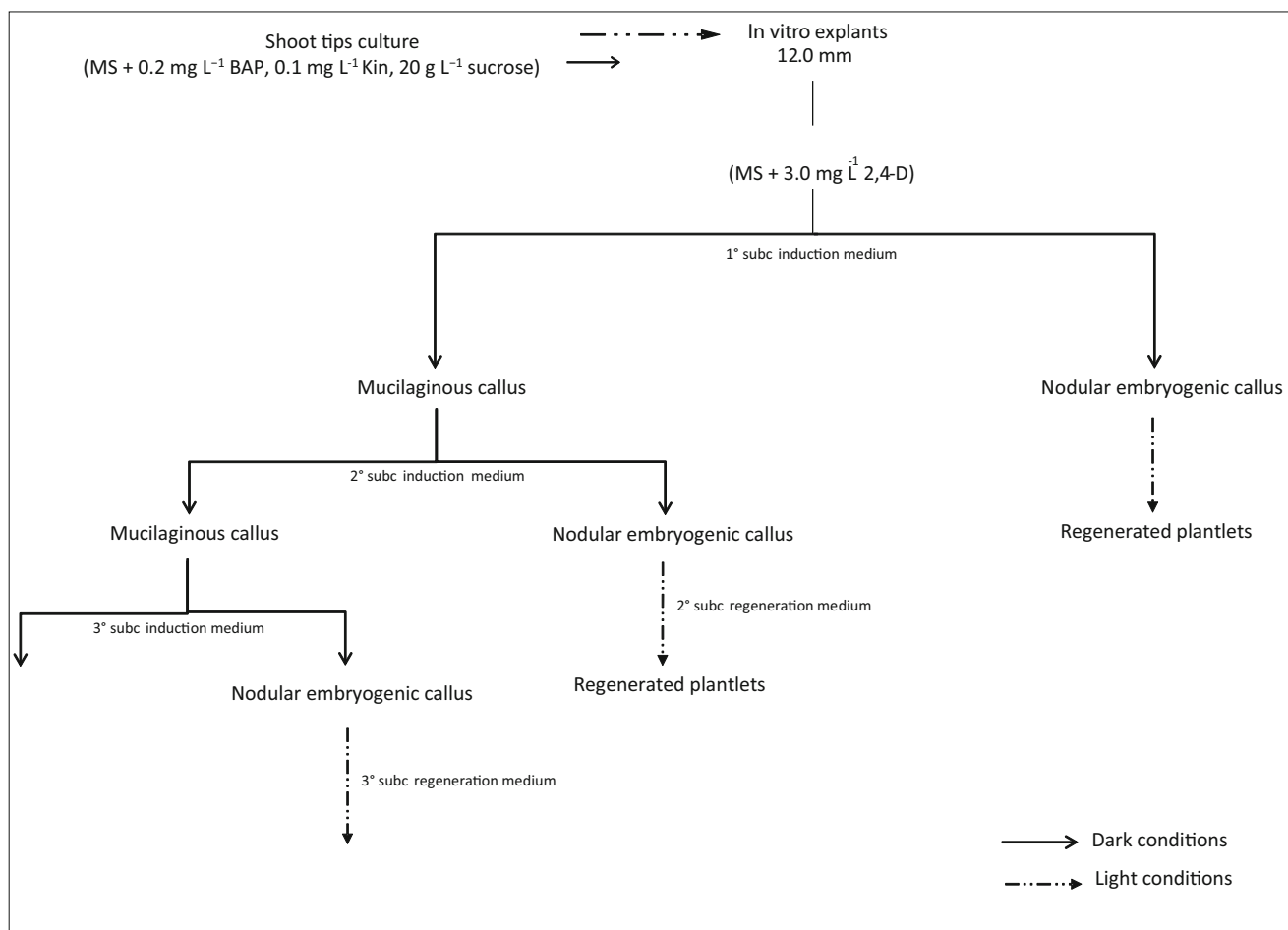
Shoot tips culture
(MS + 0.2 mg L^{-1} BAP, 0.1 mg L^{-1} Kin, 20 g L^{-1} sucrose) — · — · · · ➤ In vitro explants
12.0 mm

(MS + 3.0 mg L^{-1} 2,4-D)

