



# High-efficiency organogenesis and evaluation of the regenerated plants by flow cytometry of a broad range of *Saccharum* spp. hybrids

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## Abstract

The aim of this study was to evaluate the organogenic potential of Brazilian sugarcane varieties, in addition to verifying the in vitro multiplication rate and genetic stability by flow cytometry over monthly and consecutive subcultures. For that, stem apices of twenty-two varieties were collected in field conditions and taken to the laboratory where external layers of leaves were removed. After surface sterilization, the innermost portion of the stem segment was sectioned and placed in a MS medium supplemented with 5.0 mg L<sup>-1</sup> of NAA and 0.5 mg L<sup>-1</sup> of KIN. For the multiplication rate, ten varieties were selected and inoculated in MS medium of liquid or semi-solid consistency plus 0.10 mg L<sup>-1</sup> of KIN and 0.20 mg L<sup>-1</sup> of BAP. Subcultures were performed every 30 days for a period up to 8 months. Genetic stability was verified by flow cytometry every two subcultures. At the end of the experiment, the sprouts were rooted and acclimatized in a greenhouse. As a result, it was observed that the regeneration occurred both by direct and indirect organogenic pathway. The varieties of sugarcane differed significantly regarding the regeneration capacity and amount of adventitious shoots formed. In multiplication, a significant interaction was observed between variety, consistency of the culture medium and number of subcultures. In general, in the first subcultures, the liquid consistency medium presented similar or superior results when compared with the semi-solid medium, however, from the fourth subculture, the semi-solid medium was superior. Morphological variations were verified from the fourth subculture. In addition, in some varieties, small changes in the relative amount of DNA were detected by flow cytometry. Sprouts of normal-looking sugarcane were successfully rooted and plantlets acclimatized after the eighth subculture.

**Keywords** *Saccharum* spp. · Large-scale propagation · Morphological variation · DNA content

## Introduction

Plant tissue culture techniques have been effectively used for clonal propagation and genetic improvement of different cultures (Rocha et al. 2012), providing not only more productive and pathogen resistant plants, but also with greater vigor and adaptability to different environments (Piperidis

et al. 2010). However, these procedures require an efficient and previously established system for the selection, regeneration and proliferation of these plants of agronomic interest (Yang et al. 2010).

For sugarcane, since the approaches related to the cultivation of cells and tissues started in the 1960s, numerous techniques have been developed for the genus. Organogenic and embryogenic pathways have become the most widely used regeneration systems (Lakshmanan et al. 2006), although the competence and determination of explants varies significantly between genotypes (Garcia et al. 2007; Basnayake et al. 2011; Nogueira et al. 2019).

Currently, biotechnological methods applied to mass propagation are a very common reality in the sugarcane agribusiness because they allow the production of plants with better quality and faster than conventional methods (Snyman

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et al. 2011; Lal et al. 2014). However, plant production may have some limitations, such as microbial contamination, phenolic oxidation, vitrification, in addition to the stress of the in vitro environment that can cause genetic instability of materials (Wang and Wang 2012; Lal et al. 2014).

In general, the source of explant, the method of cultivation applied, the time and number of subcultures, as well as the concentration and type of growth regulators used, often influence the appearance of somaclonal variations under in vitro conditions (Huang et al. 2012; Krishna et al. 2016). These variations can occur at the morphological, physiological, biochemical and genetic/epigenetic level (Kaepler et al. 2000; Huang et al. 2012). In fact, the occurrence of somaclonal variation still remains one of the major problems of plant tissue culture (Bairu et al. 2011), and for sugarcane culture it is no different.

Recent research has shown that the early evaluation of the genetic stability of plants regenerated in vitro can be useful to prove the purity of the regenerants, especially during clonal micropropagation and in genetic transformation studies (Sengar et al. 2011; Pandey et al. 2012; Krishna et al. 2016). Among the various techniques available, special attention has been given to flow cytometry (Mallón et al. 2010; Prado et al. 2010). Due to the fact that the particles are analyzed individually and at high speed, large populations of cells can be measured in a short time by this tool (Shapiro 2004). For this reason, cytometry has become an excellent method for screening ploidy, detecting myxoploidy and aneuploidy and a popular approach for estimating the total DNA content (Dolezel and Bartos 2005).

In Brazil, despite the economic importance of sugarcane and the cultivation of plantlets on a large scale via tissue culture, until now no major study has been carried out to identify the most responsive Brazilian varieties in vitro and, mainly, to verify their behavior and cytogenetic stability under prolonged culture conditions.

This work aimed to evaluate the organogenic potential and the in vitro multiplication rate of Brazilian sugarcane varieties, as well as to determine the average content of nuclear DNA by flow cytometry of the regenerants over eight consecutive subcultures.

## Material and methods

### Organogenic potential of Brazilian sugarcane varieties

To verify the organogenic potential of sugarcane plants, stem tips of twenty-two Brazilian varieties were collected at Embrapa Coastal Tablelands, Rio Largo – AL, and sent to the Embrapa Genetic Resources and Biotechnology, Brasília, DF, Brazil.

In the Tissue Culture and Plant Genetic laboratory, the stem tips were washed with detergent in running water and the outer layers of leaves removed, in order to facilitate handling in the laminar flow chamber and avoid contamination of explants in vitro. After the initial cleaning, the apices were immersed in 70% alcohol for 1 minute, sodium hypochlorite (NaClO) (2.5% active chlorine) for twenty minutes, followed by triple washes in distilled and autoclaved water.

With the aid of tweezers and scalpel, about 2 to 3 leaves were removed and the innermost portion of the stem segment was sectioned transversely, with a thickness of approximately three millimeters (Fig. 1). To avoid oxidation, the explants were quickly inoculated into a culture medium containing MS salts (Murashige and Skoog 1962), supplemented with 30 g L<sup>-1</sup> of sucrose, 5.0 mg L<sup>-1</sup> of naphthalenoacetic acid (NAA), 0.5 mg L<sup>-1</sup> of kinetin (KIN) and solidified with 2.3 g L<sup>-1</sup> of Phytigel® (Sigma, St. Louis, MO, USA), according to the protocol established by Gill et al. (2006). The pH of the medium was adjusted to 5.8 ± 0.1 before sterilization, performed by autoclaving at 121 °C and 1.5 atm for 20 minutes.

After inoculation, all explants were kept under the same culture conditions, at a temperature of 25 ± 2 °C, 16 hours of photoperiod and irradiation of 30 μmol.m<sup>-2</sup>.s<sup>-1</sup>, provided by cold white fluorescent lamps (Sylvania, 40w).

In this experiment, two stem tips were used for each variety of sugarcane, which were sectioned providing 30 explants. These were distributed following a completely randomized design in 10 sterile *Petri* dishes (15 × 90 mm), each containing three sections of the explants.

After 40 days of cultivation were evaluated the percentage of regeneration, the average number of regenerated shoots, the percentage of formation of adventitious roots and calluses at the base of the explants, as well as the percentage of phenolic oxidation of the explants.

