CLAUDICÉIA RISSO PASCOTTO

Avaliação do comportamento meiótico em acessos poliplóides de *Brachiaria* (Poaceae: Paniceae) visando a seleção de genitores para cruzamentos interespecíficos.

> Tese apresentada ao Programa de Pósgraduação em Ciências Biológicas (área de concentração: Biologia Celular), da Universidade Estadual de Maringá para a obtenção do grau de Doutor em Ciências Biológicas.

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Ao meu esposo, Hilson, pelo seu amor, incentivo e compreensão durante todos esses anos.

Aos meus pais, Élio e Aparecida, pelo apoio constante na minha vida.

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APRESENTAÇÃO

Esta tese é composta por cinco artigos originados a partir da análise do comportamento meiótico em acessos de quatro espécies de *Brachiaria* (*B. ruziziensis, B. jubata, B. dictyoneura e B. bizantha*), coletados junto à coleção de germoplasma desta gramínea, alocada na Embrapa Gado de Corte (Campo Grande, MS).

Os artigos estão apresentados de acordo com as normas estabelecidas pelas revistas a que foram submetidos.

- 1. Claudicéia Risso-Pascotto, Maria Suely Pagliarini, Cacilda Borges do Valle . A NEW BASIC CHROMOSOME NUMBER FOR THE GENUS *Brachiaria* (Trin.) Griseb. (Poaceae: Panicoideae: Paniceae). *Genetic Resources and Crop Evolution* (in press).
- 2. Claudicéia Risso-Pascotto, Maria Suely Pagliarini, Cacilda Borges do Valle. MICROSPOROGENESIS IN *Brachiaria dictyoneura* (Fig. & De Not.) Stapf (Poaceae: Paniceae). *Genetics and Molecular Biology* (submetido).
- Claudicéia Risso-Pascotto, Daniela Vieira Mendes, Neide da Silva, Maria Suely Pagliarini, and Cacilda Borges do Valle. ABNORMAL CHROMOSOME ARRANGEMENT AT METAPHASE PLATE DURING MICROSPOROGENESIS IN A HEXAPLOID ACCESSION OF *Brachiaria brizantha* (Poaceae: Paniceae). *Genetic Resources and Crop Evolution* (submetido).
- Claudicéia Risso-Pascotto, Maria Suely Pagliarini and Cacilda Borges do Valle. MULTIPLE SPINDLES AND CELLULARIZATION DURING MICROSPOROGENESIS IN AN ARTIFICIALLY INDUCED TETRAPLOID ACCESSION OF Brachiaria ruziziensis (GRAMINEAE). Plant Cell Reports. 23: 522-527, 2005.
- Claudicéia Risso-Pascotto, Maria Suely Pagliarini, Cacilda Borges do Valle and Liana Jank. SYMMETRIC POLLEN MITOSIS I AND SUPRESSION OF POLLEN MITOSIS II PREVENT POLLEN DEVELOPMENT IN *Brachiaria jubata* (Gramineae). *Brazilian Journal of Medical and Biological Research* (in press).

Pós-graduanda: Claudicéia Risso Pascotto Orientadora: Dra. Maria Suely Pagliarini Co-orientadora: Dra. Cacilda Borges do Valle

RESUMO. A coleção de germoplasma de Brachiaria da Embrapa Gado de Corte compreende 465 acessos de 13 espécies coletadas nas savanas africanas em meados de 1980 pelo Centro Nacional de Agricultura Tropical - CIAT (Colômbia). Algumas espécies têm mostrado grande potencial como fonte de variabilidade genética para ser explorada em cruzamentos interespecíficos. Considerando que a maioria dos acessos analisados até o momento é poliplóide e apomítica, e que o programa de melhoramento depende do uso de plantas sexuais como genitor feminino e acessos apomíticos compatíveis com o mesmo nível de ploidia como doador de pólen, a caracterização citogenética de todos os acessos desta coleção é fundamental para direcionar o programa de hibridização. Estudos citológicos realizados sobre a microsporogênese de germoplasma de B. dictyoneura, B. brizantha, B. ruziziensis e B. jubata revelaram que alguns acessos apresentaram anormalidades meióticas severas, comprometendo a viabilidade gamética. Em B. dictyoneura, um novo número básico de cromossomos (x = 6) foi identificado para o gênero. Esta espécie está representada por apenas nove acessos na coleção, o que não permite afirmações conclusivas sobre a mesma, Porém os cinco acessos analisados mostraram-se tetraplóides (2n = 4x = 24) e as anormalidades meióticas foram típicas de poliplóides, acrescidas de fusão celular e ausência de citocinese. Em B. brizantha, um acesso hexaplóide (2n = 4x = 54) mostrou o conjunto cromossômico arranjado em duas placas metafásicas e a segregação cromossômica ocorreu em um fuso tripolar somente para o genoma haplóide, formando uma célula trinucleada ao final da primeira divisão. Um acesso sexual de B. ruziziensis tetraploidizado artificialmente (2n = 4x = 36) apresentou orientação de fuso anormal durante a primeira divisão, levando à celularização ao final da meiose. As anormalidades observadas nos acessos analisados não se microsporogênese, observadas restringiram a mas foram também durante а microgametogênese. Um acesso de B. jubata apresentou anormalidades na mitose do pólen, comprometendo o desenvolvimento do grão de pólen. Os acessos poliplóides e apomíticos de Brachiaria são pseudógamos, o que significa que o núcleo não-reduzido do saco embrionário necessita ser fertilizado por um gameta masculino para garantir o bom desenvolvimento do endosperma e sementes viáveis, embora a célula-ovo não seja fertilizada em um saco embrionário apospórico. A fertilidade do pólen deve ser adequada para garantir boa produção de sementes. Para serem usados em sistemas produtivos, os híbridos devem produzir uma quantidade razoável de sementes viáveis que atenda o estabelecimento de pastagens em áreas extensivas. Dessa forma, acessos com anormalidades meióticas que comprometam severamente a fertilidade do pólen devem ser descartados precocemente do programa de melhoramento.

Claudicéia Risso Pascotto Dra. Maria Suely Pagliarini Dra. Cacilda Borges do Valle

ABSTRACT. The Brachiaria germplasm collection at Embrapa Beef Cattle is comprised of about 465 accessions of 13 species collected in the savannas of East Africa in the mid 1980s by the 'Centro Internacional de Agricultura Tropical - CIAT' (Colombia). Some species have shown great potential as source of genetic variation to be explored in interspecific crosses. Considering that the majority of the accessions analyzed so far are polyploidy and apomictic, and that the breeding program is restricted to using sexual plants as female genitor and apomictic accessions with the same ploidy level as pollen donors, the cytogenetic characterization of all accessions of this collection is fundamental to direct the hybridization program. Cytological studies of microsporogenesis in germplasm of B. dictyoneura. B. brizantha, B. ruziziensis, and B. jubata revealed that some accessions presented severe abnormalities, impairing gamete viability. In B. dictyoneura, a new basic chromosome number (x = 6) was identified for the genus, and all five accessions analyzed were tetraploid (2n = 4x = 24) with abnormalities typical of polyploids. This species is represented by only nine accessions in the collection which impairs definite conclusions about it. Cell fusion and absence of cytokinesis were also observed in this species. In B. brizantha, one hexaploid accession (2n = 4x = 54) showed the chromosome set arranged in two metaphase plates and chromosome segregation occurred in a tripolar spindle only for haploid genome, forming a trinucleate cell at the end of the first division. One artificially tetraploidized sexual accession of *B. ruziziensis* (2n = 4x = 36) presented abnormal spindle orientation during the first division leading to cellularization at the end of meiosis. Abnormalities were not restricted to microsporogenesis, but observed also during microgametogenesis. One accession of B. jubata presented abnormal pollen mitosis impairing pollen development. The polyploid and apomictic accessions of Brachiaria are pseudogamous, which means that the unreduced nuclei of the embryo sac need to be fertilized by a male gamete for endosperm and healthy seed development, even though the egg cell is not fertilized in an aposporous embryo sac. Pollen fertility must be adequate to guarantee seed production. To be widely utilized in production systems, hybrids must produce a good amount of viable seeds to meet the large demand in pasture establishment over extensive areas. Thus, accessions identified with meiotic abnormalities which severely impair pollen viability need to be discarded early in the breeding program.

A new basic chromosome number for the genus *Brachiaria* (Trin.) Griseb. (Poaceae: Panicoideae: Paniceae)

A new basic chromosome number for the genus *Brachiaria* (Trin.) Griseb. (Poaceae: Panicoideae: Paniceae)

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Abstract. The genus *Brachiaria* (Trin.) Griseb. has been characterized as having two basic chromosome numbers to this date: x = 7 and x = 9, with the predominance of the latter. Cytological studies performed on five accessions of *Brachiaria dictyoneura* (Fig. & De Not.) Stapf revealed a new chromosome number for the genus, x = 6. The origin of x = 6 is still unknown. All accessions examined for this species presented 2n = 24 and typical meiotic abnormalities of polyploids. The use of such accessions in the *Brachiaria* hybridization breeding program is discussed.

Key words: *Brachiaria dictyoneura*, basic chromosome number, meiosis, polyploidy, grass breeding.

