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Flow cytometry reliability analysis and variations in sugarcane DNA content

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ABSTRACT. The aim of this study was to evaluate the reliability of flow cytometry analysis and the use of this technique to differentiate species and varieties of sugarcane (*Saccharum* spp) according to their relative DNA content. We analyzed 16 varieties and three species belonging to this genus. To determine a reliable protocol, we evaluated three extraction buffers (LB01, Marie, and Tris·$MgCl_2$), the presence and absence of RNase, six doses of propidium iodide (10, 15, 20, 25, and 30 µg), four periods of exposure to propidium iodide (0, 5, 10, and 20 min), and seven external reference standards (peas, beans, corn, radish, rye, soybean, and tomato) with reference to the coefficient of variation and the DNA content. For statistical analyses, we used the programs Sisvar® and Xlstat®. We recommend using the Marie extraction buffer and at least 15 µg propidium iodide. The samples should not be analyzed immediately after the addition of propidium iodide. The use of RNase is optional, and tomato should be used as an external reference standard. The results show that sugarcane has a variable genome size (8.42 to 12.12 pg/2C) and the individuals analyzed could be separated into four groups according to their DNA content with relative equality in the

genome sizes of the commercial varieties.

Key words: *Saccharum* spp; Cytometer; Standardization

INTRODUCTION

Obtaining new varieties has the technological potential to improve the quality of raw materials and increase the productivity of sugarcane (*Saccharum* spp). Genetic improvement is the production base through which new varieties that promote sharp gains in productivity and quality are obtained. Currently, there are four programs for this purpose in Brazil: IAC (Agronomic Institute of Campinas), CTC (Sugarcane Technology Center), Canavialis (Monsanto), and RIDESA (Network Inter-Sector Development Sugarcane).

In breeding species such as sugarcane for vegetative reproduction, the process of selecting superior genotypes begins immediately in the segregation of the F1 population, which is generated from the hybridization of previously selected parents. Researchers seek to improve the efficiency of the methodologies that are used in this selection process, which occurs at the individual level (Pedrozo et al., 2009).

Flow cytometry is a useful tool for this type of selection, because it allows the estimation of the content and size of genomic DNA. This technique is a new and rapid method to efficiently and reproducibly determine the relative nuclear DNA content and level of ploidy of a large number of plant and animal species (Ochatt, 2011). Intraspecific variability has been revealed by flow cytometry in *Arabidopsis thaliana* L. (Schmuths et al., 2004), *Panicum virgatum* L. (Costich et al., 2010), *Olea europaea* ssp *europaea* var. *europaea* (Brito et al., 2008), and *Chenopodium quinoa* Wild. (Kolano et al., 2012).

However, there is little knowledge regarding nuclear DNA content and genome size in sugarcane. This information can be valuable for understanding phenomena at the intersections of cytogenetic (Burner, 1997), complementary, conventional, and molecular germplasm development programs that are aimed at increasing genetic diversity and gene exchange for the selection of superior genotypes. However, in sugarcane, the benefits of flow cytometry have not been reaped (Edmé et al., 2005). Additionally, in contrast to ploidy and chromosome number, little is known regarding differences in genome size within and between *Saccharum* species (Zhang et al., 2012).

This study aimed to evaluate the reliability of flow cytometry analysis and the use of this technique to differentiate species and varieties of sugarcane according to their relative DNA content.

MATERIAL AND METHODS

Plant material

To analyze the reliability of flow cytometry analysis, experiments were performed using *Saccharum officinarum* L. DNA content was measured in 16 varieties (SP 842025, SP 801842, SP 891115, RB 925211, SP 813250, RB 867515, CTC 159, RB 925345, CTC 9, CTC 16, CTC14, CTC 7, CTC 8, CTC 1, CTC 2, and CO 413) and three species (*S. officinarum* L., *Saccharum sinense* L., and *Saccharum spontaneum* L.) of sugarcane, which were maintained by the Department of Agriculture in a panel of sugarcane cultivars at Universi-

dade Federal de Lavras (UFLA). The individuals that were assessed were from a 12-month crop of sugarcane.