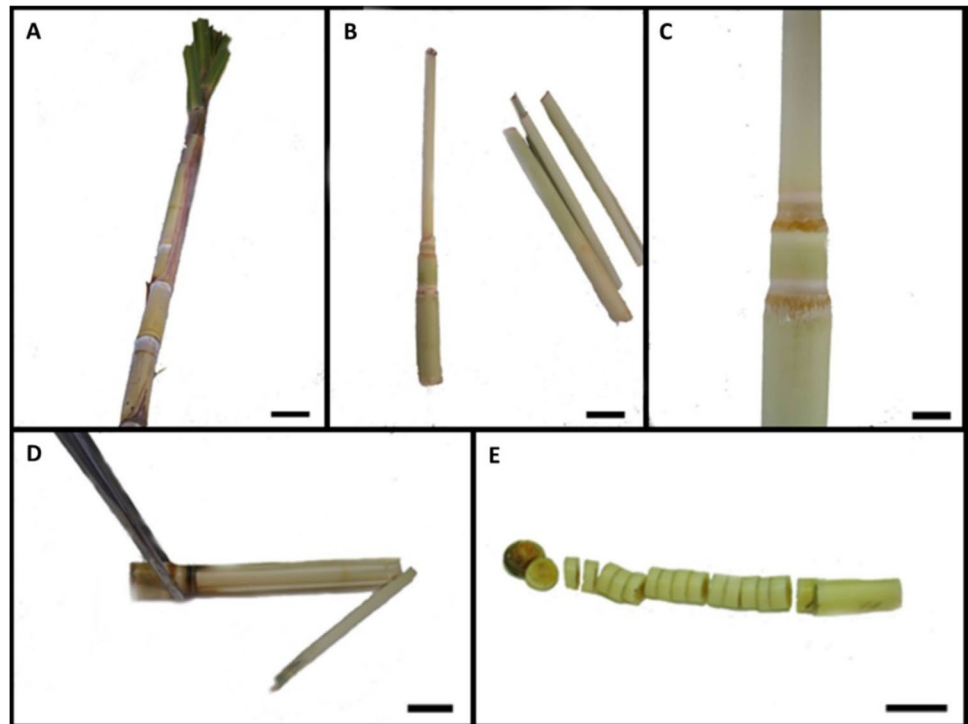
### Histological analysis

For anatomical analysis and elucidation of the regeneration route from stem tips in vitro, samples were collected at 40 days of subculture and fixed in FAA 70 (Formaldehyde, acetic acid and 70% ethanol, in a ratio of 1:1:18 v/v) (Johansen 1940).

Subsequently, the samples were dehydrated in an increasing alcoholic series (70–100%), infiltrated and included in historesin (Leica, Heidelberg, Germany) according to the manufacturer's recommendations. Transverse and longitudinal serial sections (7–10 μm) were obtained in a manual rotary microtome (Leica model RM 2125), extended and adhered to microscopic slides in a plate heated to ±40 °C.

The sections were stained with Toluidine blue (O'Brien et al. 1964), followed by assembly between slide-coverslips

**Fig. 1** Procedures used for direct organogenesis induction from the apex of adult stem of sugarcane plants. Shoot apices before asepsis (A–C) (Bar = 5.0 cm) and after asepsis in a laminar flow chamber (D and E) (Bars = 1 cm)



with Entellan. The results were recorded in a light microscope, coupled to a digital image capture system (Software ImagePro 4.0).

### In vitro multiplication rate

To evaluate the multiplication rate over consecutive subcultures, ten varieties of sugarcane regenerated via organogenesis were used as explant sources for this experiment – SP716949; SP784764; SP701143; SP854594; RB83160; RB845210; RB863129; VAT90-212; VAT90-186; VAT90-61.

For that, sprouts were excised from the matrix in a laminar flow chamber, and inoculated in MS medium added with  $30 \text{ g L}^{-1}$  of sucrose,  $0.10 \text{ mg L}^{-1}$  of kinetin (KIN) and  $0.20 \text{ mg L}^{-1}$  of 6-benzylaminopurine (BAP). Two consistencies of the culture medium were used: semi-solid (SSM) and stationary liquid (LM). Solidification of the SSM was carried out with the addition of  $2.3 \text{ g L}^{-1}$  of Phytigel.

The explants were kept in a growth room under light conditions, where the cultivation conditions were the same for all varieties.

Subcultures were carried out at intervals of 30 days and, at the end of each one, in addition to the evaluation of the multiplication rate (number of shoots larger than 0.5 cm), the height of the largest shoot until the intersection of the last leaf, the formation of rosettes and abnormalities and the contamination of the culture medium were also determined. In total, 8 consecutive subcultures were carried out.

As a repetition, three 250 mL glass vials with 5 sprouts were used for each consistency of culture medium (SSM and LM).

### Estimation of nuclear genome size through flow cytometry

For each two subcultures, newly sprouts obtained by direct organogenesis of the ten varieties were collected and evaluated for the average content of nuclear DNA by flow cytometry at the Tissue Culture Laboratory – Department of Agriculture at the Federal University of Lavras, Lavras, MG, Brazil.

As in the sugarcane samples, approximately 20–30 mg of young leaves of the plants and the external reference standard - tomato (*Lycopersicon esculentum* var. ‘Stupické polní tyčkové rané’, 1.96 pg) were used. The external reference standard samples were kindly provided by Dr. Jaroslav Doležel from the Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Olomouc, Czech Republic. The tissues were ground in a Petri dish containing 1 mL of cooled extraction buffer to release the nuclei. The buffer used for these analyzes was Marie [50 mM glucose, 15 mM NaCl, 15 mM KCl, 5 mM  $\text{Na}_2\text{EDTA}$ , 50 mM sodium citrate, 0.5% Tween 20, 50 mM HEPES (pH 7.2) and 1% (m/v) polyvinylpyrrolidone-10 (PVP-10)] (Marie and Brown 1993).

After the extraction process, the nuclei suspension was aspirated with the aid of a Pasteur pipette and filtered

through a 50 µm mesh. The nuclei were stained by adding 25 µL of a solution of 1 mg/1 mL of Propidium Iodide. The treatment of the samples with RNase was not applied, since previous results showed no differences in the results.

The nuclear DNA content of the plants in (pg) was estimated using the formula: Sample DNA = (G1 of the sample/G1 of the standard) x DNA of the standard.

The analyzes were performed using a FACS Calibur cytometer (BD, Biosciences, San Jose, CA, USA) and the histograms generated were analyzed using the WinMDI 2.8 software. The design was completely randomized with 4 repetitions per variety, each of which was composed of a sprout and two readings. Samples were collected and evaluated on the same day to prevent possible incorrect readings due to random errors.

### In vitro rooting and acclimatization in a greenhouse

At the end of the eighth subculture, sprouts were individualized, transferred to rooting medium and then acclimatized in a greenhouse.