Introduction

Two species of the genus *Brachiaria* (Trin.) Griseb. have become very important forage grasses for tropical pastures in Latin America. The two most cultivated varieties, *B. brizantha* (A. Rich.) Stapf cv. Marandu and *B. decumbens* Stapf cv. Basilisk, were selected from the natural genetic variability. Both are apomictic and lack some important agronomic traits, despite their large contribution to cattle production systems and widespread utilization. To overcome the lack of diversity and specific problems, new genetic combinations using intra-and interspecific hybridization is underway at Embrapa Beef Cattle Research Center

(Embrapa Beef Cattle, Campo Grande – MS, Brazil). This program, however, faces considerable complexities because the majority of accessions are polyploid and apomictic.

The *Brachiaria* germplasm collection at Embrapa Beef Cattle is comprised of 465 accessions of 13 species collected in the African savannas and introduced to Brazil from Centro Internacional de Agricultura Tropical (CIAT, Colombia) since 1987. Some species have shown great potential as source of genetic variability to be explored in interspecific crosses. Among these, *B. dictyoneura*, a tufted perennial species (Renvoize et al., 1996) closely related to *B. humidicola* (Rendle) Schweick. which is widely planted in poorly drained pastures, is included. Considering that the majority of the accessions of *Brachiaria* analyzed so far are polyploid and apomictic, and that the breeding program is restricted to using sexual plants as female genitors and apomictic accessions with the same ploidy level as pollen donors, the cytogenetic characterization of all accessions of this collection is fundamental to direct the hybridization program. The current cytological study reports a new basic chromosome number for the genus *Brachiaria*.

Material and Methods

Five accessions of *B. dictyoneura* (BRA005851, BRA007871, BRA007889, BRA007897, and BRA007919) from the Embrapa Beef Cattle *Brachiaria* collection (Campo Grande, state of Mato Grosso do Sul, Brazil) were cytologically analyzed. Inflorescences for meiotic study were collected and fixed in a mixture of ethanol 95%, chloroform and propionic acid (6:3:2) for 24 hours, transferred to 70% alcohol and stored under refrigeration until use. Microsporocytes were prepared by squashing and staining with 0.5% propionic carmine. Chromosome associations at diakinesis were evaluated in 20 pollen mother cells in

inflorescences collected from four plants of each accession. Meiotic behavior was also evaluated. Photomicrographs were made with a Wild Leitz microscope using Kodak Imagelink – HQ, ISO 25 black and white film.

Results and Discussion

All accessions had 2n = 4x = 24 chromosome, which corroborates a new basic chromosome number never reported for the genus *Brachiaria*: x = 6. The great majority of species in the genus *Brachiaria*, tribe Paniceae, presents x = 9 (Dujardin, 1979; Basappa et al., 1987; Honfi et al., 1990; Morrone and Zuloaga, 1992; Bernini and Marin-Morales, 2001; Mendes-Bonato et al., 2002; Risso-Pascotto et al., 2003), but also x = 7 has been reported (Basappa et al., 1987; Bernini and Marin-Morales, 2001). The origin of this new basic chromosome number needs to be clarified by a detailed karyological study. Analysis of karyotypes in the genus Brachiaria was reported only for a few accessions of five species (Valle et al., 1987; Bernini and Marin-Morales, 2001). Genomes have not been described for this genus yet. To date, no adequate taxonomical revision of Brachiaria genus is available. Although about 100 species have been recognized, the precise boundaries of the genus are still undefined. Renvoize et al. (1996) proposed a classification for the genus Brachiaria where the species were sorted into nine groups according to character associations. In this context, B. dictyoneura is an African species belonging to group 6 together with eight other species, including B. bovonei (Chiov.) Robyns, B. brevispicata (Rendle) Stapf, B. humidicola (Rendle) Schweick., B. jubata (Fig. & De Not.) Stapf, B. platynota (K. Schum.) Robyns, B. reticulata Stapf, B. stigmatisata (Mez) Stapf, and B. subulifolia (Mez) Clayton. Brachiaria dictyoneura and B. humidicola have been used for sown pastures. They are closely related and, at times, have been erroneously named

by agronomists. The major difference between these two species lies in the habit of growth: while *B. humidicola* is a true stoloniferous plant, *B. dictyoneura* is tufted and forms tussocks. One stoloniferous ecotype distributed from Zimbabwe for pasture development under the name *B. dictyoneura* (Bogdan, 1977) started the confusion. Two basic chromosome numbers, x = 9 and x = 7, have been reported for *B. humidicola* (Bernini and Marin-Morales, 2001). The results suggest that x = 6 found in *B. dictyoneura* might have arisen from x = 7.

The five accessions under analysis showed typical tetraploid configuration (2n = 4x = 24). At diakinesis, chromosome associated predominantly as bivalents (Fig. 1a, b) but uni-, tri- and quadrivalents were also recorded. According to Singh (1993), such chromosome pairing is expected based on random association of four homologous chromosomes, in true autotetraploids. Alonso and Kimber (1981) and Kimber and Alonso (1981) have made great contributions to the development of mathematical models to estimate pairing affinity relationships between genomes in polyploid species, based on diakinesis and metaphase I configuration frequencies. Estimates of affinity or preferential pairing were primarily based on the frequency of multivalent formation in relation to chiasma frequency. Increased multivalent formation may result from multiple pairing initiation. In this context, the results obtained from meiotic analysis of chromosome pairing showing few multivalents and a predominance of bivalents suggest that *B. dictyoneura* is a polyploid species with an advanced stage of diploidization of the karyotype.

Accession BRA005851, however, displayed the highest frequency of quadrivalents. In some cells of this accession, the 24 chromosomes associated as six quadrivalents, suggesting that the four genomes are homologous. The frequency of chromosome configurations observed in autotetraploids could vary. Singh (1993) compared these frequencies in autotetraploids with the same basic chromosome number and concluded that the frequencies of bivalents and tetravalents differ among species and even within a species was also found in



Figure 1. Some aspects of microsporogenesis in *B. dictyoneura* (2n=4x=24). a) Diakinesis showing 8 II (bivalent) and 2 IV (tetravalent) (arrows). b) Metaphase I with 12 II. c) Anaphase I with irregular chromosome segregation (11:13). d) Anaphase I with irregular chromosome segregation (10:12) and 2 laggards. Observe sister chromatid segregation in one laggard (arrow). e) Prophase II with micronuclei in one cell. f) Tetrad with micronuclei.

the accessions under analysis. Kuspira et al. (1985) concluded that the difference among the frequencies of quadrivalents in autotetraploid *Triticum monococcum* was due to genetic factors. The behavior in chromosome pairing in these *B. dictyoneura* accessions could reflect differences in degree of diploidization of the karyotypes.

Chromosome migration at anaphase I in autotetraploids is determined by the coorientation of their kinetochores at metaphase I. All accessions presented precocious chromosome migration to the poles and laggards in the first division (Fig. 1 d) giving rise to micronuclei in telophases, tetrads (Fig. 1 f) and microspores, generating unbalanced microspores. The percentage of abnormal tetrads among accessions ranged from 9.62 to 28.48%. Autotetraploids are partially pollen and ovule abortive because of unbalanced spore constitution. Singh (1993) presented an extensive list of species where selection for higher fertility was accompained by a higher frequency of bivalents in autotetraploids and concluded that the character is genetically controlled.

The *Brachiaria* breeding program aims at producing fertile hybrids with promising agronomical traits and good seed production is vital for new cultivars to be adopted. This presupposes compatibility between progenitors which requires sexual accessions as female genitor and apomictic compatible pollen donor with the same basic chromosome number and ploidy level. The results reported in this study indicated a severe limitation to including the present accessions of *B. dictyoneura* with x = 6 in the breeding program with other *Brachiaria* species with x = 9 or x = 7 as basic chromosome numbers. Sources of sexuality among related species with the same basic chromosome number will need to be identified and/or a search for more accessions in the center of origin/ diversification need to be pursued in order to include these materials in the breeding program.

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Microsporogenesis in *Brachiaria dictyoneura* (Fig. & De Not.) Stapf (Poaceae: Paniceae)

Microsporogenesis in *Brachiaria dictyoneura* (Fig. & De Not.) Stapf (Poaceae: Paniceae)

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Abstract. Microsporogenesis was analyzed in five accessions of *B. dictyoneura* presenting x = 6 as the basic chromosome number. All accessions were tetraploid (2n = 4x = 24) with chromosome pairing in bi-, tri-, and quadrivalents. Meiotic abnormalities recorded were those typical of polyploids, including precocious chromosome migration to the poles, laggard chromosomes, and micronucleus formation. The frequency of these abnormalities, however, was lower than those reported for polyploid accessions previously analyzed in other *Brachiaria* species. Cell fusion and absence of cytokinesis were also recorded in some accessions, leading to restitutional nucleus formation in some cells. Genetically unbalanced microspores, binucleate, and 2n microspores were found among normal meiotic products as results from these abnormalities. The limitation in using these accessions with x = 6 as pollen donor in interspecific crosses with sexual species with x = 7 or x = 9 in breeding programs is discussed.

Key words: *Brachiaria dictyoneura*, basic chromosome number, cell fusion, cytokinesis, interspecific hybridization, microsporogenesis.