Sample preparation and extraction

Sample preparation and flow cytometry analysis were performed at the Laboratory of Tissue Culture/Department of Agriculture, UFLA. To estimate the DNA content, three samples were evaluated for each variety, each of which was considered a repeat, and the design was completely randomized with three replications. The first fully expanded leaf of each individual was used to quantify DNA by flow cytometry. One leaf was collected from each plant, and a small section in the middle region was removed. The fragments were identified, packed in plastic bags with moist cotton, and taken to the laboratory.

To evaluate the reliability of the analyses, the DNA content and the coefficient of variation (CV) for each analysis were measured using approximately 20-30 mg of young leaves and an external reference standard. The fragments were crushed on a Petri dish containing 1 mL cold extraction buffer to release the nuclei (Doležel et al., 1994). After the extraction process, the nuclei suspension was aspirated through two layers of gauze using a Pasteur pipette and filtered through a 50-µm mesh. The nuclei were stained by adding 15 µL 1 mg/mL propidium iodide solution.

In all of the analyses, the external reference standard used was the tomato (*Solanum lycopersicum* L. cv. Stupické), which contains 1.96 pg DNA, except when the studied variable was the standard reference buffer Marie.

Flow cytometry reliability analysis

Three different extraction buffers (LB01, Marie, and Tris·$MgCl_2$) (Galbraith et al., 1983) were tested, and two types of propidium iodide in six doses (10, 15, 20, 25, and 30 µg) were tested, with or without the presence of RNase (0 and 200 g/mL), in five exposure times (0, 5, 10, 15, and 20 min). We tested seven external reference standard benchmarks: pea (*Pisum sativum* L. cv. Ctirad), fava bean (*Vicia faba* L.), corn (*Zea mays* L.), radish (*Raphanus sativus* L. cv. Saxa), rye (*Secale cereale* L.), soybean (*Glycine max* L.), and tomato (*Solanum lycopersicum* L. cv. Stupické) (Table 1).

Table 1. DNA content of the external reference standards.

Reference standard	DNA content (pg)	Reference
Pea cv. Ctirad	9.09	Doležel et al. (1998)
Rye	16.19	
Fava bean	26.90	Doležel et al. (1998)
Radish cv. Saxa	1.11	
Tomato cv. Stupické	1.96	
Corn	5.43	Lysák and Doležel (1998)
Soybean	2.05	Dolezel et al. (1994)

Variation in DNA content

The analyses were performed according to the most reliable method in accordance with results of experiments described above, where we used 1 mL cold Marie buffer to release

the nuclei (Doležel et al., 1998). The nuclei were stained by adding 15 µL solution of 1 mg/mL propidium iodide for five minutes.

Statistical analyses

The CVs were obtained using the BD CellQuest™ Pro software (version 5.1) (BD Biosciences, San Jose, CA, USA), and the nuclear DNA content (pg) of the plants was estimated using the following equation:

$$\text{Sample DNA (pg)} = \frac{\text{G1 sample}}{\text{G1 standard}} \times \text{DNA standard (pg)}. \quad \text{(Equation 1)}$$

The symbol C corresponds to the size of the haploid nuclear genome (in megabase pairs, Mbp), which was estimated according to the equation proposed by Bennett and Smith (1976) for converting pg = 980 Mbp (Doležel et al., 2003).

$$\text{Genomic size (Mbp)} = \frac{[\text{DNA content 2C (pg)} \times 980\text{Mbp}]}{2} \quad \text{(Equation 2)}$$

For each sample, at least 10,000 nuclei were analyzed using a logarithmic scale. The analysis was performed using a FACSCalibur™ cytometer (BD Biosciences). Histograms were obtained using the CellQuest software (BD Biosciences), and they were analyzed using the FlowJo® 10.0.6 software.

Genome size (Mbp) and DNA content (pg) CVs were subjected to an analysis of variance using the F test, and when significant ($P \leq 0.05$), the mean CVs and DNA content values were grouped using the Scott and Knott (1974) cluster test. The samples were grouped according to their DNA content similarity values, which were obtained using principal coordinates analysis (PCoA) in XLSTAT (Addinsoft, 2010), for different DNA content values, species, and varieties.

