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Nuclear DNA content and chromosome number in *Brachiaria* spp. genotypes[1]

Conteúdo de DNA nuclear e número cromossômico de genótipos de *Brachiaria* spp.

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ABSTRACT - Breeding programs for *Brachiaria* spp. use both intraspecies and interspecies crosses between sexual and apomictic plants in order to obtain new cultivars with the desired characteristics. As there are different ploidy levels both within and between species of this genus, it becomes necessary to evaluate the genotypes used in breeding programs, as a guide to breeders when adopting crossing strategies. In this work, DNA content and chromosome number were determined in order to characterise ploidy levels in *Brachiaria* spp. genotypes. In the analysis of 15 genotypes, DNA content varied with the ploidy levels (2x, 3x and 4x), and between species and/or taxon. The average DNA content was 1.74 pg (2x) in *B. ruziziensis*, 3.74 pg (4x) in *B. decumbens* and 3.52 pg (4x) for *B. brizantha*. For the genotype 86, 2.57 pg of DNA was obtained and 2n = 3x = 27, indicating a triploid accession, probably a natural hybrid. The variation in the total DNA content allowed the differentiation of *Brachiaria ruziziensis* (2n = 2x = 18) from the tetraploid species *Brachiaria Brizantha* and *Brachiaria decumbens* (2n = 4x = 36), as well as the probable hybrid triploid (2n = 3x = 18) of these species.

Key words: Germplasm bank. Cytogenetics. Flow cytometry. Forage.

RESUMO - Os programas de melhoramento de *Brachiaria* spp. utilizam os cruzamentos intraespecíficos e interespecíficos entre plantas sexuais e apomíticas para obtenção de novas cultivares com características desejadas. Como existem diferentes níveis de ploidia dentro e entre as espécies deste gênero, torna-se necessária a avaliação dos genótipos utilizados em programas de melhoramento para a orientação das estratégias de cruzamentos adotadas pelos melhoristas. Neste trabalho, o conteúdo de DNA e o número cromossômico foram determinados para caracterizar o nível de ploidia de genótipos de *Brachiaria* spp. Na análise de 15 genótipos, o conteúdo de DNA variou de acordo com o nível de ploidia (2x, 3x e 4x) e entre espécie e/ou taxon. O conteúdo de médio de DNA foi de 1,74 pg (2x) para *B. ruziziensis*, 3,74 pg (4x) para *B. decumbens* e de 3,52 pg (4x) para *B. brizantha*. Para o genótipo 86 obteve-se 2,57 pg de DNA e 2n = 3x = 27, indicando tratar-se de um acesso triplóide, provavelmente um híbrido natural. A variação no conteúdo de DNA total permitiu diferenciar *Brachiaria ruziziensis* (2n = 2x = 18) das espécies tetraploides (2n = 4x = 36) *Brachiaria decumbens* e *Brachiaria brizantha*, bem como o provável híbrido triplóide (2n = 3x = 18) dessas espécies.

Palavras-chave: Banco de germoplasma. Citogenética. Citometria de Fluxo. Forrageiras.

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INTRODUCTION

In Brazil, it is estimated that an area of 172 million hectares of pasture land is used to feed a herd of approximately 170 million head of cattle (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2006). In the Cerrado region, where approximately 35% of the cattle are bred, there is an estimated area of approximately 43 million hectares of *Brachiaria* spp. (KARIA; DUARTE; ARAÚJO, 2006).

Among the most widely cultivated species of *Brachiaria* in Brazil are *B. decumbens* Stapf and *B. brizantha* (Hochst.) Stapf, which are facultative apomictic tetraploids. *B. ruziziensis* (Germain et Evrard) is a sexual diploid, and is used as a "bridge" to enable hybridizations with apomictic species. For this it is necessary to double the chromosomes number of *B. ruziziensis* (SOUZA SOBRINHO *et al*., 2009) and thus use it as a female parent at the same ploidy level as the apomictic tetraploid plants, which have normal microsporogenesis and are therefore used as pollen donors (VALLE *et al*., 2008).

When breeding *B. ruziziensis*, an adopted strategy is collecting plants from pasture land and obtaining improved populations in open country. As *Brachiaria* species are widely distributed throughout Brazil, and *B. ruziziensis* is allogamous, contamination with pollen from other species is possible. Thus, confirmation of the ploidy level of the biological material becomes important piece of information in a breeding program.