The culture medium used for the induction of roots in vitro was that of MS reduced to half its salt concentration, plus 5.0 mg L<sup>-1</sup> of NAA (Pathak et al. 2009), 30 g L<sup>-1</sup> of sucrose and 2.3 g L<sup>-1</sup> Phytigel. The pH was adjusted to 5.8 ± 0.1 and the medium, distributed in test tubes (25 × 150 mm) before autoclaving for 20 minutes.

Sprouts were transferred in a laminar flow chamber and the origins were marked according to the multiplication medium (LM or SSM). It should be noted that, for each variety, the repetitions were composed of ten sprouts from the liquid medium and ten from the semi-solid medium. After inoculation, sprouts were kept in a growth room under light conditions.

Root induction, the height of sprouts (cm) and the formation of secondary shoots were evaluated on the 40th day of cultivation and, shortly thereafter, the processes of pre-acclimatization of explants started, still in the growth room. Initially, the PVC film used to seal the test tubes was removed and, 72 hours later, the caps were also removed.

After the period of 48 hours without any kind of sealing, the sprouts were washed in running water, to remove residues from the culture medium, and taken to a greenhouse where they were transplanted in Bioplant substrate (Nova Ponte, Minas Gerais, Brazil) and sand, in a 1:1 (v/v) ratio.

In order to provide a moist microclimate for the sprouts during the acclimatization period, they were covered by a plastic cover and irrigated daily for a period of seven days. After 21 days of acclimatization, the percentage of survival, the number of shoots and the height of the aerial part (cm) were observed.

### Statistical analysis

The data obtained in all the experiments described were subjected to analysis of variance, using the statistical program Sisvar (Ferreira 2011) and the means compared by the Scott-Knott test at 5% probability. The data expressed as a percentage were transformed by arc sine  $(x/100)^{1/2}$ .

## Results and discussion

### Organogenic potential of sugarcane varieties and histological analysis

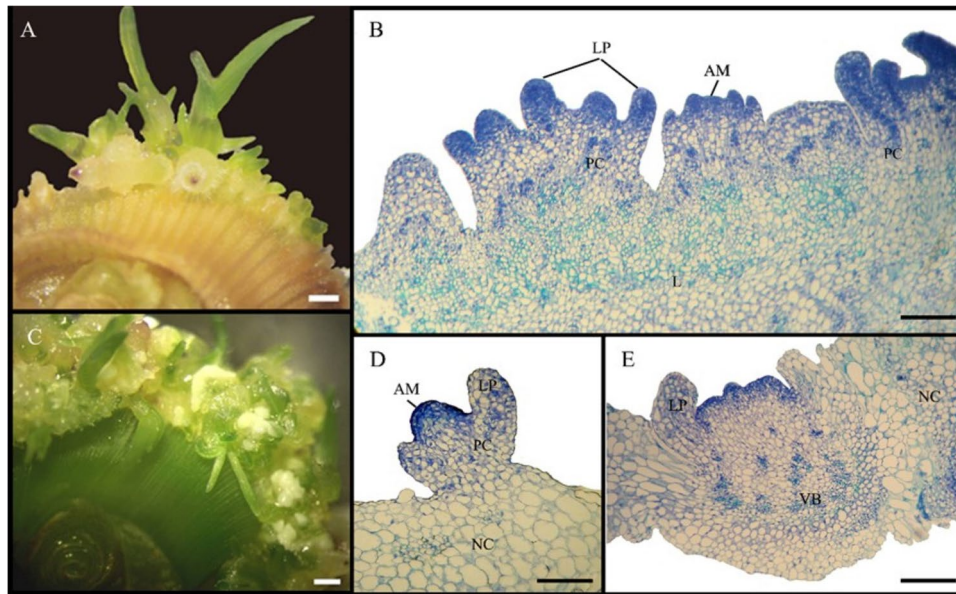
The regeneration of sugarcane in vitro from immature leaf segments occurred quickly and efficiently and allowed a large number of shoots to be obtained in a short time.

According to Gill et al. (2006), the regeneration method applied is simple and easily reproducible for inducing direct plant regeneration from immature leaf segments. However, contrary to the results found by these authors, the regeneration of the Brazilian sugarcane varieties studied in this work occurred both by the direct organogenic pathway (Fig. 2A) and by the indirect pathway (Fig. 2C) with callus formation at the base of the explants.

Histological analysis confirmed the development of adventitious buds containing apical meristem and leaf primordia formed directly from the leaf explant, in addition to the development of the procambium connecting the buds with the source explant (Fig. 2B).

Adventitious buds also developed from nodular calluses formed at the base of the original explants. In Fig. 2D, it is possible to observe an adventitious bud, in early development, containing apical meristem, leaf primordial and procambial tissues. These characteristics showed the unipolar structure of the buds, with a wide connection with the organogenic callus. In a more advanced stage, adventitious buds containing vascular bundles already developed were observed as shown in Fig. 2E.

In general, the organogenic process for all varieties studied in this work occurred asynchronously, with the presence of buds and shoots at different stages of development. Garcia et al. (2007) found that in vitro sugarcane plants when incubated in light and in the presence of a high concentration of auxin exhibited, first, direct regeneration from the outer layer of the fundamental parenchyma, followed by the development of organogenic calluses. Dibax et al. (2013) in turn, verified the regeneration of the shoots of two varieties of sugarcane (RB92579 and RB93509) from two different types of cellular organization: nodular calluses similar to somatic embryos in a globular stage or translucent organogenic calluses with intense cell division.



**Fig. 2** In vitro organogenesis of *Saccharum* sp. from adult plant stem tips. **A:** Direct regeneration after 30 days of cultivation observed in the RB845210 variety; **B:** Histological analysis showing adventitious buds at different stages of development, formed directly from the leaf explant; **C:** Aspect of indirect regeneration with callus formation in sections of explants in the VAT90-212 variety; **D:** Adventitious bud,

originating from nodular callus, in a more advanced stage of development, presenting primordial leaves and vascular bundles. **E:** Development of shoots in induction medium containing NAA + KIN after 40 days of cultivation. AM: apical meristem of the stem; LP: leaf primordia; PC: procambium; L: source leaf; NC: nodular callus; VB: vascular bundles (Bars A, C and E= 1 mm; B and D= 50 µm)

In this study, the calluses formed at the base of the explants were initially friable and had a mucilaginous aspect. Subsequently, on this tissue, a whitish-colored nodular compact callus emerged. In approximately 15 days of cultivation there was a proliferation of callogenic masses and the development of buds and adventitious shoots, evidenced by the green color.

The percentage of calluses formed at the base of the explants for each variety studied can be seen in Table 1, in addition to characteristics such as percentage of adventitious root formation and phenolic oxidation.

Regarding the percentage of regeneration, it can be seen in Fig. 3A that, among the twenty-two varieties studied, seven of them (SP784764, SP854594, RB845210, VAT90-186, VAT90-212, SP716949 and RB99395) were more responsive to the treatment used compared to the others studied and showed an average regeneration rate of at least 81.9% (Fig. 3A).