RESULTS

Flow cytometry reliability analysis

The reliability of the flow cytometry analysis was measured according to the CVs and DNA content of each analysis. There were no significant differences between the DNA contents and CVs in the flow cytometry analysis using different buffers (Table 2). The analysis performed with the Marie buffer exhibited a CV that was statistically identical to those using the LB01 and Galbraith buffers. Such buffers are capable of maintaining the integrity of the solution that is formed by the nuclei of *S. officinarum* isolates (Figure 1).

Significant differences were also found between the DNA contents and CVs obtained using different amounts of the fluorochrome propidium iodide, as presented in Figure 1 and Table 2.

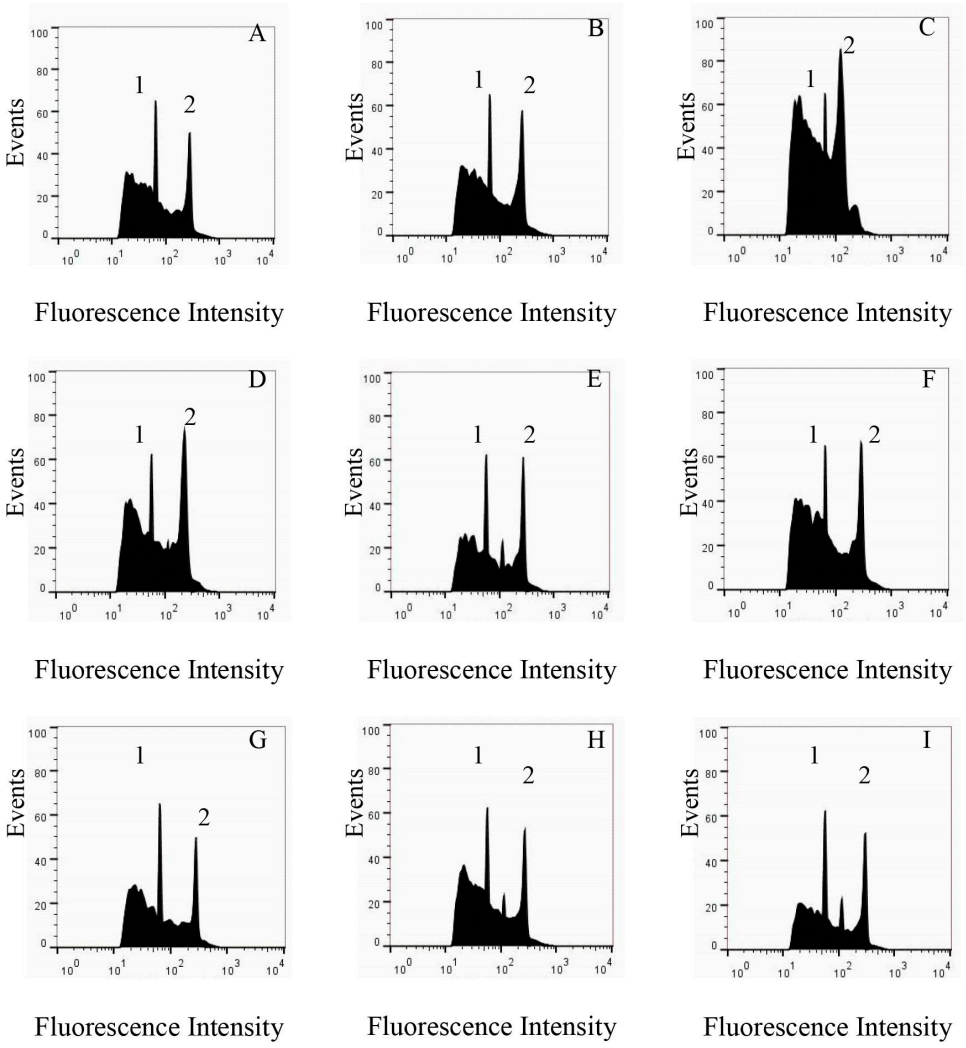

Figure 1. Flow cytometry histograms of extraction buffers: (**A**) Marie; (**B**) LB01; (**C**) Tris·$MgCl_2$; the amount of propidium iodide, (**D**) 10 µg, and (**E**) 20 µg; the exposure time of propidium iodide, (**F**) 0 min, and (**G**) 10 min; and the presence (**H**) or absence (**I**) of RNase from *Saccharum officinarum* L. Peak 1, tomato (*Solanum lycopersicum* L. cv. Stupické) and Peak 2, sample.

