



## Meiotic analysis in induced tetraploids of *Brachiaria decumbens* Stapf

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**ABSTRACT** - The meiotic behavior of three tetraploid plants ( $2n=4x=36$ ) originated from somatic chromosome duplication of sexually reproducing diploid plants of *Brachiaria decumbens* was evaluated. All the analyzed plants presented abnormalities related to polyploidy, such as irregular chromosome segregation, leading to precocious chromosome migration to the poles and micronuclei during both meiotic divisions. However, the abnormalities observed did not compromise the meiotic products which were characterized by regular tetrads and satisfactory pollen fertility varying from 61.36 to 64.86%. Chromosomes paired mostly as bivalents in diakinesis but univalents to tetravalents were also observed. These studies contributed to the choice of compatible fertile sexual genitors to be crossed to natural tetraploid apomicts in the *B. decumbens* by identifying abnormalities and verifying pollen fertility. Intraspecific crosses should reduce sterility in the hybrids produced in the breeding program of *Brachiaria*, a problem observed with the interspecific hybrids produced so far.

**Key words:** *Brachiaria*, chromosome duplication, intraspecific crosses, meiotic behavior, plant breeding.

### INTRODUCTION

*Brachiaria* (Trin.) Griseb, a tropical forage grass genus of mainly African origin, has species that are widely used in cultivated pastures in Latin America (Renvoize et al. 1996). However, the diversity of pastures is dangerously restricted to a few cultivars, especially in the case of *B. decumbens*, with a single cultivar spread from México to all of South America. These reproduce asexually through apomixis (Valle and Savidan 1996) thus embryos are parthenogenetic and progenies are identical to the mother-plant (Dusi and Williemse 1999). This lack of diversity leads to homogeneous pastures, which is advantageous for animal management but represent a serious risk when planted over wide expanses of land. Superior *Brachiaria*

cultivars are in great demand to diversify pastures with varieties well adapted to soil and climate, resistance to pests and persistence under grazing (Valle et al. 2008, Valle and Pagliarini 2009).

Diploid accessions of obligate sexual reproduction – one of *B. brizantha* and 20 of *B. decumbens* were identified upon characterization of the collection (Valle 1990, Penteadó et al. 2000). These may be used in crosses with apomictic cultivars once the ploidy barrier is overcome. Thus, doubling the chromosomes of sexual diploids ( $2x$ ) was the easiest way to produce a  $4x$  sexual plant to use as the female parent in sexual x apomictic crosses (Valle 1990). This strategy resulted in widening genetic variability across inter and intraspecific hybrids thus allowing the selection of new genotypes which may be conserved by

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apomixis and could combine nutritive value and resistance to spittlebugs, especially in the case of *B. decumbens* (Valle et al. 2008).

Chromosome duplication of sexual and diploid accessions of *B. decumbens* was carried out at Embrapa Beef Cattle Research Center, Campo Grande, Brazil, between 2004 and 2007. Effectiveness of polyploidization was low due to problems in extraction and *in vitro* meristem culture, which demanded time and large quantities of plant material. In this work, only 3.9% of the regenerated and examined plants resulted in tetraploids (Valle et al. 2008, Simioni and Valle 2009). This paper describes the meiotic behavior and pollen viability of three of the artificially tetraploidized plants of sexual reproduction from that experimentation. The objective of this detailed analysis was to identify problem genotypes which need to be discarded as genitors in the breeding program.

## MATERIAL AND METHODS

The three plants (cD24-2, cD24-27 and cD24-45) used for meiotic analyses were obtained by chromosome doubling of a diploid sexual accession of *B. decumbens*, named D24, by colchicine treatment of basal segments of *in vitro* grown plantlets. Ploidy levels and complete euploidy was confirmed by chromosome counting in root tips. The mode of reproduction was confirmed as sexual through analysis of embryo-sacs (Valle et al. 2008, Simioni and Valle 2009), using interference contrast microscopy on methyl-salicylate cleared ovules as established by Young et al. (1979).