Knowing the ploidy level can aid the *Brachiaria* spp. breeder in selecting parent plants with compatible ploidy levels and assist in verifying the efficiency of ploidy manipulation techniques. To determine the ploidy level of a genotype, a count of the chromosome number or an estimate of the DNA content can be used, requiring comparison of these results with plants of the same species whose ploidy level has already been established. For *Brachiaria* spp., Ishigaki *et al*. (2009) and Pinheiro *et al*. (2000) used these two methods for establishing the efficiency of induction protocols for chromosome duplication, and Penteado *et al*. (2000) to confirm the ploidy of some genotypes from a germplasm bank.

There are several reports in the literature in which the ploidy level was determined by estimating the DNA content, such as *Brachiaria* spp. (PENTEADO *et al*., 2000), *Dioscorea alata* (EGESI *et al*., 2002), *Musa* spp. (BARTOŠ *et al*., 2005; NSABIMANA; STADEN, 2006; PILLAY *et al*., 2006), *Koeleria* spp. (ALES *et al*., 2006), *Pennisetum* spp. (CAMPOS *et al*., 2009) and *Paspalum* spp. (SARTOR *et al*., 2011).

The objective of this study was to determine the ploidy level of *Brachiaria* spp. genotypes by analysis of the DNA content and chromosome counts.

MATERIAL AND METHODS

The determination of the ploidy level was carried out in 13 genotypes of *B. ruziziensis* and two commercial cultivars, *B. brizantha* cv. Marandú and *B. decumbens* cv. Basilisk, belonging to the breeding program for *Brachiaria ruziziensis* of Embrapa Gado de Leite in Juiz de Fora, in the Brazilian state of Minas Gerais.

The ploidy level was determined by estimation of the nuclear DNA content and by a count of the chromosome number.

For measuring the DNA content, approximately 30 mg of young leaf tissue was used for each sample, together with the same amount of leaf mass from *Pisum sativum* (ervilha) cv. Ctirad (quantity reference standard for DNA 2 C = pg 9.09, DOLEZEL; BINAROVA; LUCRETTI, 1989). With the help of a scalpel, the leaves were triturated in a petri dish containing 1 mL $MgSO_4$ nuclei extraction buffer (DOLEZEL; BARTOS, 2005; DOLEZEL; BINAROVA; LUCRETTI, 1989). The nuclei suspension was aspirated through two layers of gas, using a plastic pipette, and filtered through a 50 µm nylon mesh. The nuclei were stained with a solution containing 1 mg/mL propidium iodide and 5 µL RNase.

The samples were stored in the dark in a refrigerator, and analysed within 1 hour of preparation. For each sample, 10,000 nuclei were analyzed for fluorescence emission, employing a logarithmic scale. The analysis was carried out using the FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA). The histograms were obtained with the Cell Quest software (Becton Dickinson and Company, San Jose, CA, USA) and analysed using the WinMDI 2.8 software (TROTTER, 2000).

The nuclear DNA content (pg) of the samples were measured with equation 1:

$$DNA\ sample = (G_0/G_1 sample / G_0/G_1 standard) \times DNA\ standard \quad (1)$$

where: DNA sample = nuclear DNA content of the sample (pg); G_0/G_1 sample = position of the G_0/G_1 peak of the sample; G_0/G_1 standard = position of the G0/G1 peak for *P.sativum*; DNA standard = DNA content for *P. Sativum*, 2 C = 9.09 pg.

Based on the estimation of DNA in the sample, the C-value (gametic DNA content, with a chromosome number *n*) and the Cx value (DNA content in each genome, with a basic chromosome number *x*) were also calculated and transformed into megabase pair units (Mbp), where 1 pg of DNA is equal to 978 Mpb (DOLEZEL *et al*., 2003; GREILHUBER *et al*., 2005).

A completely randomised design was used, with three replications per genotype. The data were subjected to

variance analysis using the SISVAR statistical software, and the means of the DNA content were compared by the Tukey mean test ($p<0.05$) (FERREIRA, 2007).

For cytogenetic analysis, root tips from *Brachiaria* spp. plants were treated with 25 mg L^{-1} cycloheximide and 300 mg L^{-1} hydroxyquinoline (1:1) for 2 hours 45 minutes, rinsed in distilled water, fixed in Carnoy's solution (absolute ethyl alcohol:acetic acid, 3: 1) and stored in a freezer until used. To prepare the slides, the roots underwent enzymatic maceration in a solution of pectinase/cellulase (100/50U) (Sigma™), crushed, and stained with Giemsa at 5% for 10 minutes.

