



Chromosome duplication in *Brachiaria* (A. Rich.) Stapf allows intraspecific crosses

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ABSTRACT - *Brachiaria decumbens* cv. Basilisk is the single most important forage grass used for pastures in the tropics. Breeding to produce improved cultivars has been impossible until now due to the lack of compatible sexual ecotypes. This paper reports the success of somatic chromosome duplication of sexually reproducing diploid plants of *B. decumbens* and of a diploid hybrid between *B. decumbens* and *B. brizantha*, which should allow intraspecific crosses with natural apomictic tetraploid accessions of either species. Polyploidization was induced in explants cultured in vitro on a medium supplemented with colchicine at 0.01% for 48 hours, transferred to the same medium without colchicine until shoot regeneration occurred. Five sexual tetraploid plants (3.9% of plants recovered) were obtained. Crosses with apomictic cultivars recovered 14 seeds. The novel sexual tetraploids generated were unique and represented a major breakthrough in breeding *B. decumbens* to obtain superior hybrids.

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

INTRODUCTION

Brachiaria (A. Rich.) Stapf [(syn. *Urochloa* Hochst. ex A.Rich.) R.D.Webster] is a tropical grass genus of mainly African origin (Pereira et al. 2001). In Central Brazil basically two cultivars of two species (cv. Basilisk of *B. decumbens* and cv. Marandu of *B. brizantha*) are planted on an estimated area of some 100 million hectares (Resende et al. 2007). These commercial varieties have an apomictic mode of reproduction (Dusi and Williemse 1999) and they are polyploids ($2n=4x=36$) (Penteado et al. 2000, Mendes-Bonato et al. 2002).

In apomictic reproduction, 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 represents a serious risk when planted over wide expanses of land.

B. decumbens cv. Basilisk has exceptional adaptation to acidic soils, vigorous growth, ease of establishment, and good forage value throughout the year, but these favorable characteristics are counteracted by its susceptibility to spittlebugs (Valle 1990).

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. Breeding of *Brachiaria* was started about 20 years ago and three varieties have already been released. Interspecific hybrids tetraploids were produced and evaluations have identified promising genotypes to be released as commercial cultivars once grazing trials are completed (Valle et al. 2008). However,

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chromosome abnormalities have been described which impair seed production therefore hindering adoption by farmers (Junqueira-Filho et al. 2003, Mendes-Bonato et al. 2004, 2007, Fuzinato et al. 2007).

Characterization of accessions of *Brachiaria* identified some diploid accessions of obligate sexual reproduction – one of *B. brizantha* and 20 of *B. decumbens* (Valle 1990, Penteado et al. 2000, Mendes-Bonato et al. 2002) which can be used in crosses with apomictic cultivars. Intraspecific hybrids are usually more fertile than interspecific ones, widening genetic variability and exploring new genotypes conserved by apomixis, helping to solve the problem of spittlebug susceptibility in *B. decumbens*, adaptation to poorer soils in *B. brizantha* and of less nutritive values in both (Valle 1990).

Ploidy differences between sexual (diploid) and apomictic (tetraploid) plants impose the greatest limitation to hybrid production (Carneiro and Dusi 2002). To counteract this problem it is necessary to balance the chromosome number between genitors. Two pathways are possible: haploidization of apomictic tetraploids or duplication of the sexual diploids.

Artificial polyploid induction for different purposes in breeding programs is frequently done (Hermsen 1984) either to increase plant size, restore fertility in intra or interspecific hybrids or to allow gene transfer between individuals with different ploidy levels (Schifino-Wittmann and Dall'Agnol 2003).

This paper reports the success in chromosome duplication of sexual and diploid accessions of *B. decumbens* and of diploid interspecific hybrids between *B. brizantha* and *B. decumbens* with the purpose of hybridizing induced tetraploids with the natural tetraploids of the same species. A micropropagation protocol was developed for *Brachiaria* (Rodrigues-Otubo et al. 2000) so it was possible to generate somatic polyploids through colchicine directly in the culture medium containing the meristems.

MATERIAL AND METHODS

Plant material and *in vitro* cultivation

This work was conducted in the Plant Cytogenetics and Biotechnology Laboratories of Embrapa Beef Cattle, in Campo Grande, MS, Brazil.