For meiotic studies, young inflorescences still folded in the flag leaf were collected from the three tetraploid plants, then fixed in a solution containing absolute ethanol: acetic acid (3:1) for 24 hours, transferred to 70% ethyl alcohol and stored under refrigeration (Araújo et al. 2005). Microsporocytes were prepared by squashing and staining with 1% propionic carmine and analyzed under light microscopy.

All meiotic phases were evaluated and abnormalities recorded. Most of the gametic chromosome counts were performed in cells at diakinesis and metaphase I, since at these stages chromosome pairing is clearly observed. A minimum of 20 cells in diakinesis were analyzed.

Pollen fertility was estimated in fresh dehiscent anthers. About 1500 mature pollen grains per plant were evaluated. Pollen fertility was determined by the staining with 1% propionic carmine: full, stained grains were

classified as fertile and non-stained or weakly stained grains were considered sterile (Simioni et al. 2004, Simioni et al. 2006).

## RESULTS AND DISCUSSION

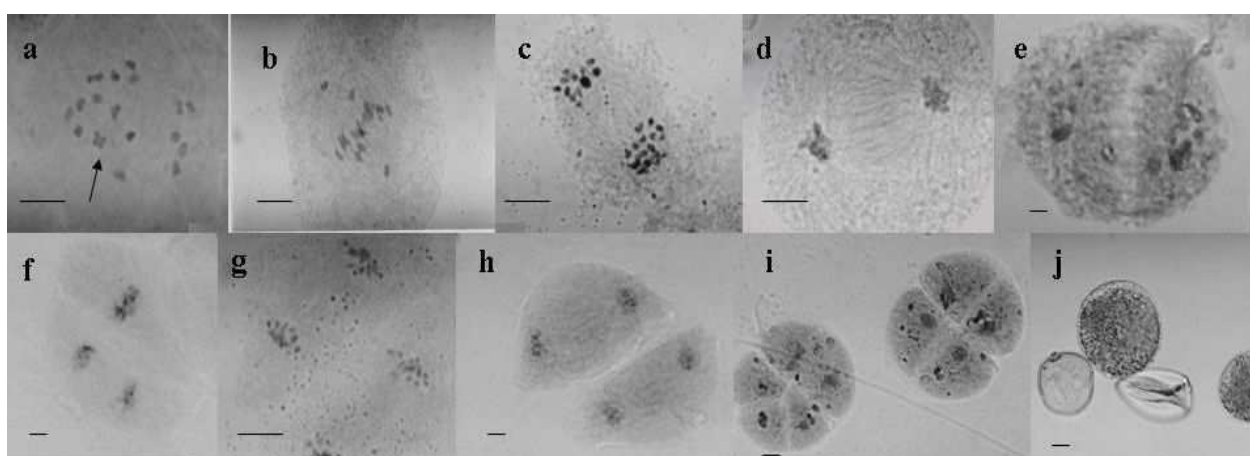
Chromosome associations at diakinesis confirmed the polyploid condition ( $2n = 4x = 36$ ) in the three plants evaluated. Meiotic behavior, mainly in meiosis I, was typical of polyploids, with chromosomes associating as univalents, bivalents and quadrivalents, with the prevalence of bivalent configurations (Table 1, Figure 1a).

The three plants underwent somatic duplication, thus becoming autotetraploid organisms possessing a combination of two genetically similar genomes (homologous). In such cases, the chromosome behavior at meiosis is expected to be random or not in any preferential form (polysomic inheritance) (Albinic et al. 2006), with a tendency to form multivalents (Ramsey and Schemske 2002). However, the predominance of bivalent associations suggests a genetic control of chromosome pairing such as has been recognized in artificially induced autotetraploids of *Trifolium riograndense* (Schifino and Moraes-Fernandes 1986, 1987), of *B. brizantha* (Araújo et al. 2005), and of *B. ruziziensis* (Risso-Pascotto et al. 2005). In 20 accessions of natural tetraploid *B. nigropedata* ( $2n = 4x = 36$ ), bivalent associations also predominated in diakinesis, suggesting that its origin was possibly as a segmental allopolyploid (Utsunomiya et al. 2005). In contrast, Caetano et al. (2006), studying autotetraploid *Panicum maximum* cv Tanzânia ( $2n = 4x = 36$ ), found 99 cells presented multivalent associations at diakinesis, with a predominance of tetravalents among the 109 diakinesis analyzed. In *B. decumbens* cv. Basilisk ( $2n = 4x = 36$ ), the meiotic process was typical of polyploids with multiple chromosome associations (Junqueira-Filho et al. 2003). Of the interspecific hybrids between the induced tetraploid sexual accessions of *B. ruziziensis* and the natural and apomictic tetraploid accessions of *B. brizantha* ( $2n = 4x = 36$ ) and *B. decumbens* ( $2n = 4x = 36$ ), only 6.3 % of the microsporocytes paired as 18 bivalents, explaining the reduction in pollen viability of these hybrids (Lutts et al. 1991, Lutts et al. 1994).