The slides were examined under a Leica DMLS microscope fitted with a camera, employing a 100X objective (immersion objective), with at least 20 metaphases of each analysed genotype being observed.

RESULTS AND DISCUSSION

The results of the variance analysis revealed differences in DNA content of the evaluated genotypes, with it being possible to separate the 15 genotypes into 3 groups by estimation of the DNA content (Table 1). There was consistency between the results obtained by evaluating ploidy level and chromosome number. This assumption can be made, based on a prior knowledge of the chromosome number and ploidy level of the evaluated species.

Using the DNA content, the diploid genotypes (*B. ruziziensis*), the tetraploid (*B. brizantha* and *B. decumbens*) and the triploid were all identified, in relation to the standard reference, *P. sativum* (Table 1 and Figures 1A, 1B, 1C and 1D). This information is important for the *Brachiaria ruziziensis* breeding program, since it makes it possible to confirm, quickly and accurately, the species and ploidy level of genotypes collected on pasture land or from bred populations obtained by intercrossings conducted in open country. This methodology is also valid when checking the efficiency of techniques for manipulating the ploidy level so as to induce chromosome doubling (PEREIRA *et al*., 2012).

The average DNA content in accessions of *B. ruziziensis* was 1.74 pg for the 2x genotypes (Figure 1B and Table). Ishigaki *et al*., (2009) working with chromosome doubling of *B. ruziziensis*, obtained estimates of 1.41 pg DNA for diploid plants and 2.77 pg for tetraploid plants. This difference in values for estimates of the DNA

Table 1 - Estimation of DNA amount, C-value, Cx value, chromosome number and ploidy level for the *Brachiaria* spp. genotypes

Genotypes	Means of DNA content in G1 (pg)	C-value (**Mbp C^{-1})	Cx value (Mbp Cx^{-1})	Chromosome number (2n)	Ploidy level	Species
Marandú	3.52 ±0.07 c	1720	860	36	4x	*B. brizantha*
Basilisk	3.79 ± 0.06 c	1855	927	36	4x	*B. decumbens*
R10	3.77 ± 0.04 c	1843	922	36	4x	*B. decumbens*
R02	3.67 ± 0.15 c	1794	897	36	4x	*B. decumbens*
Mean	3.74 ± 0.06	1829	914.5			
04	1.70 ± 0.03 a	831	831	18	2x	*B. ruziziensis*
09	1.70 ± 0.02 a	831	831	18	2x	*B. ruziziensis*
10	1.68 ± 0.01 a	821	821	18	2x	*B. ruziziensis*
11	1.69 ± 0.03 a	826	826	18	2x	*B. ruziziensis*
30	1.77 ± 0.02 a	865	865	18	2x	*B. ruziziensis*
37	1.71 ± 0.02 a	836	836	18	2x	*B. ruziziensis*
43	1.84 ± 0.08 a	900	900	18	2x	*B. ruziziensis*
69	1.80 ± 0.04 a	880	880	18	2x	*B. ruziziensis*
84	1.69 ± 0.03 a	826	826	18	2x	*B. ruziziensis*
329	1.85 ± 0.03 a	905	905	18	2x	*B. ruziziensis*
Mean	1.74 ± 0.06	852.1	852.1			
86	2.57 ± 0.23 b	1257	838	27	3x	Natural hybrid

Means followed by the same letter in a column do not differ statistically at 5% by the Tukey test. ** Mbp = Megabase pairs. C-value = gametic DNA content with a chromosome number *n*. Cx value = DNA content in each genome with a basic chromosome number *x*