A group of nine varieties showed a regeneration rate considered intermediate, where approximately 60% of the explants produced shoots. The varieties with the lowest frequency of regeneration, in turn, were: SP813804 (16%), RB93509 (14.1%), RB92579 (14.1%), CB98710 (14.6%), RB951541 (12%) and the variety RB867515, of which only 4.11% of explants were organogenic.

Lakshmanan et al. (2006) believe that although genotypes play an important role in controlling shoots regeneration in vitro, this effect can be minimized with the

concentration and type of auxin used, the polarity and orientation of the tissue, as well as the size and stage of development of explants. In this sense, the need to adapt the protocol used for less responsive varieties becomes evident.

In addition to the variation in the percentage of regeneration, it was observed that the varieties also differed significantly in relation to the number of adventitious shoots formed. With significantly higher results, the VAT90-186, VAT90-212 and SP716949 varieties stood out with an average formation of 35.3; 29.8 and 43.5 shoots per repetition, respectively (Fig. 3B).

In view of these results, it was found that the varieties most responsive to regeneration (SP854594, RB845210, VAT90-186, VAT90-212 and SP716949) were the ones with the highest percentage of explants with callus formation on the cut surface and adventitious roots, and however, with the lowest percentage of oxidation. On the contrary, the varieties with the lowest percentage of regeneration (RB951541; RB93509; RB92579 and SP813804) were the ones that presented the highest rate of explants phenolic oxidation, which certainly compromised their regeneration (Table 1 and Fig. 3).

### In vitro multiplication rate

According to the statistical analysis, the results found for the average number of shoots and height of the highest sprout

**Table 1** Percentage of sugarcane explants that presented callus at the base of the explants, formation of adventitious roots and phenolic oxidation during cultivation in MS culture medium plus 5.0 mg L<sup>-1</sup> of NAA and 0.5 mg L<sup>-1</sup> of KIN

Variety	Callus (%)	Oxidation (%)	Adventitious roots (%)
RB867515	4.1 c	20.6 c	37.2 a
RB951541	4.1 c	62.0 a	4.7 b
RB93509	0.0 c	61.5 a	4.6 b
RB92579	14.0 c	66.5 a	4.7 b
CB98710	22.1 c	29.5 b	3.6 b
SP813804	16.5 c	56.4 a	6.6 b
SP701143	45.6 b	41.2 b	16.5 b
SP791011	8.2 c	41.2 b	24.7 b
RB921003	44.3 b	22.0 c	44.0 a
SP813250	59.6 b	6.6 c	6.6 b
RB83160	28.4 c	37.8 b	23.5 b
CB45-3	49.8 b	44.0 b	38.5 a
RB863129	59.7 b	16.5 c	23.2 b
SP841431	33.0 c	6.6 c	46.4 a
VAT90-61	29.4 c	7.3 c	18.3 b
SP784764	46.4 b	13.2 c	13.2 b
SP854594	79.9 a	9.9 c	39.7 a
RB845210	83.3 a	8.2 c	50.0 a
VAT90-186	66.5 a	11.0 c	38.8 a
VAT90-212	73.2 a	6.6 c	36.4 a
SP716949	76.6 a	9.9 c	63.3 a
RB99395	29.6 c	12.3 c	20.6 b

\*Averages followed by the same letter belong to the same group, using the Scott-Knott Test at the 5% probability level

showed a significant interaction between variety, consistency of the culture medium and number of subcultures.

It was observed that the varieties showed different behaviors in relation to the potential for multiplication of shoots within and between the culture media used. In general, it was observed that in the first subcultures the liquid consistency medium (LM) presented similar or superior results when compared with the semi-solid consistency medium (SSM). However, as of the fourth subculture, SSM was the one with the highest multiplication rate for all varieties analyzed (Fig. 4).

The SP701143 variety was the one that showed the highest formation of shoots average along the subcultures in both culture media, when compared with the other varieties. According to Fig. 4, a significant increase in the number of shoots can be seen throughout the subcultures, reaching an average of 11.2 shoots/tiller in SSM and 6.9 shoots/tiller in LM in the last three subcultures. This high multiplication rate can be explained by the large number of shoots formed at the base of a main tiller, often smaller than 1.0 cm in height.

The varieties RB83160, RB845210 and SP716949 showed similar behavior in the multiplication rate, with an increase in the production of shoots in the SSM medium, but maintaining a constant in the LM throughout the cultivation time. The height of the shoots in the LM was similar, with approximately 3.4 cm for the three varieties, whereas, in the SSM, the height of the shoots of the SP716949 variety was significantly higher, with 3.7 cm in relation to the others (2.8 cm of average). It was observed that the increase in the number of sprouts for the RB845210 variety occurred at the expense of their growth.

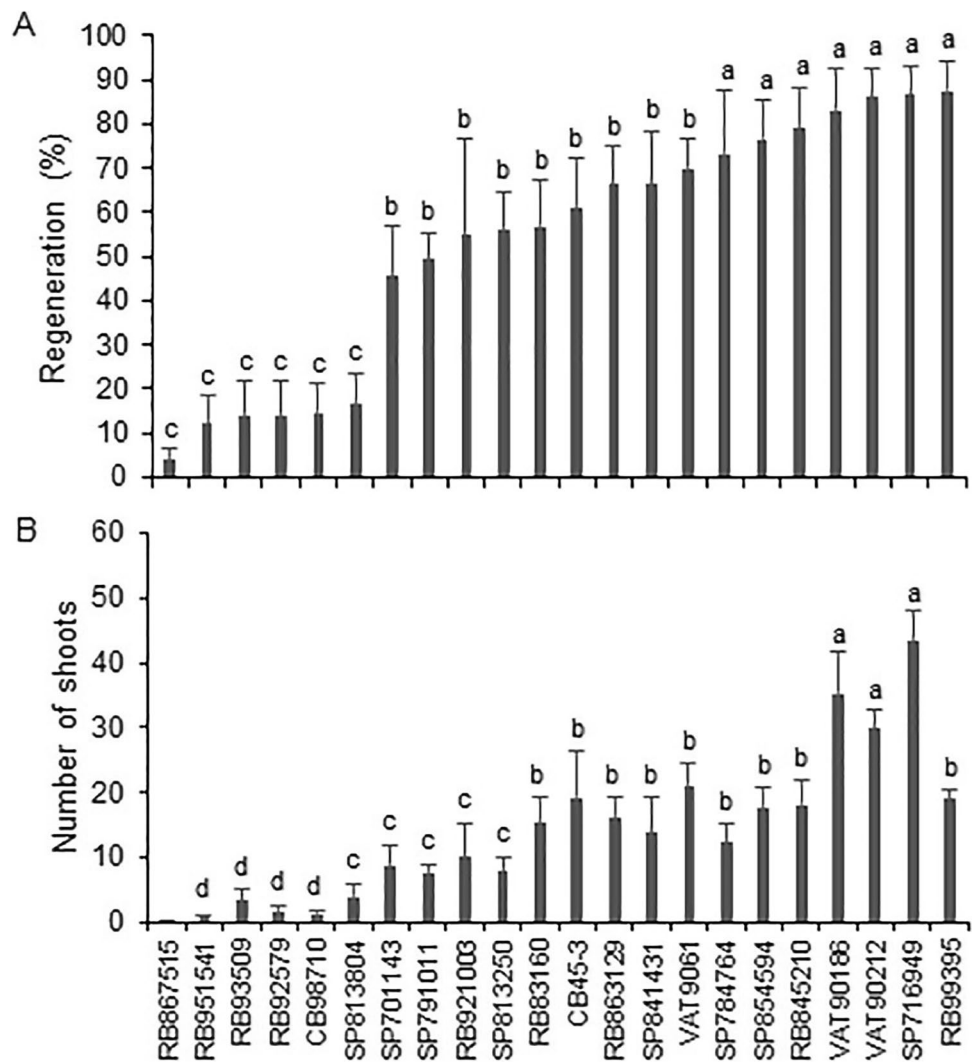
The VAT90-61, RB863129 and SP784764 varieties showed no production of multiple shoots during the experiment. The VAT90-212 and SP854594 varieties, in turn, had a good multiplication rate and, like the other varieties, the SSM medium was the one that provided the best results, after the fourth subculture (Fig. 4). The observed heights remained close to 3.0 cm.