Plants from accessions D4, D5, D6, D18, D24, D25, D27, D30, D35 and D40 of *B. decumbens* and interspecific hybrid plants from the cross between B105 (*B. brizantha*) x D4 (*B. decumbens*); B105 x D5/2 and B105 x D5/6; all diploids of sexual reproduction were used in this experiment. Cuttings from these plants were collected from the germplasm bank maintained at Embrapa Beef Cattle.

Meristems were extracted, then sterilized and cultured *in vitro* using an LS culture medium (Linsmaier and Skoog 1965) containing growth regulators: naphthaleneacetic acid, 1 mg L⁻¹, kinetin, 3 mg L⁻¹ and 6-benzylaminopurine, 3 mg L⁻¹. Once multiplied, the buds were individualized and transferred to test tubes containing modified MS medium (Murashige and Skoog 1962) with naphthaleneacetic acid, 1 mg L⁻¹ and supplemented with 45 g L⁻¹ sucrose for the rooting of the explants.

Colchicine Treatment

Basal segments of the plants cultivated *in vitro* were isolated and placed in an LS culture medium supplemented with colchicine at 0.01% for 48 hours. This concentration was the most effective in duplicating *B. brizantha* according to Pinheiro et al. (2000).

After 48 hours, the buds were again placed in an LS medium without colchicine for regrowth. In about 30 days, individual buds were isolated and placed in an MS medium for rooting. Plantlets were acclimated in the greenhouse and then transferred to the field.

Cytological analysis

Somatic chromosome numbers on root tips as well as meiosis in pollen-mother cells were studied to verify chromosome numbers on the mother plants and on the treated ones.

Root tips were pre-treated with a saturated solution of 1-bromonaphthalene for 3 hours at 16 °C, and then fixed in an absolute ethanol: acetic acid solution at a 3:1 proportion for up to 24 hours at 3 °C and stored in 70% ethyl alcohol in the refrigerator until use. For chromosome counting, the root tips were re-hydrated, treated with 2% pectinase for one hour, hydrolyzed in 1N HCl for 10 minutes at 60 °C. The color was developed in the dark with Schiff reactive and 1% propionic carmine (Pozzobon and Valls 1997). The analyses were performed under light microscope. A minimum of 20 cells were studied.

To determine chromosome numbers in meiosis, young inflorescences still folded in the flag leaf were collected, fixed in a solution containing absolute ethanol: acetic acid (3:1) for 24 hours, transferred to 70% ethyl alcohol and stored under refrigeration. The slides were prepared by squashing the anthers and staining with 1% propionic carmine (Pagliarini et al. 2002, Araújo et al. 2005) and a minimum of 20 cells in diakinesis were analyzed.

The mode of reproduction was studied using interference contrast microscopy on methylsalicylate clarified ovaries as established by Young et al. (1979) and modified by Dusi and Williemse (1999).

Hybridization

Crosses were attempted in the greenhouse using potted plants of all the duplicated plants. Flowers were emasculated in the mother plants and brushed with fresh pollen brought in Petri dishes, from the natural tetraploid and apomictic cultivars: *B. decumbens* cv. Basilisk and *B. brizantha* cv. Marandu. After pollination, the flowers were bagged and identified. Seeds were collected at least 21 days after pollination.

RESULTS AND DISCUSSION

In vitro Culture

Extraction and culture of explants had to be continuously repeated since there were large differences in genotype responses to cultivation. More responsive genotypes resulted in numerous calli (data not shown), allowing more explants to be excised and treated with colchicine.

A small quantity of samples in some accessions was caused by negative responses in the *in vitro* cultivation. Also, contamination and oxidation were common during *in vitro* culture. The D24 accession of *B. decumbens* was the most responsive, producing a large number of buds from a single meristem grown (data not shown), which continued to form calli after transplanting to other culture medium.

About 1809 meristems were extracted and from those only 421 basal segments were treated with colchicine. The losses by contamination (fungi and bacteria) and oxidation amounted to 23.27% of all meristems cultured.

Success in chromosome duplication

The original D24 accession of *B. decumbens* and the B105 x D4 hybrid were indeed diploids as determined in root tips for D24 and in pollen mother cells for the hybrid (Figures 1a, 1b). Penteado et al. (2000) also determined D24 as diploid using flow cytometry.