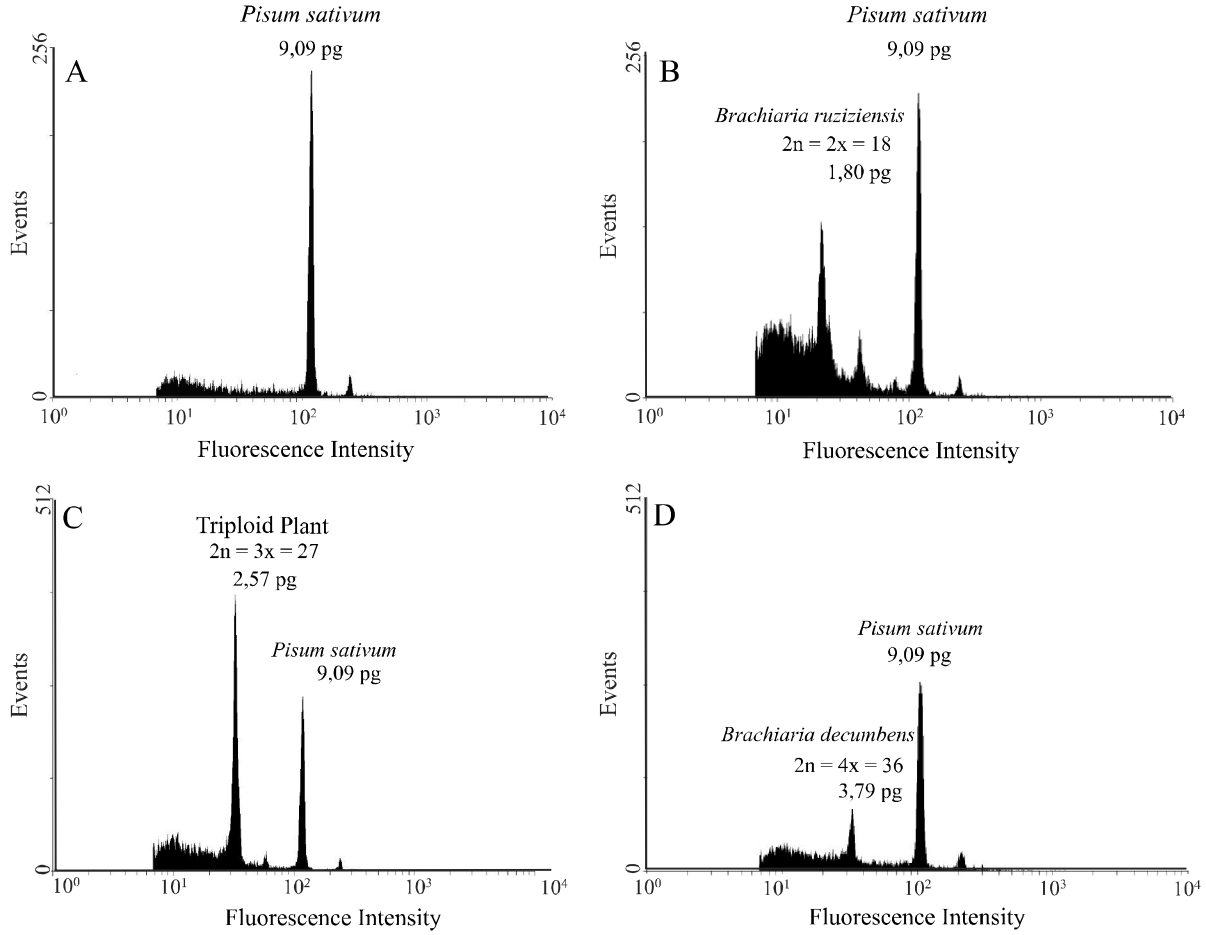
content is due to several factors, such as: instrument calibration; reference standard; plant material; nuclear extraction buffer and the fluorochrome used. These authors used a buffer containing Tris-HCl and the standard reference *Oryza sativa* (rice). In this study we used the $MgSO_4$ buffer and the standard reference, *P. sativum*.

Subsequently, Ishigaki *et al.* (2010) estimated the size of the genome of five cultivars of four species of *Brachiaria*, also employing flow cytometry. The genome size was expressed by the C-value (gametic DNA content) and the Cx value (DNA content per genome). These authors observed that the difference in C-value between the cultivars was higher for an increase in the ploidy level. However, among the apomictic polyploid cultivars, the Cx value decreased with the increase in ploidy. This work also showed an increase in C-value with the increase in ploidy, as the 2x genotypes displayed 852.1 Mbp, the 3x displayed 1.257 Mpb, and the 4x displayed 1.829 Mpb. With respect to the mean Cx value however, there was an increase for the tetraploid genotypes (914.5 Mpb) compared to the diploids (852.1 Mpb), and a decrease for the triploid (838 Mpb) in relation to both.

There was a difference of 316 and 222 Mbp between the values of C in the polyploid apomictics, *B. Brizantha* cv. Marandu and *B. decumbens* cv. Basilisk, in relation to the results observed by Ishigaki *et al*. (2010). The observed differences in these estimates were probably due to methodological and instrumental differences. In contrast to that used in this work, Ishigaki *et al*. (2010) used the nuclear extraction buffer Tris HCl and the reference standard, *Oryza sativa*, when preparating samples.