In fact, the potential for sugarcane multiplication is highly influenced by the genotype (Cheema and Hussain 2004; Cidade et al. 2006; Ali et al. 2008; Khan et al. 2008; Silva et al. 2009; Nogueira et al. 2019), which explains the wide variation in cultivation conditions found in sugarcane work, especially with regard to the studied concentration and growth regulators (Biradar et al. 2009; Khan et al. 2009; Uzma et al. 2012; Rocha et al. 2013). In most cases, the hormonal interaction between the cytokinins BAP and KIN has demonstrated better efficiency in the development of multiple shoots (Khan et al. 2009; Silva et al. 2009).

To date, no reports have been found in the literature containing information regarding the potential for multiplication of the Brazilian sugarcane varieties studied in this work, with the exception of Nogueira et al. (2019) who studied multiplication by somatic embryogenesis. However, by the results, it can be seen that the superiority of the culture medium of semi-solid consistency in relation to the production of multiple shoots in the last subcultures can be explained by the beginning of bacterial contamination of the liquid medium and by the morphological variation of sprouts formed from the fourth subculture.

Despite the ease of work and the good multiplication rate, in the culture medium with liquid consistency there was a progression in the number of contaminated repetitions, reaching a peak of approximately 45% at the end of the eighth subculture. Although tests were not carried out to detect the origin of the contaminants, it is recognized in the literature that the use of liquid medium, despite its benefits, can provide a greater frequency of contamination in cultures (Izarra et al. 2020). Added to this is the fact that sugarcane has endogenous bacteria that are important for the active growth of crops in the field (Mendes et al. 2007), a fact that could compromise the emergence of contaminants in advanced subcultures of multiplication. Contamination of

**Fig. 3** Comparison of Brazilian varieties of sugarcane regarding the percentage of regeneration (A) and average formation of shoots per repetition (B), after 40 days of cultivation in MS culture medium supplemented with 5.0 mg L<sup>-1</sup> of NAA and 0.5 mg L<sup>-1</sup> of KIN. Varieties with the same letter do not differ by the Scott-Knott test at the 5% probability level



the liquid medium had negative effects on the multiplication of sugarcane, even completely inhibiting the multiplication of some varieties, as was the case with the VAT90-186 variety. The percentage of contamination in the SSM medium was significantly lower, with an average of 10% at the end of the experiment.

Morphological variations were also observed over consecutive subcultures. From the fourth transplanting, a larger formation of small shoots (<1.0 cm) was observed at the base of a larger main sprout. This type of arrangement was called rosette in this study (Fig. 5A).

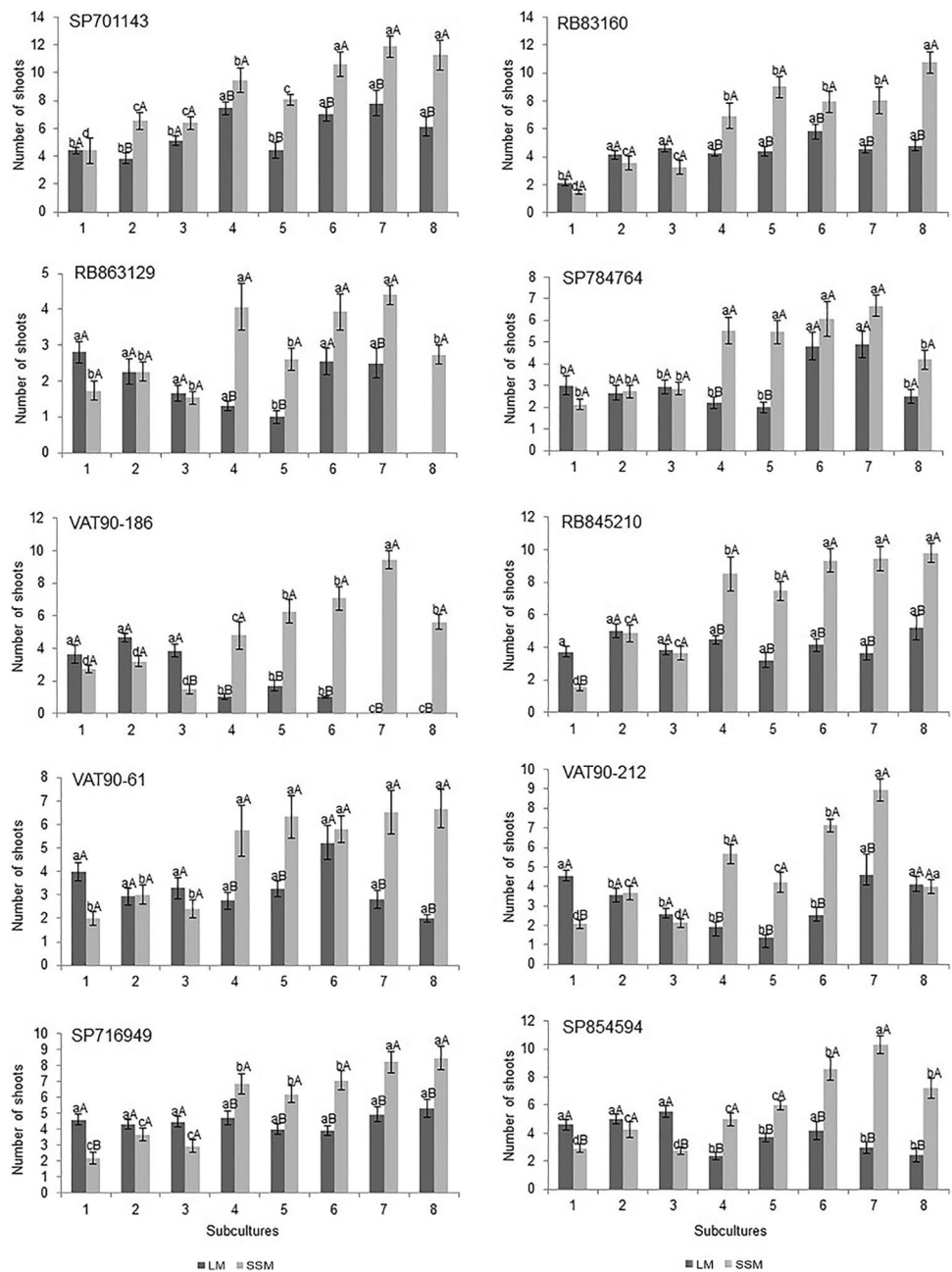
The rate of rosette formation was significantly higher when the tillers were grown in a semi-solid culture medium and, mainly, was highly influenced by the genotype. The most pronounced levels of rosette formation were observed in the varieties RB845210 and SP701143, with total averages of 37 and 34%, respectively. Then comes the variety SP716949, with an average of 19% rosette formation in the SSM. The other varieties presented this type of disposition