Introduction

The genus *Brachiaria* includes both annual and perennial species. Amongst the perennials, *B. dictyoneura* is important as forage in the humid and subhumid tropics (Lascano and Euclides, 1996). It has been used for sown pastures and it has been compared to *B. humidicola* from which it differs by its growth habit: tufted and not stoloniferous as the last (Renvoize et al, 1996). In Colombia, *B. dictyoneura* cv. Llanero was released by the Instituto Colombiano Agropecuario (ICA) in 1987. This cultivar was derived from seeds introduced from Australia (CSIRO) but was collected in Zambia in 1971. This cultivar has later been reclassified as *B.*

humidicola (Renvoize et al, 1996) since it has prostrate growth and spreads by stolons. This cultivar is well adapted to low-fertility acid soils and tolerates poor drainage. Its nutritive value is comparable to commercial *B. humidicola*. It is tolerant, but not resistant, to spittlebugs (Keller-Grein et al, 1996). In Brazil, this cultivar had relative success due to only slight advantages over the generally sown *B. humidicola*. There are no commercial cultivars of true *B. dictyoneura* on the market until today.

Some *Brachiaria* species with agronomic potential are inadequately represented in existing collections around the world. No more than 15 accessions of this species are found in the major germplasm collections. In the Brazilian *Brachiaria* collection at Embrapa Beef Cattle (Campo Grande, MS), only nine accessions are available. Some of these accessions have shown great potential as source of genetic variability to be explored in interspecific crosses. Considering that the majority of the accessions of *Brachiaria* analyzed so far is polyploid and apomictic, and that the breeding program is restricted to using sexual plants showing the same ploidy level as the pollen donors, the cytogenetic characterization of accessions of this collection becomes fundamental to direct the choice of progenitors in the hybridization program. Preliminary studies performed in *B. dictyoneura* by Risso-Pascotto et al (2005) showed a new basic chromosome number (x = 6) for the genus *Brachiaria*. The current paper reports a detailed study on the microsporogenesis in five promising accessions from the Embrapa *Brachiaria* collection.

Material and Methods

Five accessions of *B. dictyoneura* (BRA005851, BRA007871, BRA007889, BRA007897, and BRA007919) from the Embrapa Beef Cattle *Brachiaria* collection (Campo Grande, state of

Mato Grosso do Sul, Brazil) were cytologically analyzed. Site characteristics are: climate type Aw: tropical humid savanna; average annual precipitation = 1526 mm; average temperature = 22° C; altitude 520 m; latitude = 20° 28' S; longitude = 55° 40' W; poor Dark Red Latossol (59% sand; 8% silt; 33% clay; pH = 4.2).

Inflorescences for meiotic study were collected in the plot of 16 plants representing each accession and fixed in a mixture of ethanol 95%, chloroform and propionic acid (6:3:2) for 24 hours, transferred to 70% alcohol and stored under refrigeration until use. Microsporocytes were prepared by squashing and staining with 0.5% propionic carmine. Photomicrographs were made with a Wild Leitz microscope using Kodak Imagelink – HQ, ISO 25 black and white film.

Results and Discussion

Chromosome countings during microsporogenesis showed all accessions presenting 2n = 24 chromosomes which corroborates a new basic chromosome number never reported for the genus *Brachiaria*. This genus has been characterized as having two basic chromosome numbers, x = 7 and x = 9, with the predominance of the latter (Basappa et al, 1987; Honfi et al, 1990; Morrone and Zuloaga, 1992; Valle and Savidan, 1996; Mendes-Bonato et al, 2002 a, 2005; Utsunomiya et al, 2005). *Brachiaria dictyoneura* and *B. humidicola* are closely related and, at times, have been erroneously named by agronomists. Two basic chromosome numbers, x = 7 and x = 9, have been reported for *B. humidicola* (Bernini and Marin-Morales, 2001), suggesting that x = 6 found in *B. dictyoneura* might have arisen from x = 7. However, the origin of this new basic chromosome number needs to be clarified in a detailed karyological study.

Chromosome pairing at diakinesis was typical of tetraploidy (2n = 4x = 24). Chromosome associated predominantly as bivalents, but uni-, tri, and quadrivalents were also recorded (Table 1; Fig 1 a and b). In the accession BRA005851, the frequency of quadrivalents was higher than that in the other accessions. In some cells, the 24 chromosomes associated as six quadrivalents, suggesting that the four genomes are homologous. Thus, the differences found in relation to chromosome pairing among accessions could reflect differences in degree of diploidization of the karyotypes along the evolution of the species.

The overall meiotic behavior in these accessions showed to be typical of polyploids (Table 2) with irregular chromosome segregation in both divisions. The accession BRA005851, which presented chromosomes associated mainly as bivalents or quadrivalents, showed lower frequencies of abnormal meiocytes. Figure 1 illustrates meiotic abnormalities recorded in the accessions. Precocious chromosome migration to the poles in metaphases (Fig. 1 c), laggards in anaphases (Fig. 1 e, f, and j), micronuclei in telophases (Fig. 1 g and k), and tetrads (Fig. 1 l) were the main abnormalities. The percentage of abnormal tetrads among accessions ranged from 9.62% in BRA005851 to 21.66% in BRA007919. Abnormalities such as these, but in much higher frequencies, have been previously reported among polyploid accessions of different species of *Brachiaria* (Mendes-Bonato et al, 2002 a, 2005; Risso-Pascotto et al, 2003; Utsunomiya et al, 2005) and are responsible for pollen sterility due to unbalanced microspores.

Cell fusion detected among three *B. dictyoneura* accessions (Table 3) was also reported in other *Brachiaria* species (Mendes-Bonato et al, 2001, 2002 b, 2003; Risso-Pascotto et al, 2003; Utsunomiya et al, 2004, 2005). In the present accessions, only two or three cells were involved in the fusion, whereas in the other *Brachiaria* species up to ten cells were recorded forming syncytes. As in the present accessions, cell fusions reported in the other *Brachiaria* species are more common in prophase I, when the callose wall is not

Accession	Chromosome association								
code	Range					Average association/cell			
	Ι	Π	III	IV		Ι	II	III	IV
BRA005851	0-2	0-12	0-1	0-6		0.20	5.45	0.10	3.15
BRA007871	0-6	3-12	0-1	0-3		0.95	9.20	0.15	1.05
BRA007889	0-2	4-12	0-0	0-4		0.20	9.10	0.00	1.40
BRA007897	0-2	5-12	0-0	0-3		0.05	8.85	0.00	1.55
BRA007919	0-4	3-12	0-1	0-3		1.40	7.15	0.30	1.85

 Table 1. Chromosome associations at diakinesis

 Table 2. Meiotic abnormalities related to irregular chromosome segregation

Dhago	No. of analyzed cells (% of abnormal cells)							
I hase	BRA005851	BRA007871	BRA007889	BRA007897	BRA007919			
Metaphase I	149 (8.72)	139 (37.41)	142 (23.24)	152 (26.32)	128 (32.81)			
Anaphase I	161 (16.77)	141 (25.53)	135 (24.44)	139 (23.74)	142 (48.59)			
Telophase II	144 (3.47)	159 (25.78)	125 (8.80)	117 (6.84)	114 (15.79)			
Prophase II	154 (7.14)	128 (14.06)	139 (41.73)	114 (9.65)	157 (12.10)			
Metaphase II	168 (9.52)	124 (25.00)	137 (26.28)	155 (32.90)	139 (58.27)			
Anaphase II	139 (8.63)	116 (18.97)	125 (23.20)	119 (16.81)	143 (41.26)			
Telophase II	151 (7.95)	118 (15.25)	126 (13.49)	124 (18.55)	129 (21.71)			
Tetrad	156 (9.62)	136 (13.97)	161 (17.39)	151 (15.23)	167 (21.66)			
Microspores	481 (7.90)	502 (8.96)	503 (12.92)	517 (14.70)	521 (17.27)			



Figure 1. Aspects of abnormal microsporogenesis in polyploid accession of *B. dictyoneura*. a) Diakinesis presenting 6 II and 3 IV (arrowheads). b) Metaphase I with 10 II and 1 IV (arrowhead). c) Metaphase I with precocious chromosome migration to the pole (arrowhead). d to f) Anaphase I with irregular chromosome segregation and laggards In d one pole contains 13 chromosomes and the other 11 (arrowhead). In e notice laggard chromosome (arrowhead). Observe also irregular chromosome segregation in f: 12 chromosomes in one pole, 10 in the other, and four laggard chromatids (arrowhead). g) Telophase I with micronuclei (arrowhead). h) Prophase II with micronucleus in both cells (arrowhead). i) Metaphase II with micronuclei (arrowhead). j) Anaphase II with micronuclei (arrowhead) and laggards. k) Telophase II with micronuclei (arrowhead). l) Tetrad with micronuclei (arrowhead). Scale bar = 1 μ m.

completely formed around the meiocyte. In *B. dictyoneura*, two types of cell fusion were observed: those completely fusionned, where the genomes were in the same cytoplasm (Fig. 2 a and b), and those connected by a thin or thick cytoplasmic channel (Fig. 2 c to i). In some meiocytes, the proximity of genomes after cell fusion led to the formation of a restitutional nucleus by rejoining of the chromosomes (Fig. 2 f to i). If the meiotic process evolves normally in the fusionned cells, restitutional nuclei will give rise to 2n gametes. Despite the majority of cells being connected by cytoplasmic channels typical of those recorded during cytomixis, chromosome transfer between meiocytes was not detected in *B. dictyoneura*. Similar cytoplasmic channels promoted cytomixis in tetraploid accessions of *B. nigropedata* (Utsunomiya et al, 2004).