From a total of 5502 microsporocytes analyzed in the three duplicated plants of this experiment, 1053 presented irregularities from prophase I until meiotic products, representing 19.14% of the total cells analyzed (Table 2). The most frequent abnormalities in the two

**Table 1.** Meiotic chromosome configurations at diakinesis phase (prophase I) of three tetraploid plants of *Brachiaria*

Plant number	Chromosome number	Nr. of cells analyzed	Mean nr. of observed associations (per cell) (range)			
			I	II	III	IV
cD24-2	36	52	0.11 (0-2)	16.92 (12-18)	0.11 (0-2)	0.42 (0-3)
cD24-27	36	30	0.77 (0-10)	15.26 (8-18)	0.5 (0-3)	0.73 (0-3)
cD24-45	36	70	0.19 (0-2)	16.2 (8-18)	0.13 (0-2)	0.76 (0-4)

**Figure 1.** Meiotic aspects of the tetraploidized accession D24 of *B. decumbens*. **a**) Microsporocytes in diakinesis (plant cD24-2) (note the prevalence of bivalent associations; tetravalent association (arrow)); **b**) metaphase I with precocious migration (plant cD24- 2); **c**) normal anaphase I (plant cD24-2); **d**) normal telophase I (plant cD24- 45); **e**) normal prophase II (cD24-45); **f**) metaphase II with asynchrony (cD24- 45); **g**) normal anaphase II (cD24-27); **h**) normal telophase II (cD24-2); **i**) Microspores (tetrads and polyads with micronuclei) (cD24-2); **j**) fertile pollen grains (stained) and sterile (not stained), plant D24-27. Scale Bar = 10 mm.

meiotic divisions were those related to chromosome segregation. In the first division, some precocious chromosome migration to the poles at metaphase (Figure 1b), laggards and bridges at anaphase were observed. Only a few meiocytes displayed some abnormalities in the second division: asynchrony in metaphase (Figure 1f) was the most common, but did not compromise the final meiotic product because all cells completed meiosis in time to form a regular tetrad and eventually forming normal pollen grains (Risso-Pascotto et al. 2004).

The final product of meiosis was characterized by normal tetrads, but a few dyads, triads, polyads and tetrads with micronuclei (Figures 1i, 1j) were also observed. Failure of cytokinesis in the first and second meiotic division leading to dyad and triad formation was also reported for *Brachiaria* (Valle and Pagliarini 2009). This phenomenon occurred in these plants, but in low frequencies (data not

shown in the Table 2), thus not compromising the predominance of tetrads.

A mean percentage of meiotic abnormalities for each plant were: 18.4 % (plant cD24-2), 20.9 % (plant cD24-27) and 18.2 % (plant cD24-45), whereas the total number of cells evaluated (Table 2), indicating that such abnormalities occurred in similar frequencies in these induced polyploids.

Pollen viability was estimated to be 64.86 % for plant cD24-2, 61.36 % for plant cD24-27 and 63.25 % for plant cD24-45 (Table 3, Figure 1j). This index was considered high for treated plants artificially induced to chromosomal duplication. Since around 63.16 % of pollen was viability, the progenies of these might be evaluated in order to select promising genotypes.