less frequently, at rates that varied up to 10% during subcultures, regardless of the culture medium used.

The arrangement of the shoots and the phenotypic variations observed over time have practical implications when considering the large-scale production of healthy and uniform plantlets. In these cases, the individualization of the tillers and the rhizogenesis process can be compromised.

Generally, this shortening is reversible when the shoots are transferred to basic media without the presence of growth regulators, particularly cytokinins, before the rhizogenesis phase Scherwinski-Pereira et al. 2012). The addition of exogenous cytokinins in the culture medium is often required for the differentiation and formation of adventitious shoots. However, morphological abnormalities have been reported when plants are grown in their presence. Ramage and Williams (2004) verified the formation of rosettes in tobacco plants grown in the presence of BAP. Vitri-fied shoots (Sivanesan and Jeong 2012), anatomical and ultrastructural variations (Quiala et al. 2012), stunting and

**Fig. 4** Multiplication rates in liquid (LM) and semi-solid (SSM) media of the ten Brazilian sugarcane varieties in eight consecutive subcultures. \* Lower case letters compare the formation of shoots within each medium and upper-case letter compare between the media, indicating that averages followed by the same letter belong to the same group by the Scott-Knott Test at the 5% probability level



excessive branching (Honda et al. 2011) were also detected as typical changes caused in response to cytokinin.

In this research, circumstantially, the appearance of green masses was observed, characterized by the excessive formation of small and apparently abnormal sprout with twisted, disorganized leaves and a light green color (Fig. 5B). These were observed during the multiplication of the RB845210 variety from the fifth subcultivation. In addition, it is possible to verify, in some cases, the formation of isolated sprouts or at the base of the clumps showing oxidized and/or necrotic leaves (Fig. 5C). Probably, the excess of growth regulator, resulting from

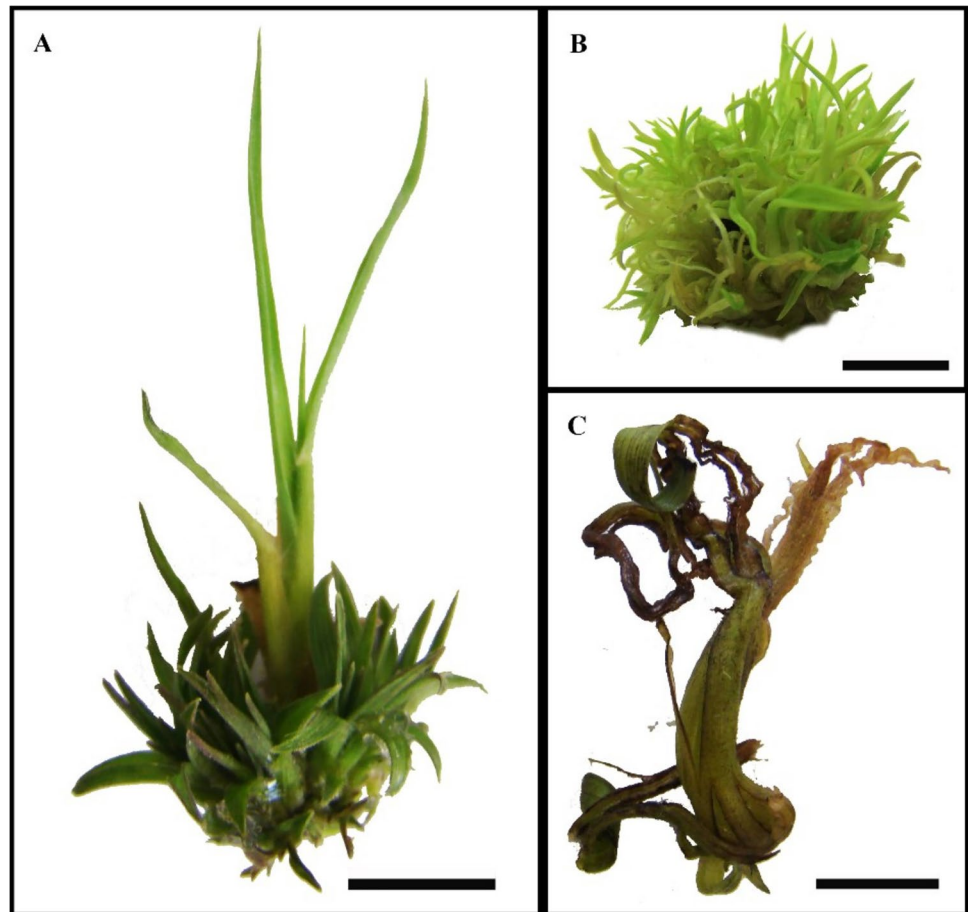
numerous multiplication cycles, may have caused such variations.

In vitro cultivation of sugarcane, in some cases, presents considerable levels of somaclonal variation (Larkin and Scowcroft 1981; Zucchi et al. 2002). Taylor et al. (1995) found morphological variations such as abundant tillering and leaf blade folding in sugarcane plants regenerated from embryogenic calluses, however, analysis by molecular marker detected few polymorphic bands indicating that genetic variations were uncommon.