Absence of the first or the second cytokinesis were also recorded in two accessions of *B. dictyoneura*, but the frequency was higher in BRA007897 and observed in all the phases from prophase II to the end of meiosis (Table 4, Fig. 3). When failure of cytokinesis occurred only in the first division (Fig. 3 a to c), with normal cytokinesis in the second division, a dyad of microspores was formed (Fig. 3 d). The two nuclei of one cell in the dyad followed two distinct ways: they remained isolated, giving rise to binucleated microspores (Fig. 3 e and g) or they were rejoined, originating a restitutional nucleus that developed into a 2n gamete (Fig. 3 d and f). When the first division was normal and the failure of cytokinesis occurred only in the second division, dyads and triads were formed depending if the failure affected one or both cells. Triads (Fig. 3 e) were formed when cytokinesis occurred in only one cell of the dyad. The frequency of binucleated microspores and 2n microspores in both accessions, BRA005851 and BRA007897, was similar but higher in the last.

Absence of cytokinesis leading to 2n gamete formation has been reported in many plant species (Veilleux, 1985), and also in *B. brizantha* (Risso-Pascotto et al, 2003). 2n gametes have had an important role in evolution through polyploidization and in plant

Dhaga	No. of analyzed cells (% of abnormal cells)							
rnase	BRA005851	BRA007871	BRA007889	BRA007897	BRA007919			
Zygotene	140 (8.57)	132 (3.78)	-	-	-			
Pachytene	185 (5.94)	205 (3.90)	-	126 (2.30)	-			
Diplotene	-	180 (3.88)	-	-	-			
Diakinesis	-	131 (4.58)	-	154 (3.25)	-			
Metaphase I	-	139 (12.94)	-	-	-			
Anaphase I	-	141 (8.51)	-	-	-			
Telophase I	-	159 (5.66)	-	-	-			

Table 3. Number of analyzed cells and percentage of cell fusion among meiocytes

Table 4. Number of analyzed cells and percentage of cells with absence of cytokinesis

Dhaga	No. of analyzed cells (% of abnormal cells)							
rnase	BRA005851	BRA007871	BRA007889	BRA007897	BRA007919			
Prophase II	-	-	-	114 (6.15)	-			
Metaphase II	-	-	-	155 (3.22)	-			
Anaphase II	-	-	-	119 (1.68)	-			
Telophase II	-	-	-	124 (4.84)	-			
Dyad	136 (2.21)	-	-	151 (7.95)	-			
Triad	-	-	-	151 (5.30	-			
Binucleated Microspores	502 (0.60)	-	-	517 (1.16)	-			
Microspores 2n	502 (0.55)	-	-	517 (1.35)	-			



Figure 2. Cell fusion among meiocytes. a, b) Fusionned meiocytes in prophase I. c, d) Fusionned meiocytes in metaphase I through cytoplasmic channel (arrowhead). In both, observe precocious chromosome migration to the poles. e, f) Telophase I (e) and metaphase II (f) fusionned by cytoplasmic channel. Observe that in one cell of metaphase II the two chromosome sets were rejoined forming a restitutional nucleus. g, h) Fusionned cells in metaphase I showing restitutional nucleus. i) Anaphase I forming a restitutional nucleus in the cytoplasmic channel. Scale bar = $1 \mu m$.

breeding (Veilleux, 1985; Bretagnolle and Thompson, 1995). The majority of *Brachiaria* species is polyploid and reproduces by apomixis which by circumventing meiosis and fertilization in the megagametophyte followed by parthenogenetical production of the non-reduced embryo, preserves the genetic constitution of the mother plant indefinitely (Valle and Savidan, 1996). The meiotic behavior in polyploidy accessions of *Brachiaria* species studied by Mendes-Bonato et al (2002, 2005) and Utsunomiya et al (2005) provided evidence that polyploidy in this genus may have originated predominantly by chromosome doubling instead of interspecific hybridization. In this context, 2n gamete could have had a fundamental role in the evolutionary history of the genus.

The main objective of the *Brachiaria* breeding program is to produce fertile hybrids by interspecific hybridization, combining alleles for agronomic traits, thus amplifying the genetic diversity in the genus and options as new forage cultivars. Crosses between *Brachiaria* species presuppose compatibility between progenitors: sexual accessions as female genitor and apomictic pollen donor of the same basic chromosome number and ploidy level. Sexuality in the *Brachiaria* agamic complex being bred was reported only in diploid accessions of basic chromosome number x = 9, particularly in *B. ruziziensis* (Valle and Savidan 1996). The cytogenetic findings in these accessions of *B. dictyoneura*, related to a new basic chromosome number x = 6, limits their use as pollen donor in crosses with other sexual *Brachiaria* species with x = 7 or x = 9, particularly to the taxonomically related *B. humidicola* x = 9. Continued cytogenetic studies in the *Brachiaria* collection are essential for these accessions to be included as pollen source in interspecific hybridization program. For that, new sources of compatible sexual species with the same basic chromosome number need to be identified.



Figure 3. Aspects of absence of cytokinesis during microsporogenesis. a, b, c) Metaphase II, anaphase II, and telophase II with absence of first cytokinesis. Observe laggard and micronucleus in b and c, respectively (arrowhead). d) A dyad with restitutional nucleus in both cells and one normal microspore (arrowhead). e) A triad with a binucleated microspore (arrowhead) and micronucleus in two microspores. f) A normal microspore and a restitutional one (arrowhead). g) A normal microspore and one with a binucleated nucleus (arrowhead). Scale bar = 1 μ m.

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Abnormal chromosome arrangement at metaphase plate during microsporogenesis in a hexaploid accession of *Brachiaria brizantha* (Poaceae: Paniceae)

Abnormal chromosome arrangement at metaphase plate during microsporogenesis in a hexaploid accession of *Brachiaria brizantha* (Poaceae: Paniceae)

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Abstract. In the hexaploid (2n = 6x = 54) accession B176 of *Brachiaria brizantha*, one cytological characteristic differentiated it from the other accessions previously analyzed with the same ploidy level. Near 40 % of meiocytes presented the chromosome set arranged into two metaphase plates at the poles of the cell, close to the membrane. In these cells, both metaphase plates were arranged in an angle to form a typical tripolar spindle. Therefore, cells did not show normal chromosome segregation at anaphase I. Only nine univalent chromosomes migrated from each plate to the opposite pole with the remainder staying immobile in the plate. As the major chromosome sets did not segregate, they gave rise to two telophase nuclei, instead of four nuclei. As a result of such spindle orientation and chromosome behavior, trinucleate telophases I were recorded. After telophase, cytokinesis eliminated the small nuclei into a microcyte. The second division proceeded normally, with the presence of microcytes in all phases. The origin of such abnormality was explained based on the hexaploid nature of the accession. The high percentages of meiotic abnormalities recorded in this accession, compromises fertility and renders it inadequate for the breeding program.

Key words: *Brachiaria brizantha*, forage grass, pollen viability, polyploidy, microsporogenesis.

Introduction

The improvement of *Brachiaria* grasses through breeding is complex and laborious. In this genus, the majority of species are polyploid (Basappa et al. 1987, Honfi et al. 1990, Bernini and Marin-Morales 2001, Mendes-Bonato et al. 2002, 2005, Utsunomiya et al. 2005) and polyploidy is correlated with apomixis (Valle and Savidan 1996). Apomictics in this genus reproduce by apospory of the *Panicum* type and are pseudogamous, which means that the unreduced polar nucleus of the embryo sac need to be fertilized for correct development of

endosperm (Alves et al. 2001). The two *Brachiaria* cultivars most widely used in Brazilian pastures, *B. brizantha* cv. Marandu and *B. decumbens* cv. Basilisk, are tetraploid and apomictic, and were directly selected from natural genetic variability. The major objective of the *Brachiaria* breeding program underway at the Embrapa Beef Cattle Research Center is the creation of new cultivars involving intra- and interspecific hybridization to introgress agronomic characteristics lacking in these cultivars, and also to enlarge the genetic variability in the genus.

To participate in a cross, the parental accessions need to both reproduce sexually or the plant used as female reproduce sexually and the pollen donor by apomixis but with high pollen fertility. The first condition is difficult to accomplish in the genus because compatible sexual accessions with desirable agronomic characters are rare or non-existent. The second, in turn, has been extensively employed in several crosses where sexual artificially tetraploidized accessions of *B. ruziziensis* are crossed with apomictic genotypes of two closely related species, *B. brizantha* and *B. decumbens*. The success of hybridization, however, depends on the pollen fertility of the apomictic accession (Valle and Savidan 1996, Miles et al. 2004).