1° subc induction medium

Mucilaginous callus   Nodular embryogenic callus

2° subc induction medium

Mucilaginous callus   Nodular embryogenic callus   Regenerated plantlets

2° subc regeneration medium

3° subc induction medium

Nodular embryogenic callus   Regenerated plantlets

3° subc regeneration medium

➤ Dark conditions
— · · — · ➤ Light conditions

Figure 3. Diagrammatic representation of stages involved in establishment of *Saccharum* spp. hybrids (sugarcane) embryogenic callus culture and regeneration process, started from shoot tips pre-established *in vitro*. Each stage described, whether on induction or regeneration medium, was equal to a 40-d cultivation period.

actively divides within an external polysaccharide layer that provides physical isolation from adjacent cells. Nonetheless, the simultaneous occurrence of single-cell- and multi-cell-origin embryos has already been reported for other sugarcane varieties (Alcantara *et al.* 2014) in several scientific texts (Almeida *et al.* 2012; Scherwinski-Pereira *et al.* 2012).

It was subsequently found that somatic embryos, formed after the globular stage, had a cylindrical morphology and an initial development of the radicular pole and protoderm (*RP* and *PT*, respectively; Fig. 4*E*). Visualized embryos at the cotyledonary stage displayed an apical region containing scutellum (*ES*; Fig. 4*F*) and coleoptile (*CL*; Fig. 4*F*) formation, and a basal region where the radicular tip lay (*RT*; Fig. 4*F*). This feature was consistent with the cytological description of Brisibe *et al.* (1993) during the process of somatic embryo differentiation in sugarcane. The initial development of procambium bundles was also detected (*PC*; Fig. 4*F*).

As a rule, somatic embryos at different stages of development were found in the same nodular tissue, typifying an asynchronous embryogenic process (Fig. 4*G*). Further, the formation of adventitious buds from nodular calluses

simultaneously with the development of somatic embryos was detected. This fact revealed the occurrence of indirect organogenesis during the embryogenic process (Fig. 4*H*) that, although not quantitatively determined, was of low frequency due to the types of callus used, which only followed to the stage of regeneration when presenting embryogenic characteristics. These results agreed with those by Falco *et al.* (1996), who analyzed the formation of single-pole adventitious buds, evidenced by the ample vascular connection with the originating tissue jointly with the formation of somatic embryos. Dibax *et al.* (2013) disclosed that the origin of regenerated shoots in two sugarcane cultures (RB92-579 and RB 93-509) took place solely through indirect organogenesis, even after detecting a nodular callus formation with structures similar to somatic embryos.

Development of regenerated plants and acclimatization
Regenerated sugarcane plantlets developed and produced a larger number of adventitious roots when transferred to culture medium lacking growth regulators (Fig. 2*E*), and acclimatized after having grown taller than 5.0 cm. Furthermore,