However, genetic variations have been reported previously for the species and, in general, should be taken into



**Fig. 5** Morphological variations observed in different varieties of sugarcane after consecutive subcultures in semi-solid medium. Highlight for the rosette type arrangement, with thickening of shoots in the basal region (A), formation of green masses (B) and the aspect of abnormality (C) (Bars = 1 cm)



account. Heinz and Mee (1971) working with sugarcane plants (variety H50-7209) regenerated from the callus culture, detected clones with variation in the number of chromosomes from 94 to 120 (the overall range was  $2n = 94–120$ ). Genetic variations between regenerators of distinct morphogenic routes (direct and indirect) have been reported by Suprasanna et al. (2010). In general, these studies have shown that some sugarcane genotypes are more prone to variation than others and that instability is possibly a consequence of the interaction between genotype and culture medium (Zucchi et al. 2002; Snyman et al. 2011). This fact is also proven through the results found in this work, where the ten Brazilian varieties of sugarcane studied showed different levels of phenotypic variation, when grown under the same environmental conditions.

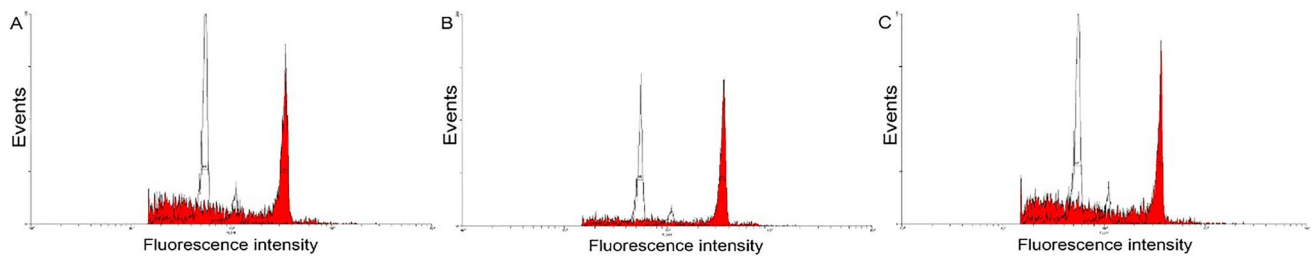
Variations at different levels have also been found for other species in the *Poaceae* family. Chromosomal aberrations, variations in the DNA nucleotide sequence (insertions and deletions), as well as morphological variations have already been detected in rice plants (*Oryza* spp.) regenerated in vitro (Kharabian and Darabi 2005; Rasheed et al. 2005; Miyao et al. 2012). Similarly, variations in barley plants have also been reported (Bednarek et al. 2007).

Some authors believe that somaclonal variation is an alternative way of creating variants and thus expanding the variability of the species' germplasm (Li et al. 2010). However, it is undesirable in the clonal multiplication of genetically uniform plantlets for commercial purposes.

### Genetic stability through flow cytometry

Sugarcane has proved to be an excellent candidate for studies of somaclonal variation, due to a combination of factors such as in vitro efficiency, morphological variations, genetic complexity and economic importance. In general, the analysis of in vitro sprouts by flow cytometry resulted in a G1 peak of DNA with good resolution and coefficients of variation below 3.5%. Representative histograms of three varieties of sugarcane are shown in Fig. 6.

For the 10 varieties studied, seven (RB83160, VAT90-186, VAT90-212, SP701143, SP854594, RB845210 and SP716949) did not show variations in the relative amount of nuclear DNA over eight consecutive subcultures. However, for varieties RB863129, VAT90-61 and SP784764, statistical differences were detected in response to the eight consecutive subcultures (Table 2).



**Fig. 6** Histograms showing flow cytometry analysis of the amount of DNA in sugarcane plants obtained using the Marie buffer. The first and second peak corresponds to the tomato plant standard (peaks at G0/G1 and G2 phases), and the third peak corresponds to sugarcane nuclei in G0/G1 phase of cell cycle. **A:** SP701143 variety; **B:** VAT90-212 variety; **C:** RB83160 variety

**Table 2** Estimated average relative DNA content (pg) of the sugarcane varieties verified over consecutive subcultures

N°. Subcultures	Variety									
	RB83160	VAT90186	VAT90212	VAT90-61	SP701143	RB863129	SP854594	SP784764	RB845210	SP716949
2°	12.4904 a	12.3874 a	12.5700 a	12.3613 b	12.3511 a	12.2605 a	12.3848 a	12.6137 a	12.7171 a	12.7889 a
4°	12.4528 a	12.1324 a	12.6606 a	12.5385 ab	12.3980 a	12.3104 a	12.4279 a	12.6131 a	12.7029 a	12.9319 a
6°	12.6707 a	12.4170 a	12.4829 a	12.7256 ab	12.5341 a	11.8797 b	12.3752 a	12.4103 ab	12.7583 a	12.8185 a
8°	12.5127 a	12.4583 a	12.5730 a	12.7602 a	12.2788 a	11.7498 b	12.4327 a	12.2249 b	12.8551 a	12.9703 a

\*Averages followed by the same letter, belong to the same group, using the Scott-Knott Test at the 5% probability level

The varieties RB863129 and SP784764 showed a reduction of approximately 0.45 pg in the amount of DNA, starting from the fourth subculture. In contrast, the VAT90-61 variety exhibited an increasing trend in relative DNA content over time in vitro cultivation.

Variations in the relative amount of DNA, even if small, occurred in 30% of the varieties from the fourth subculture and coincided with the appearance of morphological changes in the explants. These two characteristics observed demonstrate the need to control the number of consecutive subcultures for sugarcane. The size of the

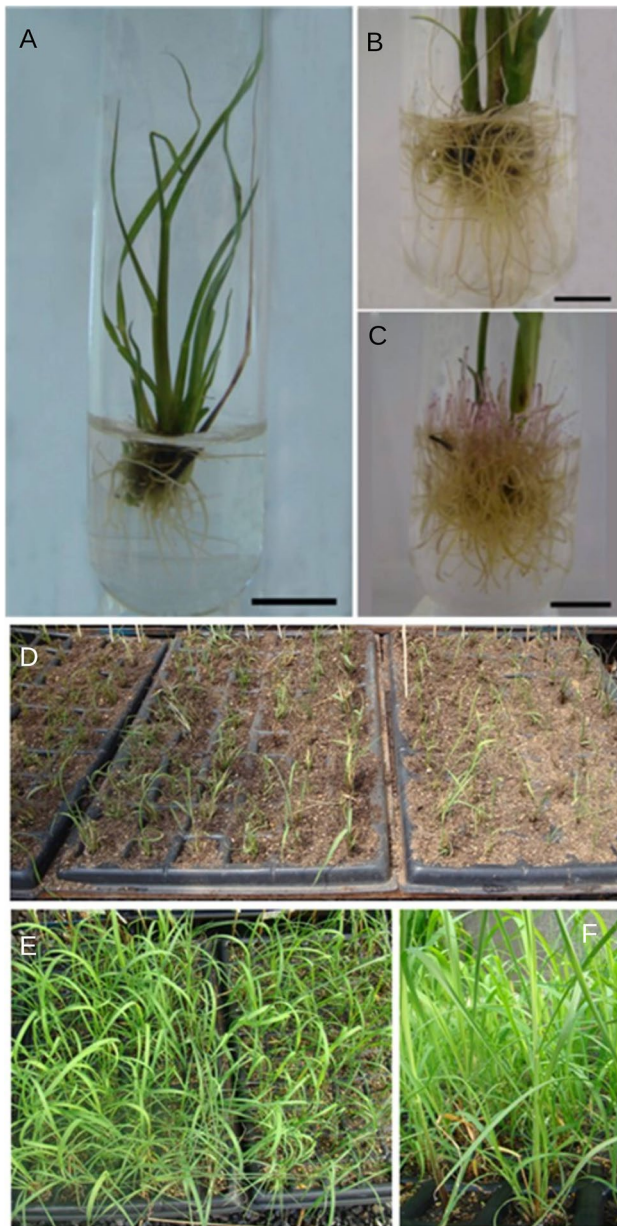
plant genome or the 2C content of nuclear DNA is highly plastic in response to environmental conditions (Msogoya et al. 2011). According to Bennetzen et al. (2005), the increase in the size of the genome may be caused by phenomena such as polyploidy and by transposable elements (transposons). On the contrary, the decrease frequently occurs due to deletions or chromosomal breaks (Petrov 2001).