Cytological characterization of accessions of *B. brizantha* of the Embrapa Beef Cattle germplasm collection has revealed accessions with a low frequency of meiotic abnormalities (Mendes-Bonato et al. 2002, 2005, Utsunomiya et al. 2005). Nonetheless, several accessions showed abnormalities besides those typical of polyploidy (Mendes-Bonato et al. 2001a,b; Risso-Pascotto et al. 2003, Mendes 2004). This paper reports abnormalities recorded in one accession of *B. brizantha* (B176) which displayed an abnormality never before reported in any other *Brachiaria* species.
Material and Methods

The accession B176 (BRA004189) of *B. brizantha* from the Embrapa Beef Cattle germplasm collection (Campo Grande, state of Mato Grosso do Sul, Brazil) collected in the wild African savannas in the 80s by CIAT (Colombia) was thoroughly analyzed under light microscopy. Site characteristics of cultivation in the field were: climate type Aw: tropical humid savanna; average annual precipitation = 1526 mm; average temperature = 22° C; altitude 520 m; latitude = 20° 28' S; longitude = 55° 40' W; poor Dark Red Latossol (59% sand; 8% silt; 33% clay; pH = 4.2).

Inflorescences for meiotic study were collected in a single plant representing the accession and fixed in a mixture of ethanol 95%, chloroform and propionic acid (6:3:2) for 24 hours, transferred to 70% alcohol and stored under refrigeration until use. Microsporocytes were prepared by squashing and staining with 0.5% propionic carmine. Photomicrographs were made with a Wild Leitz microscope using Kodak Imagelink – HQ, ISO 25 black and white film.

Results and Discussion

Due to its interesting agronomic characteristics, the *B. brizantha* germplasm collection at Embrapa Beef Cattle is represented by 233 accessions collected in different countries of eastern Africa. According to indices of flow cytometry established for 222 of these accessions (Penteado et al. 2000), the great majority of them (220 accessions) are polyploid. The accession B176, whose index of cytometry indicated ploidy level of 5n (2n = 5x = 45) is, indeed, a hexaploid accession (2n = 6x = 54) with x = 9, the predominant basic chromosome

number in the genus *Brachiaria* followed by x = 7 (Basappa et al. 1987, Honfi et al. 1990, Bernini and Marin-Morales 2001, Mendes-Bonato et al. 2002, 2005, Utsunomiya et al. 2005).

The percentage of cells with meiotic abnormalities related to polyploidy, including precocious chromosome migration and laggards generating micronuclei (Figure 1) was high as expected in this accession (Table 1). In several other polyploid accessions of *B. brizantha* previously analyzed different frequencies of meiotic abnormalities related to polyploidy have been recorded (Mendes-Bonato et al. 2002, Risso-Pascotto et al. 2003, Mendes 2004). Chromosome stickiness, also recorded in some cells in this accession, had been described in *B. brizantha* (Mendes-Bonato et al. 2001 b, Mendes 2004) and in other *Brachiaria* species (Mendes-Bonato et al. 2001 a, Utsunomiya et al. 2005).

In this accession, one cytological characteristic differentiated it from the other hexaploid accessions of *B. brizantha* previously analyzed (Mendes-Bonato et al. 2002, Mendes 2004). In those, chromosomes were always arranged in a single metaphase plate and presented normal segregation at anaphase I. In B176, however, near 40 % of meiocytes (Table 1) presented the chromosome set arranged into two metaphase plates (Fig. 2), while the remainder of the cells were normal (Fig. 1). In the abnormal cells, the two chromosome sets were arranged in metaphase plates that occupied a polar place close to the membrane (Fig. 2 a to d). Both metaphase plates did not present a parallel arrangement, but instead were arranged in an angle to form a typical tripolar spindle (Fig. 2 a to d). These cells did not show normal chromosome segregation at anaphase I. Only a few chromosomes migrated from each metaphase plate to the opposite pole with the remainder staying stationary in the plate. Chromosome counting in the small segregated group, suggested that nine univalent chromosome are migrating to opposite poles (Fig. 2 d). As a result of such spindle orientation and chromosome behavior, telophases I with three nuclei, with one of them being smaller, were recorded (Fig. 2 e, f). In some cells, the spindles were not totally convergent

and two small nuclei were observed in telophases I (Fig. 2 g). As the major chromosome set did not segregate, two instead of four telophase nuclei were formed. Cytokinesis eliminated the small nuclei into a microcyte after telophase (Fig. 2 h, i). The second division proceeded normally, with the presence of microcytes in all phases.

Phase	No. of cells analyzed	Abnormality - No. of cells (%)
Metaphase I	224	Precocious chromosome migration - 196 (87.5)
		Two metaphase plates - 83 (37.1)
Anaphase I	289	Laggards - 144 (49.8)
		Chromosome stickiness - 9 (3.1)
		Tripolar spindles - 112 (38.8)
Telophase I	432	Micronuclei - 140 (32.4)
		Chromosome stickiness - 52 (12.1)
		Multinucleate cells - 169 (39.2)
Prophase II	294	Micronuclei - 143 (48.6)
		Microcytes - 26 (8.8)
		Abnormal cytokinesis - 32 (10.9)
		Chromosome stickiness - 12 (4.1)
Metaphase II	289	Precocious chromosome migration - 135 (46.7)
		Microcytes - 30 (10.4)
Anaphase II	241	Laggards - 197 (81.7)
		Microcytes - 42 (17.4)
Telophase II	299	Micronuclei - 229 (76.6)
		Microcytes - 37 (12.4)
Tetrad	375	Micronuclei - 301 (80.3)
		Microcytes - 30 (8.0)
		Polyads - 16 (4.3)

Table 1. Meiotic abnormalities observed in the accession B176 of Brachiaria brizantha.



Figure 1. a) Anaphase I showing 54 segregated chromosomes. b) Precocious chromosome migration to the pole in metaphase I. c) Anaphase I with laggards. d) Prophase II with micronucleus in each cell. e) Late anaphase II with laggards. f) Tetrad with several micronuclei. g) Anaphase I with chromosome stickiness. h) Chromosome bridge resulting from stickiness. i) Telophase I with nuclei of different sizes resulting from stickiness. (Scale bar = $1\mu m$)



Figure 2 a, b) Metaphases I with chromosomes arranged in two metaphase plates. Observe the proximity of the metaphase plate to the membrane. c, d) Anaphases I with nine univalents migrating to one pole. Observe the tripolar arrangement of the spindle. e, f) Trinucleate telophases I. Observe that one nucleus is smaller than the others. g) Telophase I with two micronuclei resulting from spindles not totally convergent. h) Telophase I forming a microcyte by irregular plane of cytokinesis. i) Metaphase II with a microcyte. (Scale bar = 1μ m)

The origin of such abnormality could be related to the hexaploid nature of this accession. Hexaploidy might have originated by chromosome doubling of a triploid that, in turn, could have originated by the union of an n gamete (n = 9, sexual parent) and an unreduced gamete (2n = 18, apomictic parent). The genitors of the triploid could be of different species or wide genetic origin that did not display the same behavior for spindle organization. Chromosome doubling in triploids could occur in order to restore the fertility through the correct chromosome pairing. However, in this accession, chromosomes were separated after doubling, therefore fertility could not be re-established. It is interesting that only the nine univalent chromosomes of each triploid set migrated to the opposite pole and the other genome remained unsegregated.

The combination of two genomes in an interspecific hybrid has frequently resulted in aberrant meiotic divisions, including those aspects related to spindle orientation. Evidence of natural hybridization in a *B. brizantha* accession with genomes not closely related was first observed by Mendes et al. (2005). Also abnormal spindle orientation was reported in an artificially tetraploidized accession of *B. ruziziensis* (Risso-Pascotto et al. 2005). Absence of genome affinity in relation to chromosome distribution in the metaphase plate was recorded in an interspecific hybrid between a sexual artificially tetraploidized accession of *B. ruziziensis* and *B. brizantha* (Mendes-Bonato et al. unpublished). In that case, both parental genomes were separated into two metaphase plates that behaved asynchronously during microsporogenesis.

Relatively little is known about the development of the meiotic spindle in plants. It is known, however, that after the nuclear envelope breaks down, randomly oriented microtubule bundles in the cytoplasm are seen to assume a bipolar orientation and form an anastral spindle (Hepler et al. 1993). In higher plant cells devoid of centrioles, the centrosome has not yet been identified as a morphological structure and the mechanisms of anastral division spindle

formation remain unclear (Shamina et al. 2000). There is no information on the literature about the influence of genetic factors on spindle formation. However, taking into account that the equilibrium for bipolar spindle formation is generally broken in hybrids, it is plausible to suggest that this character is genetically controlled, and the union of divergent genomes in a common cytoplasm could disrupt the abnormal spindle orientation, as observed in the present hexaploid accession of *B. brizantha*. Because of its high percentage of meiotic abnormalities, this accession is ineligible to participate in crosses and might be discarded from the breeding program.