In the *Brachiaria* genus, Penteado *et al*. (2000) estimated the ploidy level by flow cytometry, for a collection of 435 accessions from 13 species. For a quantification of the DNA, the indices observed for the diploid accessions

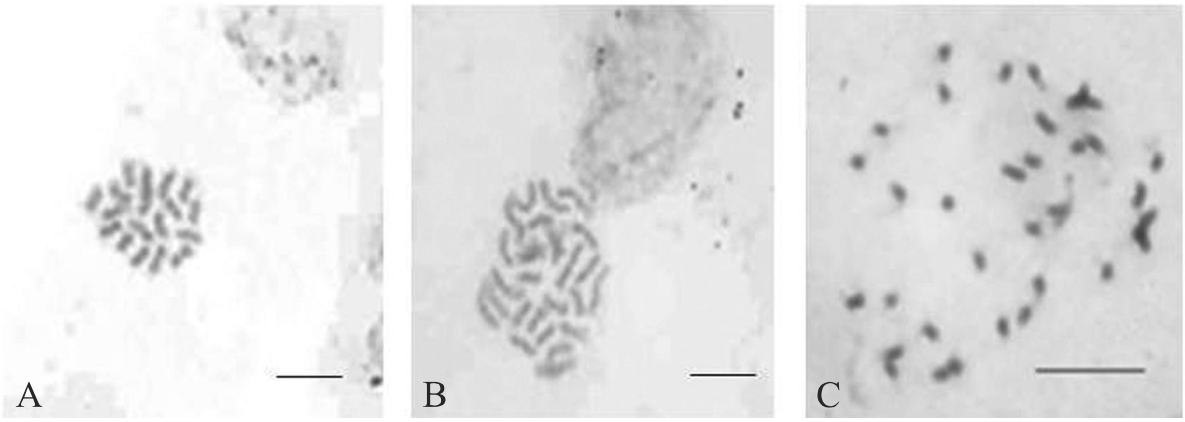
Figure 1 - Histograms of flow cytometry for DNA quantifation: A) *Pisum sativum* L. - The G1 peak is a DNA standard reference quantity of 9.9 pg; B) diploid plant - *B. ruziziensis* (Genotype 69); C) triploid plant (Genotype 86); D) tetraploid plant - *B. decumbens* (cv. Basilisk)



of each species, and the inferences drawn about ploidy levels, were made based on the indices related to the diploid standard. When there were no diploid accessions, the authors considered each chromosome complement (x) of the species as having contributed an index of 0.5, as in the case of the standard (i.e, 2x = 18 = index 1.00). Using this criterion, various ploidy levels were determined between accessions and within the studied species. As only cytometry indices were reported and the authors did not advise the DNA content, it was not possible to make comparisons with the results obtained in the present work. Confirmation of the results of the estimation of the DNA content obtained with flow cytometry (Table 1) was made by counting the chromosome number, with 18, 27 and 36 chromosomes being observed for the diploids, triploids and tetraploids respectively (Figure 2).

For genotype 86, the DNA content (2.57 pg) and somatic chromosome number (27) show that it is a triploid. This genotype probably originates from a natural cross between *B. ruziziensis* (2x) and *B. decumbens* (4x) or *B. Brizantha* (4x). This is because the DNA content for this genotype corresponds to the mean of the sum of the estimated DNA content of the gametes from these likely parent plants: 0.87 pg for *B. ruziziensis* which added to the 1.77 pg of *B. Brizantha* gives rise to a hybrid with 2.64 pg, or from the crossing of *B. ruziziensis* (0.87 pg) with *B. decumbens* (1.87 pg), giving a hybrid with 2.74 pg of DNA. These two values are within the range estimated in this work for the DNA content of the triploid hybrid of 2.34 to 2.80 pg of DNA (Table 1). Natural triploid hybrids of the genus *Brachiaria* have already been reported in the literature by Mendes *et al*. (2006) and Risso-Pascotto, Paglianini e Valle (2005).

Figure 2 - Chromosome number of different genotypes of *Brachiaria* spp: A) *B. ruziziensis* (84) with 2n = 2x = 18; B) natural hybrid (86) with 2n = 3x = 27; C) *B. ruziziensis* (Iracema) with 2n = 4x = 36. Bar = 15 µm



CONCLUSION

The variation in the DNA content distinguishes *Brachiaria ruziziensis* from the tetraploid species, *Brachiaria decumbens* and *Brachiaria Brizantha*, and from the triploid hybrids among those species collected on pasture land and from populations bred in open country.

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