In vitro, the stress condition caused by constant subcultures, the use of growth regulators and the cultivation for a prolonged period may have supposedly contributed to the

**Table 3** Response of sugarcane sprouts multiplied in liquid medium (LM) and semi-solid medium (SSM) to the process of in vitro rhizogenesis

Variety	Rooting (%)		Root number		Shoot number	
	LM	SSM	LM	SSM	LM	SSM
RB 845210	60 bB	100 aA	27.0 bA	22.4 bA	3.9 aB	7.3 aA
SP 784764	90 aA	80 aA	36.0 bA	20.0 bB	3.7 aA	3.3 bA
SP 701143	90 aA	90 aA	45.0 aA	28.0 bB	3.6 aA	5.4 aA
RB 83160	100 aA	80 aA	54.0 aA	34.0 aB	3.2 aA	2.8 bA
VAT 90-212	100 aA	90 aA	25.8 bA	26.2 bA	3.8 aA	4.9 aA
SP 716949	100 aA	90 aA	41.0 aA	36.0 aA	2.5 aA	3.9 bA
RB 863129	–	100 aA	–	19.1 bA	–	2.7 bA
VAT 90-61	–	100 aA	–	41.0 aA	–	4.2 bA
SP 854594	–	100 aA	–	23.8 bA	–	6.0 aA
VAT 90-186	–	100 aA	–	41.0 aA	–	6.8 aA

\*Averages followed by the same lower-case letter in the column do not differ statistically from each other by the Scott-Knott test at the 5% probability level. Different capital letters within each variable differ from each other by the F-test



**Fig. 7** Visual aspect of sugarcane sprouts after 40 days in rooting medium and during the acclimatization period. **A:** Overview of the aerial part and developed root system; **B:** Proliferation of roots by sprouting from the semi-solid medium; **C:** Proliferation of roots by sprouting from the liquid medium; **D:** Day of transfer of plantlets to substrate; **E, F:** Plantlets growth and development 21 days after transplantation (Bars A-C = 1 cm)

occurrence of these phenomena, since, in three varieties (RB863129, SP784764 and VAT90-61) genetic instability was observed (Table 2). Previously, Nogueira et al. (2015) found that the storage of sugarcane genotypes for long periods in vitro, even under conditions of minimal growth, can lead to changes in the estimated amount of nuclear DNA and, thus, increase the risk of somaclonal variations.

It should also be noted that variations in the global DNA content in response to in vitro culture were also detected by flow cytometry for other plant species. The combination of 2,4-D with kinetin caused a reduction in the relative amount of DNA, corresponding to the loss of 4 to 5 chromosomes, in some *Gossypium hirsutum* plants regenerated by callus culture (Jin et al. 2008).

Banana micropropagation resulted in the formation of somaclones with significantly lower leaf DNA content (1.72 picograms) when compared to normal plants, with an estimated value of 1.82 picograms. It is noteworthy that these plants were detected in field condition for presenting morphological variations (Msogoya et al. 2011). However, based on results found by Loureiro et al. (2007), morphological variations are not always directly associated with genetic changes. These authors found that *Juniperus phoenicea* plants micropropagated in vitro, although presenting different morphotypes, did not differ in the amount of DNA.

Given the above, flow cytometry analysis demonstrates the potential to quickly detect the genetic instability of materials grown in vitro and suggest further studies, when necessary.

**In vitro rooting and acclimatization in a greenhouse**

The rooting process of sugarcane sprouts from in vitro multiplication occurred efficiently, as can be seen in Table 3. Sprouts from the liquid medium showed a rate of 90 to 100% rooting, except for the variety RB845210, which had significantly lower root formation (60%). It should be noted that, among the varieties studied, four did not have enough plants to be rooted and then acclimatized.

Regarding the rooting of sprouts from the semi-solid medium, it can be observed that there was no significant difference between the 10 varieties evaluated. Root proliferation in vitro occurred between 80 and 100% of sprouts (Table 3). The average number of roots formed was high (above 20 per plant), however, it diversified between the varieties and their medium of origin. The production of new shoots was also observed during the period of in vitro rhizogenesis.

The roots quality is essential for success in the acclimatization stage. In this work, the formation of vigorous roots and the presence of many root hairs were observed. There was no callus formation at the base of the explants and adventitious roots were produced directly from the basal portion of the plants (Fig. 7A-C).

The survival of shoots multiplied and rooted in vitro was greater than 80% when transplanted to substrate in the greenhouse. In general, all the plants that produced roots managed to establish themselves ex vitro, demonstrating the importance of this stage for the completion of the micropropagation system of the species. With three weeks

in substrate, the plantlets adapted well to the external environment and showed a significant increase in the length of the aerial part of all the sugarcane varieties evaluated (Fig. 7D-F). As in the rhizogenesis phase, the source media did not interfere with the development of plantlets in the greenhouse.

## Conclusions remarks

The regeneration of stem tips of Brazilian varieties of sugarcane occurs by direct and indirect route in a period of approximately 30 to 40 days. However, the organogenic potential is genotype dependent. Among twenty-two varieties studied, sixteen had a regeneration rate equal to or greater than 60% of the explants. Regarding the multiplication of sugarcane, in general, it was observed that during the first subcultures the liquid consistency medium presented similar or superior results when compared to the semi-solid consistency medium (SSM) and from the fourth subculture, the SSM showed the highest multiplication rate for all the varieties analyzed. However, *in vitro* cultivation, for a period of more than four subcultures, induces the formation of morphological variations and alters the relative content of nuclear DNA in sugarcane plants.

In this sense, it can be concluded that despite the high rate of multiplication *in vitro* of the sugarcane varieties, the variations observed from a certain number of subcultures can make the process of obtaining plantlets unfeasible and clearly demonstrate the periodic need to renew the material to be propagated.

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**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Conflict of interest** All authors declare that they have no conflict of interest.

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