Figure 4. Histological analysis of *Saccharum* spp. hybrids (sugarcane) somatic embryogenesis. (*A*) Aspect of mucilaginous calluses containing vacuolated and meristematic (*arrow*) cells; (*B*) nodular callus (*NC*) formed on mucilaginous tissue; (*C*) linear stage pro-embryos; (*D*) globular pro-embryo with suspensor cells (*SP*); (*E*) somatic embryo with radicular pole (*RP*) and protoderm (*PT*, *arrow*); (*F*) globular embryo (*GE*, *right*) and cotyledonary embryo (*left*) containing scutellum (*ES*), coleoptile (*CL*), procambium (*PC*), and radicular tip (*RP*); (*G*) nodular callus (*NC*) with embryos in different stages of development; (*H*) adventitious bud with apical meristem (*AM*), leaf primordia (*LP*), and procambium (*PC*) formed from the nodular callus (*NC*). *Scale bars A*, *B*, *D*, *E*, *F*, and *G* = 100 μm; *H* = 50 μm; *C* = 10 μm.
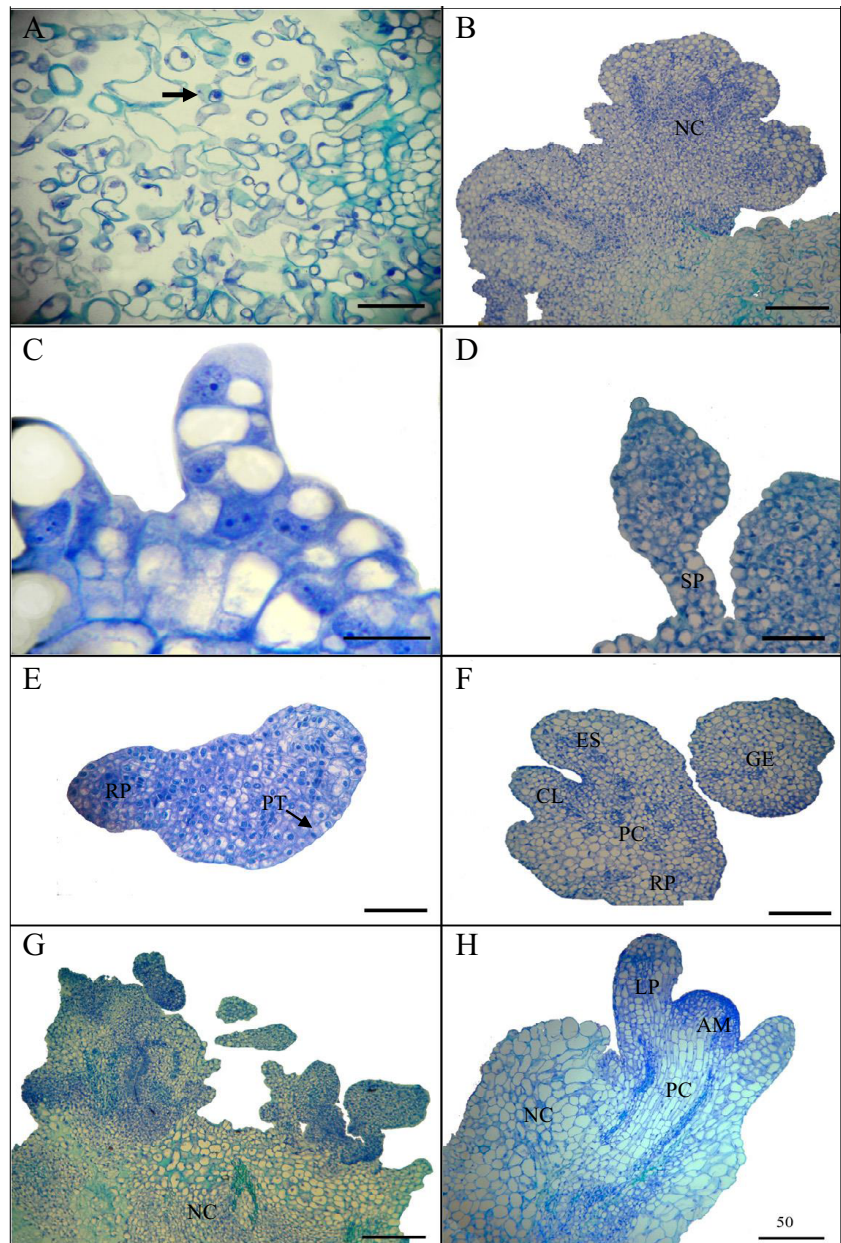
survival rates above 80% were recorded in a greenhouse for the regenerated genotypes, seven of which achieved 100% plant survival after a 21-d acclimatization (Fig. 2*F*).

The current study described an efficient sugarcane somatic embryogenesis protocol featuring high plant regeneration rates, reduced culture time, and contamination-free plantlet production. In this protocol, *in vitro* plants were employed as a source of initial material to induce embryogenic calluses. The meristem region was the most responsive and the best callus induction rates arose from explants of roughly 12 mm in length. Further, the 13 sugarcane varieties assessed displayed embryogenic potential for regenerating complete plants after 6 wk of cultivation, and several varieties continued differentiating somatic embryos for as long as 22 wk,

provided that appropriate development conditions and monitoring of materials were complied with during the different stages of cultivation. Sugarcane somatic embryogenesis as established in this current report should be useful for future efforts involving *Saccharum* spp., including plant mass production and/or genetic transformation of the species.

Author's contribution GFN performed experiments, analyzed and interpreted data, and wrote the manuscript. ZGL helped with the morpho-anatomical and histochemical analyses. MP analyzed data and wrote the manuscript. JESP assisted in the selection and collection of

sugarcane material in the field, designed experiments, analyzed and interpreted data, and wrote the manuscript. All authors agreed on the final version for publication.

Compliance with ethical standards

Conflict of interest   The authors declare that they have no conflicts of interest.