Analyses with varying amounts of propidium iodide between 15 to 30 µg yielded statistically identical results for both variables, possibly due to the saturation of the dye; i.e., at a certain point, all of the nuclei were stained and the addition of more fluorochromes did not provide an increase in intensity.

The variables also exhibited statistically significant differences after different exposure times of the solution to the nuclear fluorochrome. The CV obtained immediately after adding the dye had the highest value, resulting in estimates less reliable than those determined at other times (Figure 1 and Table 2).

Table 2. DNA content (pg) and coefficients of variation (CVs) obtained from flow cytometry analysis of sugarcane (*Saccharum officinarum* L.), with different extraction buffers, with different amounts of propidium iodide, for different exposure times (minutes) to the nuclear fluorochrome propidium iodide solution, as a function of RNase (0 or 200 µg/mL), and referencing the external reference standard.

	DNA content (pg)	CV (%)
Buffers		
Marie	8.32[a]	4.22[a]
LB01	7.62[b]	5.04[a]
Tris.$MgCl_2$	3.84[c]	7.91[b]
Propidium iodide (µg)		
10	7.26[a]	5.01[b]
15	7.93[b]	4.00[a]
20	8.05[b]	4.13[a]
25	8.16[b]	4.30[a]
30	8.16[b]	4.36[a]
Exposure Time (min)		
0	8.24[a]	5.17[b]
5	8.27[a]	4.00[a]
10	8.42[a]	4.33[a]
15	8.59[b]	3.83[a]
20	8.64[b]	4.17[a]
RNase		
Presence	7.73[b]	4.04[a]
Absence	8.30[a]	4.06[a]
External reference standard	DNA content (pg) *S. officinarum*	CV (%) reference standard
Rye	7.16[a]	2.72[a]
Tomato cv. Stupické	8.09[b]	3.09[a]
Pea cv. Ctirad	**	**
Radish cv. Saxa	8.69[b]	5.55[c]
Fava Bean	9.30[c]	5.53[c]
Corn	10.24[c]	4.54[b]
Soybean	10.34[c]	4.79[b]

Means followed by the same lowercase letter belong to the same group, using Scott-Knott analysis ($P \leq 0.05$). **Overlapping peaks of the sample and the external standard.

The results from using RNase were not different from those that were obtained in the absence of the enzyme, when only considering the CVs (Figure 1 and Table 2). The same was not true of the DNA content. In the absence of RNase, the results of the DNA content were as expected, and it is therefore recommended that sugarcane samples for flow cytometry are prepared without RNase.

Significant differences were found when analyzing the influence of the external reference standard in the reliability of the flow cytometry analysis, for both variables. When radishes were used, intermediate results were obtained for the two variables. However, the CV of this analysis was not accepted, and the bean was statistically equal to the radish (5.53 and 5.55%, respectively) (Table 2). The analysis of the pea demonstrated that overlapping peaks were due to the proximity of the DNA content values and those of the reference standard and the sample analyzed (Figure 2 and Table 2). Fava beans (26.90 pg) and rye (*Secale cereale*, 16.19 pg) should be avoided as reference standards in sugarcane analysis, because the DNA content of both species was much higher than that of sugarcane, which could lead to errors in the results. Table 1 lists the DNA contents (pg) of the benchmarks that were used in this experiment.