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MULTIPLE SPINDLES AND CELLULARIZATION DURING MICROSPOROGENESIS IN AN ARTIFICIALLY INDUCED TETRAPLOID ACCESSION OF Brachiaria ruziziensis (GRAMINEAE)

MULTIPLE SPINDLES AND CELLULARIZATION DURING MICROSPOROGENESIS IN AN ARTIFICIALLY INDUCED TETRAPLOID ACCESSION OF Brachiaria ruziziensis (GRAMINEAE)

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Abstract. The genus *Brachiaria* is characterized by a majority of polyploid accessions, mainly tetraploid, and apomictic reproduction. Sexuality is found among diploids. To overcome the incompatibility barriers, accessions with the same ploidy level are necessarily used in hybridization. Thus, sexual diploid accessions were tetraploidized to be used as female genitors. This paper reports microsporogenesis in an artificially induced tetraploid accession of *Brachiaria ruziziensis*. Chromosome pairing at diakinesis ranged from univalents to tetravalents, with predominance of bivalents. Irregular chromosome segregation was frequent in both meiotic divisions. During the first division, multiple spindles showing different arrangements were recorded. The spindle position determined the plane of first cytokinesis and the number of chromosomes determined the size of the cell. Meiotic products were characterized by polyads with spores of different sizes. Pollen sterility was estimated at 61.38%. The limitations of using this accession in the breeding program are discussed.

Key words: *Brachiaria ruziziensis* - Induced tetraploidy – Microsporogenesis - Pollen fertility – Tropical grass.

Introduction

The genus *Brachiaria*, native to the African tropical savannas, has species that are widely used in cultivated pastures in Latin America. Two polyploid (2n = 4x = 36) and apomictic cultivars, *Brachiaria brizantha* cv. Marandu and *Brachiaria decumbens* cv. Basilisk, are estimated to cover over 60 million hectares in the Brazilian savannas, but both exhibit

deficiencies that justify genetic improvement (Miles and Valle 1996). Developing more efficient cultivars in terms of edaphic adaptation, resistance to pests (especially spittlebugs), and pasture persistence is of particular interest in the *Brachiaria* breeding work. Programs aiming at producing new cultivars have previously been confronted with lack of sexuality and of genetic diversity. Until two decades ago, genetic improvement depended entirely on selection among the few naturally existing genotypes introduced from Africa. Because the two commercial species are predominantly apomictic, genetic improvement based on the combination of attributes from these two parental genotypes by conventional hybridization is not possible.

To date, with the introduction of a large germplasm collection from Africa in the late 1980s, and the basic studies on mode of reproduction and ploidy levels (Valle and Savidan 1996; Penteado et al. 2000), the potential of apomictic reproduction is becoming better appreciated, and cross-compatible sexual genotypes are being identified or produced (Valle et al. 2000). Artificial hybridization in *Brachiaria* was attempted in the early 1970s (Ferguson and Crowder 1974) by pollinating a diploid sexual accession of *Brachiaria ruziziensis* with a tetraploid apomictic accession of *B. decumbens*. This effort was unsuccessful due to the difficulty in crossing the ploidy barrier. The authors suggested the development of a sexual compatible plant by artificially doubling the chromosome number of the naturally diploid sexual *B. ruziziensis*, which was accomplished by work done in Belgium in the early 1980s (Gobbe et al. 1981) involving the successfully developing of an obligate sexual tetraploid by colchicine treatment, which has allowed apomixis to be exploited in breeding of *Brachiaria* (Gobbe at al. 1981; Swenne et al. 1981).

The Belgian material is still the basis of all ongoing breeding work in *Brachiaria*, although efforts to produce additional fully sexual tetraploids by conventional colchicine treatment or by tissue culture of sexual diploids have succeeded at Embrapa in Brazil (Pinheiro

et al. 2000). The potentiality of genetic hybridization became a reality in applied breeding programs at the Embrapa Beef Cattle Center in 1988 (Valle and Miles 2001) using the artificially induced tetraploid sexual accessions of *B. ruziziensis* as the female genitors in interspecific hybridization with *B. decumbens* and *B. brizantha*. During cytological analysis of microsporogenesis in one of these accessions, meiotic abnormalities that could compromise pollen fertility were found. This paper focused on the origin of the multiple spindles and their influence on cellularization.

Material and Methods

The *B. ruziziensis* accession (R 050) under analysis was tetraploidized by colchicine in Belgium (Sweene et al. 1981). This accession presents sexual reproduction and is used as a female genitor in interspecific hybridization with apomictic tetraploid accessions of *Brachiaria* species in the same agamic complex: *B. brizantha* and *B. decumbens*. This accession is maintained *in vivo* in the *Brachiaria* collection at the Embrapa Beef Cattle Research Center, Campo Grande, MS, Brazil.

Inflorescences for meiotic study were fixed in a mixture of ethanol (95%):chloroform:propionic acid (6:3:2) for 24 h, transferred to 70% alcohol and stored under refrigeration until use. Microsporocytes and pollen grains were prepared by squashing and staining with 0.5% propionic carmine. All meiotic phases and pollen fertility were evaluated in inflorescences collected from four plants of this accession. Pollen fertility was estimated with 1.0% propionic carmine in fresh dehiscent anthers. Photomicrographs were made with a Wild Leitz microscope using Kodak Imagelink – HQ, ISO 25 black and white film.

Results

The behavior of the chromosomes at diakinesis ranged from univalent to tetravalent, with a predominance of bivalent chromosomes (Fig. 1 a, b). Precocius chromosome migration to the poles and laggards were found during both meiotic divisions (Table 1). Abnormal chromosome disposition in the metaphase plate was also recorded in metaphase I. In this phase, chromosomes were arranged in several plates (Fig. 1 c). As a consequence, abnormal multipolar spindle shapes, from tripolar to indeterminate (Fig. 1 d-i), were found among a high number of meiocytes at meiosis I. The spindle position determined the plane of first cytokinesis. The most common plane divided the meiocyte into three equal or non-equal sized cells (Fig. 1 j-o). The second cytokinesis fractionated the cells into tetrads with non-equal microspores (Fig. 2 a, b) or polyads with spores of different sizes (Fig. 2 d-h). The number of different sizes (Fig. 2 i). Pollen sterility was estimated at 61.38%.

Discussion

In autotetraploids, pairing is characterized by multiple forms of chromosome association (Singh 1993), but chromosome segregation generally occurs in a single bipolar spindle. In *B. ruziziensis*, however, despite multiple types of association, chromosomes were allocated in several metaphase plates and each one formed an individual spindle. Multipolar spindles have been produced in plants and animals by various experimental treatments (e.g., chloral hydrate, ethyl- ether, low temperature and hydrostatic pressure) and also by spontaneous occurrence

Phases	Number of cells analyzed	Number of abnormal cells (%)	Abnormalities	Number of cells
Diakinesis	45	45 (100.0)	Multiple chromosome associations	45
Metaphase I	149	64 (42.95)	Precocious chromosome migration	59
			Non-congressed bivalent	5
Anaphase I	155	134 (86.45)	Laggard chromosomes	97
			Multipolar spindles	37
Telophase I	145	126 (86.90)	Micronuclei	94
			Multipolar spindles	32
Prophase II	123	79 (64.23)	Micronuclei	52
			Abnormal cytokinesis	27
Metaphase II	146	121 (82.88)	Precocious chromosome migration	88
			Product of abnormal cytokinesis	33
Anaphase II	122	103 (84.43)	Laggard chromosomes	81
			Product of abnormal cytokinesis	22
Telophase II	124	102 (82.26)	Micronuclei	76
			Product of abnormal cytokinesis	26
Meiotic product	276	148 (53.62)	Tetrad with micronuclei	86
			Triad	4
			Polyad	58

 Table 1. Meiotic abnormalities recorded in the tetraploidized accession of Brachiaria ruziziensis.



Figure 1. a-o Meiotic aspects of a tetraploidized accession of *Brachiaria ruziziensis*. a) Microsporocyte in diakinesis showing bivalents and tetravalents (arrowheads). b) Precocious metaphase I with bivalents and tetravalents (arrowheads). c) Metaphase I with chromosomes arranged in three plates. d-i) Different aspects of abnormal spindles in the first meiosis. j-o) Meiocytes in the second division. Observe the irregular planes of cytokinesis, Note also, that the cytoplasm is, in general, proportional to the number of chromosomes. Bars 1 µm.



Figure 2. a-i Meiotic products. a,b) Tetrads with differently sized microspores. c-h) Polyads with different number and sizes of spores. i) Pollen grains of different sizes. Bars 1 μ m.

(Walters 1960; Mendes-Bonato et al. 2002). In the latter cases, a greater frequency was reported among interspecific and intergeneric hybrids.

On the basis of comparative studies of sporogenesis, Brown and Lemmon (1992b) proposed the hypothesis that the direct control of the division plane in microsporogenesis is a function of nuclear-based radial arrays of microtubules that define spore domains. According to Brown and Lemmon (1992a), the cytoplasmic domain model for cleavage of the spore follows a pattern that reflects the position and number of nuclei. This model assumes that spindle alignment, and therefore final arrangement of nuclei, is closely regulated. In this *B. ruziziensis* accession, both the arrangement, and number of cells resulting from first cytokinesis, were abnormal. Similar abnormalities were reported in *Magnolia* (Brown and Lemmon 1992b), where the arrangement and number of microspores cleaved from microsporocytes treated with griseofulvin were also abnormal. The authors suggested that these abnormalities could be traced to displaced spindles and to spindles with multiple poles. They also hypothesized that griseofulvin affected microtubule associated proteins (MAPs), and that target MAPs played a role in spindle alignment and spindle pole consolidation through positioning and consolidation of microtubule organizing centers (MTOCs).