References

Ahloowalia BS, Maretzki A (1983) Plant regeneration via somatic embryogenesis in sugarcane. Plant Cell Rep 2:21–25

Alcantara GB, Dibax R, Oliveira RA, Bespalhok Filho JC, Daros E (2014) Plant regeneration and histological study of the somatic embryogenesis of sugarcane (*Saccharum* spp.) cultivars RB855156 and RB72454. Acta Sci Agron 36:63–72

Almeida M, Almeida CV, Graner EM, Brondani GE, Abreu-Tarazi MF (2012) Pre-procambial cells are niches for pluripotent and totipotent stem-like cells for organogenesis and somatic embryogenesis in the peach palm: a histological study. Plant Cell Rep 31:1495–1515

Basnayake SWV, Moyle R, Birch RG (2011) Embryogenic callus proliferation and regeneration conditions for genetic transformation of diverse sugarcane cultivars. Plant Cell Rep 30:439–448

Brasil (2016) Ministério da Agricultura. Cana-de-açúcar. http://www.agricultura.gov.br/vegetal/culturas/cana-de-acucar. Accessed 24 Aug 2016

Brisibe EA, Nishioka D, Miyake H, Taniguchi T, Maeda E (1993) Developmental electron microscopy and histochemistry of somatic embryo differentiation in sugarcane. Plant Sci 89:85–92

Brisibe EA, Miyake H, Taniguchi T, Maeda E (1994) Regulation of somatic embryogenesis in long-term callus cultures of sugarcane (*Saccharum officinarum* L.). New Phytol 126:301–307

Chen WH, Davey MR, Power JB, Cocking EC (1988) Control and maintenance of plant regeneration in sugarcane callus culture. J Exp Bot 39:251–261

Conab, Companhia Nacional de Abastecimento (2018) Acompanhamento da safra brasileira: cana-de-açúcar, v.4, Safra 2017/18. Brasília, 73 p

Dibax R, Alcantara GB, Machado MP, Bespalhok Filho JC, Oliveira RA (2011) Plant regeneration of sugarcane cv. RB931003 and RB98710 from somatic embryos and acclimatization. J Biotechnol Biodivers 2:32–37

Dibax R, Alcantara GB, Machado MP, Bespalhok Filho JC, Oliveira RA (2013) Protocol optimization and histological analysis of *in vitro* plant regeneration of 'RB92579' and 'RB93509' sugarcane cultivars. Ciência Rural 43:49–54

Falco MC, Mendes BMJ, Tulmann Neto A, Appezzato da Glória B (1996) Histological characterization of *in vitro* regeneration of *Saccharum* sp. Rev Bras Fisiol Veg 8:93–97

Ferreira DF (2011) Sisvar: a computer statistical analysis system. Ciencia e Agrotecnologia 35:1039–1042

Garcia R, Cidade D, Castellar A, Lips A, Magioli C, Callado C, Mansur E (2007) *In vitro* morphogenesis patterns from shoot apices of sugarcane are determined by light and type of growth regulator. Plant Cell Tissue Organ Cult 90:181–190

Gosal SS, Thind KS, Dhaliwal HS (1998) Micropropagation of sugarcane-an efficient protocol for commercial plant production. Crop Improv 25:167–171

Ho WJ, Vasil IK (1983) Somatic embryogenesis in sugarcane (*Saccharum officinarum* L.) I. The morphology and physiology of callus formation and the ontogeny of somatic embryos. Protoplasma 118:169–180

Hsie BS, Brito JZ, Vila Nova MX, Borges-Paluch LR, Silva MV, Donato VMST (2015) Determining the genetic stability of micropropagated sugarcane using inter-simple sequence repeat markers. Genet Mol Res 14:17651–17659

Johansen DA (1940) Plant microtechnique. McGraw-Hill Book, New York

Joyce P, Kuwahata M, Turner N, Lakshmanan P (2010) Selection system and co-cultivation medium are important determinants of *Agrobacterium*-mediated transformation of sugarcane. Plant Cell Rep 29:173–183

Kaur R, Kapoor M (2016) Plant regeneration through somatic embryogenesis in sugarcane. Sugar Tech 18:93–99

Krikorian AD, Berquam DL (1969) Plant cell and tissue cultures: the role of Haberlandt. Bot Rev 35:59–67

Landell MGA, Campana MP, Figueiredo P, Xavier MA, Anjos IA, Dinardo-Miranda LL, Scarpari MS, Garcia JC, Bidóia MAP, Silva DN, Mendonça JR, Kanthack RAD, Campos MF, Brancalião SR, Petri RH, Miguel PEM (2012) Sistema de multiplicação de cana-de-açúcar com uso de mudas pré-brotadas (MPB), oriundas de gemas individualizadas. Ribeirão Preto: Instituto Agronômico de Campinas, 17 p. (IAC. Documentos, 109)

Lee TSG (1987) Micropropagation of sugarcane (*Saccharum* spp.). Plant Cell Tissue Organ Cult 10:47–55