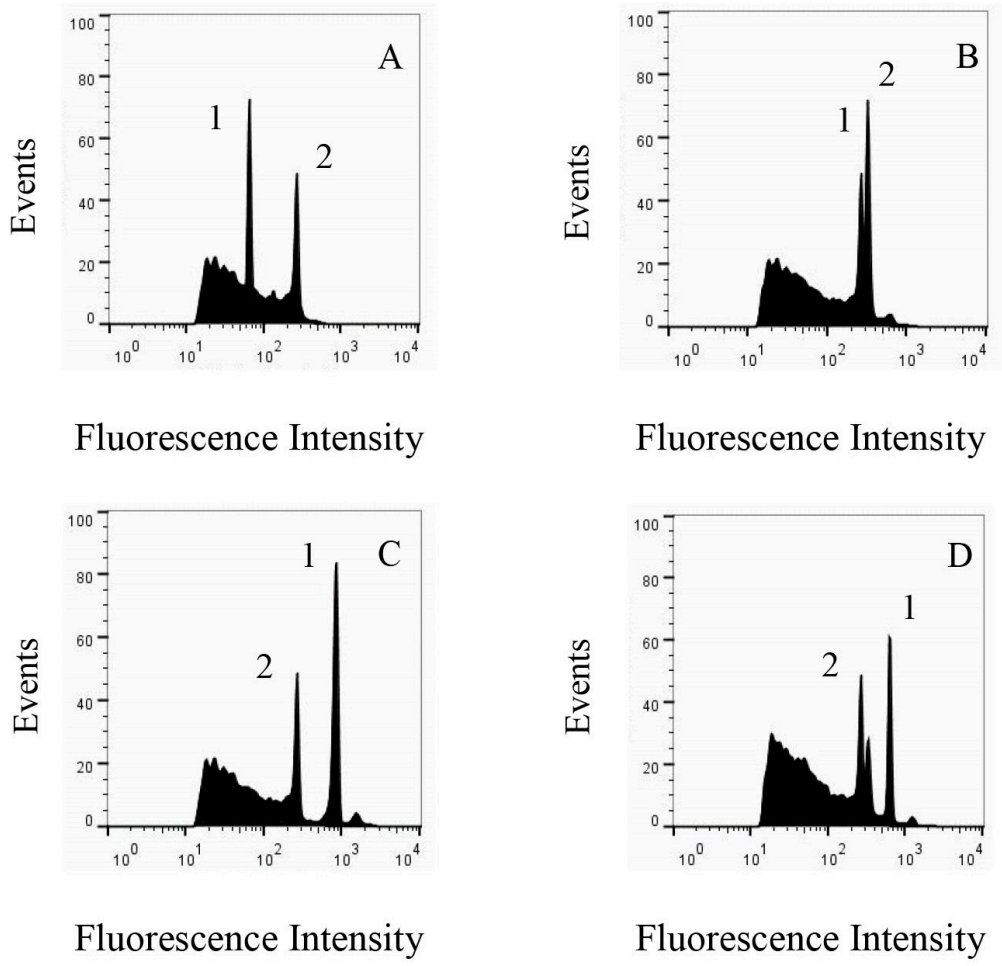

Figure 2. Flow cytometry histograms of different external standards. Peak 1, *Saccharum officinarum* L. and Peak 2, (**A**) tomato (*Solanum lycopersicum* L.), (**B**) pea (*Pisum sativum* L.), (**C**) bean (*Vicia faba* L.), and (**D**) soybean (*Glycine max* L.).

Variation in DNA content

The *Saccharum* spp could be divided into four groups using the Scott-Knott test. The largest group (CTC 7, CTC 9, CTC 2, SP 801842, CTC 8, SP 813250, SP 842025, RB 925211, RB CTC 14, and RB 925345) was also the group with the highest average DNA content and genome size, ranging from 12.12 to 11.27 pg/2C and from 5938.8 to 5522.3 Mpb/1, respectively. In descending order, the next group was composed of RB 867515, *S. sinensis*, CTC 15, CTC16, CTC 1, SP 891115, and CO 143, in which DNA content and genome size ranged from 11.09 to 10.15 pg/2C and from 5434.1 to 4973.5 Mpb/1C. *S. officinarum* formed a group with 8.42 pg/2C and 4125.8 Mbp/1C (Table 3).

Table 3. DNA content (pg) and genome size of varieties and species of sugarcane (*Saccharum* spp).