The levels of viable pollen grains in these tetraploid plants (average 63.16 %) are similar to those observed by Risso-Pascotto et al. (2004), in tetraploid hybrids from the

**Table 2.** Meiotic abnormalities recorded in three tetraploidized plants of accession D24 of *B. decumbens*

Plant number	Phases	Nr. of analyzed cells	Nr. of abnormal cells (%)	Main abnormalities (nr. of cells and %)
<b>cD24-2</b>	Prophase I (diakinesis)	52	24 (46.2)	Multiple chromosome associations: 24 (46.2)
	Metaphase I	351	156 (44.4)	Precocious migration: 99 (28.2)
	Anaphase I	202	33 (16.3)	Bridges: 17 (8.4) Laggards: 16 (7.9)
	Telophase I	241	6 (2.5)	Laggards: 6 (2.5)
	Prophase II	222	7 (3.2)	Abnormal cytokinesis: 5 (2.2)
	Metaphase II	148	54 (36.5)	Asynchrony: 14 (9.5) Precocious migration: 13 (8.8)
	Anaphase II	129	22 (17.1)	Asynchrony: 12 (9.3)
	Telophase II	82	10 (12.2)	Asynchrony: 5 (6.1) Absence of cytokinesis: 3 (3.6)
	Meiotic product	494	42 (8.5)	Micronuclei: 4 (0.8) Polyads: 27 (5.5) Triads: 8 (1.6)
Total nr. of cells		1921	354 (18.4)	
<b>cD24-27</b>	Prophase I (diakinesis)	30	20 (66.7)	Multiple chromosome associations: 20 (66.7)
	Metaphase I	315	72 (22.8)	Precocious migration: 60 (19.0)
	Anaphase I	129	9 (7.0)	Laggards: 8 (6.2)
	Telophase I	176	9 (4.5)	Aderence bridges: 7 (4.0)
	Prophase II	142	5 (3.5)	Laggards: 5 (3.5)
	Metaphase II	226	106 (46.9)	Precocious migration: 43 (19.0) Asynchrony: 40 (17.7)
	Anaphase II	219	77 (35.2)	Bridges: 26(11.9) Laggards: 24 (10.9)
	Telophase II	168	4 (2.4)	Asynchrony: 24 (10.9) Bridges: 2 (1.2)
	Meiotic product	313	58 (18.5)	Micronuclei: 8 (2.5) Triads: 46 (14.7)
Total nr. of cells		1718	360 (20.9)	
<b>cD24-45</b>	Prophase I (diakinesis)	70	42 (60.0)	Multiple chromosome associations: 42 (60.0)
	Metaphase I	330	90 (27.3)	Precocious migration: 68 (20.6)
	Anaphase I	206	29 (14.1)	Laggards: 18 (8.7)
	Telophase I	240	13 (5.4)	Bridges: 7 (2.9) Laggards: 6 (2.5)
	Prophase II	225	-	-
	Metaphase II	226	75 (33.2)	Asynchrony: 33 (14.6) Precocious migration: 19 (8.4)
	Anaphase II	132	49 (37.1)	Asynchrony: 20 (15.1) Bridges: 17 (12.9)
	Telophase II	153	12 (7.8)	Asynchrony: 7 (4.6)
	Meiotic product	281	29 (10.32)	Micronuclei: 7 (2.5) Diads: 9 (3.2) Triads: 6 (2.1)
Total nr.of cells		1863	339 (18.2)	

cross between *B. ruziziensis* ( $2n = 4x = 36$ ) and *B. brizantha* ( $2n = 4x = 36$ ), where pollen viability reached 52.76%.

In *B. brizantha*, tetraploid and hexaploid accessions displayed irregular chromosome segregation resulting in the formation of micronuclei in the tetrads (Mendes-Bonato et al. 2002a). In induced autotetraploids of *B.*

*ruziziensis*, irregular chromosome segregation in the two meiotic divisions led to the development of polyads as the final meiotic product and pollen sterility reached 63.38% (Risso-Pascotto et al. 2005). In the natural tetraploid *B. decumbens* cv Basilisk, many cells with precocious chromosome migration to the poles at metaphase I and