Similar abnormalities in tetrads have been reported in the *dv* meiotic mutant of *maize* (Staiger and Cande 1990; Shamina et al. 2000) and in plants with abnormal meiosis due to hybridism (Brown and Lemmon 1989) and polyploidy (Brown and Lemmon 1991). In all these cases, as tetrad formation was severely altered, Brown and Lemmon (1992b) suggested that the radial systems of microtubules defined volumes of cytoplasm proportional to their size. Since it is unlikely that the number and placement of lagging chromosomes could be anticipated by the cell, the logical assumption is that the final division planes are determined by placement of nuclei. The planes of cytokinesis observed after first meiosis in the accession of *B. ruziziensis* used here corroborate the above assumption, i.e., that the division planes are

influenced by the size and position of nuclei. In rare cases, nuclei with few chromosomes gave rise to a big cell. In this cases, micronuclei gave rise to microcytes after first cytokinesis.

The basis of spindle and chromosome movements during cell division has been the object of considerable speculation in recent years. The forces required for spindle assembly and movements of the spindle and chromosomes have been attributed to microtubules dynamics, together with microtubule motors. Many of the kinesin proteins identified in the spindle dividing cells were reported in mutants with abnormal or monopolar spindles and highly polyploid cells caused by duplication of chromosomes (Endow 1999). Ncd, a meiotic motor protein, was localized in oocytes spindle of Drosophila (Hatsumi and Endow 1992) and has been proposed to act in meiosis I spindle assembly by cross-linking microtubules. Mutant oocytes show highly abnormal multipolar spindles with diffuse poles. The additional role of chromosomes in meiotic spindle formation has also demonstrated in several cases. The fact that chromatin promotes microtubule organization and that the presence of several individual groups of chromosomes can result in the formation of multiple independent spindles in meiosis was demonstrated in oocytes from a variety of evolutionarily diverse animal species (Woods et al. 1999). According to Brown and Lemmon (1997), multiple spindles seem to develop from initial nucleation of microtubules in association with chromatin and the migration of the nucleating site together with microtubule reorganization.

Multiple spindles have been reported in several plant species both in first and second meiosis. In *Agropyron cristatum* (Tai 1970), *Carthamus tinctorius* (Carapetian and Rupert 1977), and *Fuchsia* (Tilquin et al. 1984), multiple spindles were reported only in meiosis I, while in *Thunbergia mysorensis* (Pagliarini 1990), *Aloysia lycioides* (Corazza-Nunes et al. 1993), and *B. decumbens* (Mendes-Bonato et al. 2002), they occurred only in meiosis II. It is remarkable that while in *B. decumbens*, multiple spindles occurred only in the second division and originated from micronuclei of the first division formed by irregular chromosome

segregation in metaphase and anaphase, in this *B. ruziziensis* accession, they were found only in the first division. In addition, they probably originated from the polyploidization process. Multiple spindles as a consequence of high ploidy levels were reported in *Rubus* (Thompson 1962) and *Fuchsia* (Tilquin et al. 1984). According to Tai (1970), polyploid species and wide hybridization present higher chances of producing multiple spindles, indicating that some chromosomes may be somewhat linked to the formation of spindle fibers.

Occurrence of additional cytokinesis causing cellularization is always associated with multiple spindles. Thus, the meiotic product is generally a polyad with unbalanced microspores. Reduced pollen fertility is a common consequence of this abnormality (see Mendes-Bonato et al. 2002). In this accession, multiple spindle and abnormal cytokinesis did not affect enough meiocytes to explain the high frequency of pollen sterility (61.38%). Irregular chromosome segregation forming unbalanced microspores was widely observed in this accession during both divisions, and may also have compromised pollen fertility, as reported for other accessions of the genus (Mendes-Bonato et al. 2001, 2002; Risso-Pascotto et al. 2003a, b).

Induced autotetraploidy has been used in several cultivated species, but the results have not always been satisfactory. Autotetraploids are partially pollen and ovule abortive because of unbalanced spore constitution. Pollen sterility in *B. ruziziensis* was obviously caused by genetic unbalance of microspores, due to irregular segregation of chromosomes, multiple spindles followed by abnormal cytokinesis and cellularization. The use of this induced tetraploid sexual accession as female genitor in the *Brachiaria* breeding program could seriously compromise hybridization and seed set in the F₁ and subsequent generations. An efficient female genitor needs a high frequency of viable ovules to generate the substantial numbers of seeds required to supply *Brachiaria* establishment in the beef production system.

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Symmetric pollen mitosis I and supression of pollen mitosis II prevent pollen development in *Brachiaria jubata* (Gramineae).

Symmetric pollen mitosis I and supression of pollen mitosis II prevent pollen development in *Brachiaria jubata* (Gramineae).

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Abstract. Microsporogenesis and pollen development were analyzed in a tetraploid (2n=4x=36) accession of the forage grass Brachiaria jubata (BRA 007820) from the Embrapa Beef Cattle Brachiaria collection that showed partial male sterility. Microsporocytes and pollen grains were prepared by squashing and staining with 0.5% propionic carmine. The meiotic process was typical of polyploids, with precocious chromosome migration to the poles and laggards in both meiosis I and II, resulting in tetrads with micronuclei in some microspores. After callose dissolution, microspores were released into the anther locule and appeared to be normal. Although each microspore initiated its differentiation into pollen grain, in 11.1% of them nucleus polarization was not observed, i.e., pollen mitosis I was symmetric and the typical hemispherical cell plate was not detected. After a central cytokinesis, two equal sized cells showing equal chromatin condensation and the same nuclear shape and size were formed. Generative cell and vegetative cell could not be distinguished. These cells did not undergo the second pollen mitosis and after completion of pollen wall synthesis each gave rise to a sterile and uninucleate pollen grain. The frequency of abnormal pollen mitosis varied among flowers and also among inflorescences. All plants were equally affected. The absence of fertile sperm cells in a considerable amount of pollen grains in this accession of B. jubata may compromise its use in breeding and could explain, at least in part, why seed production is low when compared with the amount of flowers per raceme.

Key words: *Brachiaria jubata*, male sterility, microgametogenesis, pollen mitosis, sperm cell.

Introduction

The events that culminate in the formation of the pollen grain involve an intricate and tightly controlled set of structural and molecular gene expression events in both the gametophytic and sporophytic tissues of the anther. Firstly, pollen mother cells undergo meiosis, giving rise

to a tetrad of haploid cells. The individual cells of the tetrad are released as free microspores by the action of callose, an enzyme produced by the tapetum layer of the anther (1).

After an initial burst of growth and exine synthesis, the haploid microspores undergo cytological reorganization in preparation for a key event in pollen development, i.e., the asymmetric mitotic division (2). This mitotic division, also known as 'pollen mitosis one' (PM I) results in the formation of two dimorphic cells, the vegetative cell and the generative cell, with very different fates (1-5). The smaller generative cell produces two sperm cells (male gametes) after 'pollen mitosis two' (PM II), whereas the larger vegetative cell, which constitutes the bulk of the pollen grain, produces an elongated pollen tube (a gametophytic cell) to deliver the male gametes to the embryo sac (5). The generative/sperm cells have very little cytoplasm and sparse nuclear chromatin, suggesting that they have lower transcriptional activity (6).

Defective mutants operating at various stage of microspore development have been reported in *Arabidopsis* (7-16) and *maize* (17-20). In *Brachiaria decumbens* cv. Basilisk, a variety extensively used for forage in Brazil, a possible mutation affecting pollen development has recently been reported (21). In the current paper we describe an abnormal pollen mitosis division in one accession of *Brachiaria jubata*, a species of interest in the forage breeding program.

Materials and Methods

Cytogenetic studies were carried out on the BRA 007820 accession of *Brachiaria jubata* from Embrapa Beef Cattle *Brachiaria* collection kept in Campo Grande (state of Mato Grosso do Sul, Brazil), which comprises 475 accessions of 15 species. Inflorescences for microsporogenesis and microgametogenesis studies were collected and fixed in a mixture of 95% ethanol, chloroform and propionic acid (6:3:1) for 24 h, transferred to 70% alcohol and stored under refrigeration until use. Microsporocytes and pollen grains were prepared by squashing and staining with 0.5% propionic carmine. All meiotic phases and stages of pollen development were evaluated in inflorescences collected from four plants of this accession obtained by vegetative propagation. Photomicrographs were obtained with a Wild Leitz microscope using Kodak Imagelink – HQ, ISO 25 black and white film.