Variety/species	DNA content (pg)	Genome size (Mbp)	Scott-Knott (5%)
CTC 7	12.12 ± 0.36	5938.8	a
CTC 9	12.05 ± 0.21	5904.5	a
CTC 2	11.99 ± 0.40	5875.1	a
SP 801842	11.79 ± 0.35	5777.1	a
CTC 8	11.77 ± 0.37	5767.3	a
SP 813250	11.75 ± 0.55	5757.5	a
SP 842025	11.63 ± 0.26	5698.7	a
RB 925211	11.53 ± 0.26	5649.7	a
CTC 14	11.31 ± 0.48	5541.9	a
RB 925345	11.27 ± 0.39	5522.3	a
RB 867515	11.09 ± 0.45	5434.1	b
S. sinense	11.07 ± 0.38	5424.3	b
CTC 15	11.10 ± 0.40	5439.0	b
CTC 16	10.50 ± 0.30	5145.0	b
CTC 1	10.47 ± 0.13	5130.3	b
SP 891115	10.46 ± 3.76	5125.4	b
CO 143	10.15 ± 0.30	4973.5	b
S. officinarum	8.42 ± 0.50	4125.8	c
S. spontaneum	7.70 ± 0.32	3773.0	c

Means followed by the same lowercase letter belong to the same group, using Scott-Knott analysis ($P \leq 0.05$).

For the PCoA, a two-dimensional plot was constructed (Figure 3). We included the first two components provided by the analysis, which explained as much as 100.00% of the variation between the samples: 62.22% (F1) and 37.78% (F2). The groupings differed between the PCoA and the Scott-Knot test, which classified the varieties differently.

All of the species and varieties that were analyzed were well represented in this plan, and they formed a shape that was close to the unit circle. The variables that were in the same quadrant were strongly correlated, but those that were in different quadrants were not. Using the PCoA method, we found a natural clustering of genotypes due to their DNA content similarity, demonstrating the efficiency of flow cytometry.

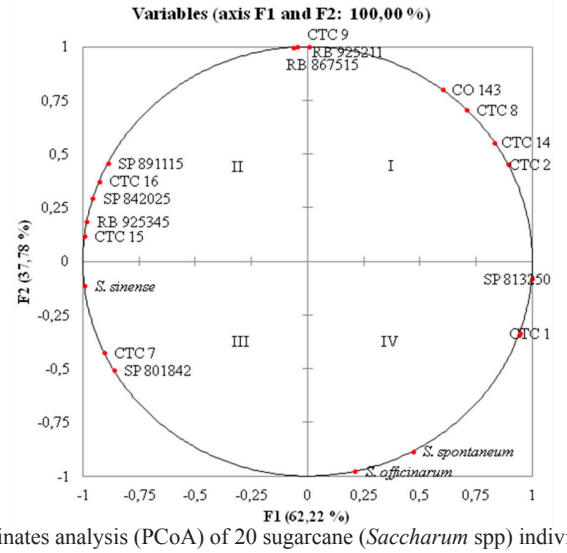

Figure 3. Principal coordinates analysis (PCoA) of 20 sugarcane (*Saccharum* spp) individuals analyzed according to their DNA content (pg) and genomic size (Mbp).

DISCUSSION

Flow cytometry reliability analysis

Galbraith et al. (2001) stated that the CV is an elementary criterion that reflects the reliability of flow cytometric analysis; the two parameters (CV and DNA content) are inversely proportional. The DNA content that was obtained using Marie's buffer differed statistically from that of the other buffers, and more closely resembled that found in the literature (8.32 pg/2C, Edmé et al., 2005).

Marie's buffer is composed of Marie glucose (nuclear glucose that maintains its integrity and prevents the formation of agglomerates of cores) and EDTA (which is used to bind to divalent cations as cofactors that serve nucleases, enzymes that carry out the degradation of the nucleic cell), and some inorganic salts (KCl and NaCl) are added to the solution as stabilizing ions. The pH of the solution varied within narrow limits (7.0-8.0). The presence of two non-ionic detergents, Triton-X-100 and Tween 20, facilitated the release of the cytoplasm and nuclei; the cytoplasm removes debris from the surface of the nuclei, and the chloroplasts disperse and reduce the tendency of the nucleus and cytoplasmic fragments to aggregate (Marie and Brown, 1993). These characteristics are essential to obtain a reliable analysis, as observed in this experiment.

Other studies have been conducted with *Vitis vinifera* (Leal et al., 2006) and *Quercus suber* L. (Loureiro et al., 2006a), and have obtained similar results to those of the present study, with the results obtained with the buffer Marie being the most reliable.

When using 10 µg propidium iodide, the averages of the variables differed from those at other doses. In this study, the CV regarding the amount of dye was greater than the accepted value in the literature, which is 5% (Guimarães et al., 2009). Cytosolic compounds interfere with the binding of the fluorochrome with the DNA dye, inhibiting fluorescence; this interference is common in plants (Price et al., 2000), and can occur due to the greater amounts of dye needed for accurate readings (Table 2).