**Table 3.** Pollen viability (%) of three tetraploidized plants of accession D24 of *B. decumbens*

Plant number	Viable pollen (%)	Pollen sterility (%)	Nr. of pollen grains analyzed
cD24-2	64.86	35.14	629
cD24-27	61.36	38.64	1030
cD24-45	63.25	36.75	928
Mean	63.16	36.84	

laggards at anaphase gave rise to micronuclei in telophase I generating a high frequency of abnormal products in meiosis (Mendes-Bonato et al. 2002b). Some irregularities in *Brachiaria* were never before observed in other plants. Among the irregularities, abnormal chromosome segregation, desynapsis, chromosome stickiness, fusional syncytes, multipolar spindles and chromosome transfer among microsporocytes were reported (Mendes-Bonato et al. 2001a, Mendes-Bonato et al. 2001b, Risso-Pascotto et al. 2003b, 2005, Risso-Pascotto et al. 2003c, Utsunomiya et al. 2005, Boldrini et al. 2006), and some of them caused partial or total pollen sterility, compromising the use of those genotypes in breeding programs. In several meiotic studies carried out in accessions of *Brachiaria*, the described meiotic abnormalities have been attributed to mutations (Risso-Pascotto et al. 2003a).

In autotetraploid *Panicum maximum* cv Tanzânia, meiotic abnormalities occurred in a significant frequency. The most common were precocious migration and laggard chromosomes at metaphase and anaphase, respectively; micronuclei in telophase, tetrads with unbalanced microspores and microcytes were also quite frequent. Cell fusion in different phases of the meiotic process was also observed (Caetano et al. 2006).

Cytological analyses like the ones presented here are extremely important tools in the breeding programs in order to select compatible and fertile genitors, both by determining the level of ploidy and the absence of meiotic

abnormalities that could compromise the viability of the gametes.

All three artificially polyploidized plants of *B. decumbens*, accession D24, displayed some chromosome abnormalities but those did not impair gamete fertility. The frequencies of abnormalities varied both with the genotypes evaluated and the phase of meiosis analyzed. Pollen sterility remained below 37 % in all three genotypes. Therefore, all three could be used as female or male genitors both in a sexual crossing block to recover more fertile progenies, or in crosses with apomictic *B. decumbens* cv. Basilisk to recover sexual and apomictic hybrids. These intraspecific crosses were never before attempted in *B. decumbens*. Hybrids of these duplicated plants crossed to natural apomicts should result in a progeny segregating for sexual and apomictic plants in a 1: 1 proportion, as previously reported for interspecific hybrids of *Brachiaria* (Valle and Savidan 1996). Whereas sexual hybrids may be used in further crosses in the breeding program, the apomictic ones can enter agronomic performance in field trials. Those displaying superior agronomic traits are immediate candidates as new cultivars to promote pasture diversification with increased productivity, insect resistance and improved nutritive value. Polyploidization of *B. decumbens* is thus a major breakthrough in the breeding of this species of indisputable significance to animal production in the tropics.

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## Análise meiótica em tetraplóides induzidos de *Brachiaria decumbens* Stapf

**RESUMO** - O comportamento meiótico de três plantas tetraplóides ( $2n = 4x = 36$ ) originadas de duplicação cromossômica somática de uma planta diplóide de reprodução sexual de *Brachiaria decumbens* foi avaliado. Todas as plantas analisadas apresentaram anormalidades relacionadas à poliploidia, tais como segregação cromossômica irregular, levando à migração precoce dos cromossomos para os polos e formação de micronúcleos durante as duas divisões meióticas. Entretanto, as anormalidades observadas não comprometeram os produtos meióticos, que foram caracterizados por tétrades regulares e satisfatória fertilidade dos grãos de pólen, que variou de 61,36 a 64,86%. Os cromossomos parearam principalmente como bivalentes na diacinese, mas univalentes até tetravalentes também foram observados. Estes estudos contribuíram para a escolha de genitores sexuais férteis e compatíveis para cruzamento com apomíticos naturais de *B. decumbens*, identificando anormalidades e conferindo a viabilidade do pólen. Com cruzamentos intraespecíficos, espera-se reduzir a esterilidade nos híbridos produzidos no programa de melhoramento, problema observado nos híbridos interespecíficos obtidos até então.

**Palavras-chave:** *Brachiaria*, comportamento meiótico, cruzamentos intraespecíficos, duplicação cromossômica, melhoramento vegetal.

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