Results

Cytological characterization of the accession was carried out from meiosis to pollen formation. The chromosome number scored at diakinesis revealed tetraploid (2n=4x=36). The meiotic process was typical of polyploids (Table 1), with chromosomes associating in bi-, triand quadrivalents. Precocious chromosome migration to the poles, laggards and micronucleus formation were found in both meiosis I and II, resulting in tetrads with micronuclei in some microspores (Figure 1 a). After callose dissolution, microspores were released into the anther locule and seemed to be normal. Each microspore then initiated PM I. In most microspores, pollen mitosis developed normally, with the characteristic polarization of the nucleus and its subsequent asymmetric division (Figure 1 b) into two unequal cells (Figure 1 c). However, in 11.1% of the microspores, after callose dissolution (Figure 1 d), the released microspores underwent symmetric pollen mitosis, i.e., the nucleus was not displaced from the cell center to one side of the cell (Figure 1 e). The typical hemispherical cell plate, like that observed in normal pollen grain (Figure 1 c), was not seen in these cases, and all mitotic stages from prophase to telophase occurred without polarization (Figure 1 f and g). After cytokinesis, two equal-sized cells were observed as a result of symmetric division (Figure 1h). Both cells also showed equal chromatin condensation and the nuclei had the same spherical shape and were of equal sizes. (Figure 1h), i.e., the generative nucleus and vegetative nucleus could not be differentiated. After this stage, each cell developed into a pollen grain (Figure 1i). These cells did not undergo PM II and completed the synthesis of the pollen wall (Figure 1j). Each pollen grain was half the size of a normal pollen grain (Figure 1j). The frequency of abnormal pollen mitosis varied among flowers and also among inflorescences. All plants were equally affected. Sterile pollen grains resulted from this abnormal pollen development (Figure 1k). Pollen sterility, however, was much higher due to abnormal chromosome segregation caused by polyploidy.

Phases	N° of analyzed cells	N° of abnormal cells (%)	Abnormalities	N° of cells
Metaphase I	155	52 (33.55)	Precocious chromosome migration	46
			Fusion	6
Anaphase I	172	45 (26.16)	Laggard chromosomes	43
			Fusion	2
Telophase I	158	15 (9.50)	Micronuclei	13
			Fusion	2
Prophase II	161	22 (13.66)	Micronuclei	18
			Microcytes	4
Metaphase II	245	63 (25.71)	Precocious chromosome migration	54
			Microcytes	9
Anaphase II	155	26 (16.77)	Laggard chromosomes	24
			Microcytes	2
Telophase II	154	13 (8.44)	Micronuclei	8
			Microcytes	5
Meiotic product	158	22 (13.92)	Tetrad with micronuclei	18
			Tetrad with microcytes	4

 Table 1. Frequency of abnormalities recorded during microsporogenesis



Figure 1. Pollen development in *Brachiaria jubata* (BRA 007820). a) Normal tetrad with several micronuclei in microspores. b) Normal pollen mitosis (PM I): telophase. Note the polarized nuclei position. c) Pollen grain after a normal and asymmetric PM I. Observe the typical hemispherical cell plate between the generative (arrow) and the vegetative cells. d) Tetrad after callose dissolution. e) Abnormal released microspore without nucleus polarization. f-g) Symmetric PM I: metaphase (f) and telophase (g). Observe the lack of displacement of cell division from the cell center. In telophase (g), note that both nuclei are of equal sizes and chromatin is equally condensed. h) Cytokinesis after telophase dividing the microspore into two equal-sized cells. i) Two uninucleate sister pollen grains. j) Two sister pollen grains flanked by a normal one (arrow). Note that the normal pollen grain is double the size of those resulting from symmetric PM I. k) Sterile pollen grain.

Discussion

The pollen grain is released from the anther when it consists of just generative and vegetative cells in approximately 70% of plant families, and therefore PM II occurs while the pollen tube grows through the female pistil. In other plant families, including the Gramineae, this second mitotic division occurs before the pollen is shed from the plant (1). Therefore, in the genus *Brachiaria*, normal pollen production should display this mitosis before pollen shedding from the anther. In the accession of *B. jubata* under analysis, abnormal pollen mitosis (PM I) impaired the generative and vegetative cell differentiation, and the absence of PM II prevented sperm cell formation in over 10% of the microspores, causing pollen sterility.

Pollen development and male gametogenesis are critically dependent upon cell polarization which contributes to a highly asymmetric cell division (PM I). In normal microspores, the nucleus is displaced from the cell center to one side of its long axis at the G_1 phase of the microspore cell cycle. Displacement continues throughout the mitotic cycle (22) and the nucleus polarization is maintained by microtubule cytoskeleton (4). In the present accession, displacement of the nucleus did not occur in abnormal microspores. A similar behavior was also reported to occur naturally in *Brachiaria decumbens* (21), whereas the symmetric divisions observed in *Tulipa gesneriana* (23), tabacco (24), and *Arabidopsis* (15) were induced.

Asymmetric cell division relies on the formation of a typical hemispherical cell plate, in which spindle orientation is very important for normal differentiation of the generative and vegetative cells (25). An asymmetric, cone-shaped spindle perpendicular to the plasma membrane appears in this division (26, 27). The generative pole lies adjacent to the plasma membrane and the vegetative pole is located in the inner cytoplasm of the microspore. In the present study, a hemispherical cell plate was not recorded in the first pollen mitosis of this *B*. *jubata* accession, as also reported by Junqueira Filho et al. (21) for a *B. decumbens* accession. Tanaka (4) postulated that microtubules are involved in nuclear displacement and in the maintenance of the nuclear position of the hemispherical phragmoplast, and both are prerequisites for asymmetrical cell division in microspores. Based on this assumption, we suggest that the abnormal plants found in both *Brachiaria* species display anomalies in microtubule organization since nuclear displacement did not occur, nor was the hemispherical cell plate formed.

In normal plants, the vegetative and generative cells have different structures and developmental fates (4, 15, 28). While the large vegetative cell accumulates an abundance of stored metabolites required for rapid pollen tube extension, the smaller cell is enclosed by vegetative cell cytoplasm and contains relatively few organelles and stored metabolites. A peculiar feature of the abnormal microspores of B. jubata, similar to the abnormal microspores of B. decumbens reported by Junqueira Filho et al. (21) was the lack of differentiation between the vegetative and the generative cell. Both were of equal-sized. According to Tanaka (4), when the asymmetric cell division is inhibited or altered into a symmetric division, differentiation of the generative cell is prevented, and a cell-within-a cell is never formed thereafter. This might indicate that there is an intimate relationship between asymmetric cell division and the subsequent fates of the two daughter cells. In B. jubata, the lack of differentiation between the two daughter cells resulting from the first pollen mitosis compromised the pollen fate. After an evident cytokinesis among them, PM II did not occur, and each cell immediately differentiated into pollen grains. Thus, two half-sized pollen grains with a single nucleus were originated from each microspore. In contrast, in the abnormal microspore of *B. decumbens* reported by Junqueira Filho et al. (21), the two daughter cells underwent another symmetric pollen mitosis after symmetric PM I, the result being four

uninucleate and sterile pollen grains at the end of PM II. The occurrence of PM II in *B*. *decumbens* (21) is the main differential characteristic between the two *Brachiaria* species.

Another characteristic of PM I for both *Brachiaria* species is the similar chromatin condensation in both resulting cells, i.e., there is no difference between the nuclei. In normal asymmetric PM I division, the nuclei of the vegetative cell and the generative cell respectively diffuse and highly condensed chromatin, visible just after PM I. Such striking difference in the structure of chromatin is assumed to be associated with the difference in gene activity between the two types of nuclei, the generative cell nucleus being less transcriptionally active than the vegetative one (28). The absence of chromatin differentiation between nuclei resulting from PM I in *B. jubata* suggests that both are equally active.

The production of a functional pollen grain involves a complex series of regulated processes and requires the expression of a large number of sporophytic and gametophytic genes (20). First pollen mitosis appears to be a critical point for gametophytic development (1), because a specific set of genes is activated at this time. Mutations that affect the nuclear division pattern at the gametophytic stage have been scarcely registered (15,16,20,30,31). An abnormality similar to that found in the present accession of *B. jubata*, causing symmetric PM I and suppressing PM II has not been described in other species. This abnormality was not regularly expressed in the microsporocytes of all flowers or inflorescences examined. Only 11.1% of the microspores presented abnormal pollen mitosis. An incompletely penetrant phenotype resulting in equal, unequal and partial divisions at PM I has been reported in the gametophytic mutation gemini pollen l of Arabidopsis thaliana (15), and in *B. decumbens* (21), both disrupting microspore polarity, division asymmetry and pollen cell fate.

For double fertilization in angiosperms, two sperm cells are necessary. One sperm cell fuses with the egg cell to form the embryo and the other fuses with the central cells to form the endosperm. Abnormalities that cause male sterility in plants are considered to be of great interest in breeding programs because they might prevent self-polination of sexual genitors but also cause lack of seed set in genoptypes with important breeding traits. The *Brachiaria* breeding program aims at producing hybrids by intra- and interspecific crosses using sexual accessions or hybrids as mother plants and apomictic accessions as pollen parents. Polyploid accessions of *Brachiaria* are pseudogamous (apomictic), which means that the central-cell nucleus needs to be fertilized for endosperm and healthy seed development, even though the egg cell is not fertilized in an aposporous embryo-sac. The absence of fertile sperm cells in a considerable amount of pollen grains in this apomictic accession of *B. jubata*, caused by symmetric pollen mitosis (PM I), added to the unbalanced gametes generated by irregular chromosome segregation during meiosis due to polyploidy, may compromise its use in breeding and could explain, at least in part, why seed production is low when compared with the amount of flowers per raceme.

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