Loureiro et al. (2006b), in analyzing *Pisum sativum*, found that 1 mL of a suspension containing nuclei was saturated with 150 µg of propidium iodide, using five times the highest amount that was used in this experiment. However, such a solution may have been sufficiently dyed long before this point. For Barre et al. (1996), an incubation period of 5-10 min is usually sufficient to saturate all DNA sites. Longer periods of staining can decrease the fluorescence and/or increase levels of waste, which significantly compromises flow cytometry analysis.

Loureiro et al. (2006b) found that in *P. sativum*, after 60 min of exposure to a nuclei-containing suspension, the fluorescence began to decrease. This decrease was not observed in this study, and this may not have occurred due to the long test times, as the time interval that was used by Loureiro et al. (2006b) is not achieved in routine analyses.

RNase, also called endonuclease, is responsible for the degradation of RNA (Brown, 1999). Enzyme treatment may be necessary for the determination of DNA content, because some dyes such as propidium iodide also bind to RNA, leading to an overestimation of DNA content and justifying the use of this protein in this type of analysis. Frequently, however, RNase may not be effective due to low amounts of RNA in the sample. This often occurs in leaves, for example, where the use of RNase may seem necessary. However, it is worth highlighting that treatment with RNase is essential when analyzing RNA-rich tissues, such as seeds and meristems (Doležel et al., 2007). This fact explains the results in Table 2.

Price and Johnston (1996) recommended that the reference standard used in flow cy-

tometry must have a value close to that of a DNA sample whose content is unknown to avoid non-linearity, but they should not have overlapping peaks.

Variation in DNA content

The flow cytometry analysis showed only one peak corresponding to the G0/G1 level (2C) of the cell cycle. Peaks corresponding to the G2 + M (M = mitosis) level (4C) were not detected, indicating a lack of cell division or endoploidy (an increase in the number of chromosomes due to replication, without further cell division).

The results presented here show that sugarcane has a variable genome size (8.42 to 12.12 pg/2C). The increase in DNA content of some commercial varieties may not necessarily affect the phenotype of these interspecific populations, and this was probably caused by self-duplication (duplication of the genome that occurs without cell division).

The varieties were classified in virtually the same group, indicating that the size of the genome is stable in commercial varieties (the other 15 varieties were divided into two groups). According to the literature, this stability can be observed even after 12 generations of interbreeding of advanced materials (Edmé et al., 2005). Such genome stability can be explained by the fact that most varieties of sugarcane present in the world are derived from the intercross progeny of a few relatives, or by indirect selection pressure. A similar stability is observed in other crops such as banana (*Musa* spp) (Lysák et al., 1994).

The differences in DNA content and genome size may be attributed to the nucleotipic effect. This affects variations in both development and adaptation, through its effects on parameters such as nuclear and cellular volume, and the timing of mitotic and meiotic cycles (Nunes et al., 2009).

Williams et al. (2002) found a slight variation in DNA due to small deletions or amplifications of DNA sequences in chromosomes during the hybridization process. Therefore, hybridization may eventually change the size of the genome without a simultaneous change in the number of chromosomes.

The use of more than one clustering method classified them such that each technique prevented erroneous inferences that are adopted in the allocation of material within a particular subgroup of varieties (Arriel et al., 2006; Silva et al., 2012). The varieties that were grouped in quadrant I shared similar values in rusticity, high fiber content, and a higher incidence of lodging. Those present in the second quadrant were considered late, and those in quadrant III had a high productivity. Those in the fourth quadrant were early and more tolerant to stress (Figure 3) (Sugarcane Technology Center, 2002; Hoffmann, 2008; Marin, 2013).

CONCLUSIONS

It is possible to establish a reliable analysis of flow cytometry in sugarcane using the Marie extraction buffer and at least 15 µg propidium iodide; the samples should not be analyzed immediately after the addition of the buffer and propidium iodide.

Flow cytometry classified the different varieties and species that were analyzed into four groups, according to their genomic DNA size and content.

Conflicts of interest

The authors declare no conflict of interest.

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