Manickavasagam M, Ganapathi A, Anbazhagan VR, Sudhakar B, Selvaraj N, Vasudevan A, Kasthurirengan S (2004) *Agrobacterium*-mediated genetic transformation and development of herbicide-resistant sugarcane (*Saccharum* species hybrids) using axillary buds. Plant Cell Rep 23:134–143

Martínez-Estrada E, Caamal-Velázquez JH, Salinas-Ruíz J, Bello-Bello JJ (2017) Assessment of somaclonal variation during sugarcane micropropagation in temporary immersion bioreactors by intersimple sequence repeat (ISSR) markers. In Vitro Cell Dev Biol - Plant 53:553–560

Moutia M, Dookun A (1999) Evaluation of surface sterilization and hot water treatments on bacterial contaminants in bud culture of sugarcane. Exp Agric 35:265–274

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nogueira GF, Pasqual M, Scherwinski-Pereira JE (2013) Survival of sugarcane shoot tips after cryopreservation by droplet-vitrification. Pesq Agrop Brasileira 48:1524–1527

Nogueira GF, Pio LAS, Pasqual M, Amaral A, Scherwinski-Pereira JE (2015) An approach on the in vitro maintenance of sugarcane with views for conservation and monitoring of plant nuclear DNA contents via flow cytometry. In Vitro Cell Dev Biol - Plant 51:220–230

O'Brien TP, Feder N, McCully ME (1964) Polychromatic staining of plant cell walls by toluidine blue. Protoplasma 59:368–373

Pola S, Mani S, Ramana T (2009) Long-term maintenance of callus cultures from immature embryo of *Sorghum bicolor*. World J Agric Sci 5:415–421

Ramage CM, Williams RR (2004) Cytokinin-induced abnormal shoot organogenesis is associated with elevated *Knotted1*-type homeobox gene expression in tobacco. Plant Cell Rep 22:919–924

Sané D, Aberlenc-Bertossi F, Gassama-Dia YK, Sagna M, Trouslot MF, Duval Y, Borgel A (2006) Histocytological analysis of callogenesis and somatic embryogenesis from cell suspensions of date palm (*Phoenix dactylifera*). AoB Plant 98:301–308

Scherwinski-Pereira JE, Guedes RS, Silva RA, Fermino JRPCP, Luis ZG, Freitas EO (2012) Somatic embryogenesis and plant regeneration in

açaí palm (*Euterpe oleracea*). Plant Cell Tissue Organ Cult 109: 501–508

Seema G, Pande HP, Lal J, Madan VK (2001) Plantlet regeneration of sugarcane varieties and transient gus expression in calli by electroporation. Sugar Tech 3:27–33

Sengar RS, Sengar K, Garg SK (2011) Biotechnological approaches for high sugarcane yield. Plant Sci Feed 1:101–111

Simões Neto DE (2009) Variedades de cana–de–açúcar no Estado de Pernambuco contribuição do melhoramento Clássico da Ridesa–Ufrpe. Anais da Academia Pernambucana Ciên Agron 5–6:125–146

Sindaçucar (2018) Sindicato da Indústria do Açúcar no Estado de Pernambuco. http://www.sindacucar-al.com.br/variedades/. Accessed 20 Dec 2018

Singh G, Sandhu SK, Meeta M, Singh K, Gill R, Gosal SS (2008) *In vitro* induction and characterization of somaclonal variation for red rot and other agronomic traits in sugarcane. Euphytica 160:35–47

Singh RK, Kumar P, Tiwari NN, Rastogi J, Singh SP (2013) Current status of sugarcane transgenic: an overview. Adv Genet Eng 2:2–7

Sobhakumari VP (2012) Assessment of somaclonal variation in sugarcane. Afr J Biotechn 11:15303–15309

Taparia Y, Gallo M, Altpeter F (2012) Comparison of direct and indirect embryogenesis protocols, biolistic gene transfer and selection parameters for efficient genetic transformation of sugarcane. Plant Cell Tissue Organ Cult 111:131–141

Tiwari AK, Bharti YP, Tripathi S, Mishra N, Lal M, Rao GP, Sharma PK, Sharma ML (2010) Biotechnological approaches to improve sugarcane crop with special reference to disease resistance. Acta Phytopath Entomol Hung 45:235–249

von Arnold S, Sabala I, Bozhkov P, Dyachok J, Filonova L (2002) Developmental pathways of somatic embryogenesis. Plant Cell Tissue Organ Cult 69:233–249