A total of 216 adult plants were regenerated and 41 had root tips analyzed. One plant, named cD24-2 was successfully duplicated. When flowering occurred, pollen mother cells of 87 plants could be analyzed. Duplication was confirmed in cD24-2 (Figures 1c, 1d) and two other plants derived from D24 of *B. decumbens*: cD24-27 and cD24-45 (Figures 1e, 1f, 1g, 1h) and two derived from the hybrid B105 x D4: cH4-8 and cH4-100 (Table 1).

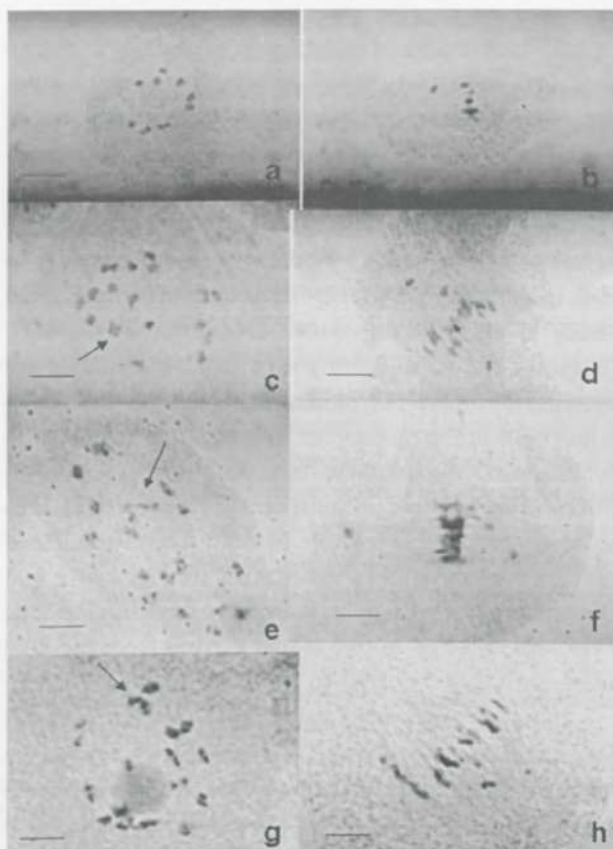


Figure 1. Some meiotic cell aspects of the diploid hybrid B105 x D4: (a) Diakinesis (prophase I) with nine bivalents; (b) Metaphase I with precocious migration. (c - h) Some meiotic cell aspects of tetraploidized plants of *Brachiaria decumbens*: (c,d) Plant cD24-2, diakinesis (prophase I) and metaphase I, respectively. (e,f) Plant cD24-27: diakinesis (prophase I) and metaphase I, respectively. (g,h) Plant cD24-45: diakinesis (prophase I) and metaphase I, respectively. Note the prevalence of bivalent associations in diakinesis, tetravalent association (arrows), metaphase I with precocious migration (d, f) and lagging chromosomes and bridges (h). Scale: 10µm

Table 1. Adult plants recovered in *Brachiaria decumbens* accessions and in hybrids between *B. brizantha* and *B. decumbens* (B105 X D4) evaluated for effectiveness of chromosome duplication (mitosis and meiosis)

Accession	Number of plants evaluated	Number of diploid (2X) plants	Number of tetraploid (4X) plants	Number of plants with chimeras*
D6	02	02	0	-
D18	01	01	0	-
D24	50	47	03	-
D25	20	20	0	-
D30	03	01	0	02
D35	12	12	0	-
(H4) B105 X D4	22	20	02	-
(H5) B105 X D5/2	16	10	0	06
(H6) B105 X D4/6	02	01	0	01
Total	128	114	05	09

* Chromosome number undefined

Effectiveness of polyploidization is low due to problems in extraction and *in vitro* meristem culture, which demand time and large quantities of plant material. In this case, only 3.9% of the regenerated and assessed plants resulted in tetraploids.

Six of the plants from the hybrid B105 x D5/2 (cH5-61, cH5-77, cH5-78, cH5-94, cH5-97 e cH5-101) and one plant from B105 x D5/6 (cH6-61) resulted in chimeras, thus ploidy levels were impossible to determine (Table 1). Chimeras and consequently mixoploid individuals are common in artificial polyploidization since only mitotically active cells at the moment of treatment are subjected to duplication. The affected tissue is disorganized leading to chromosome number irregularities (Sybenga 1992), thus some tissues or even whole plants present duplicated sectors among others not duplicated (Taylor and Quesenberry 1996). Chimeras in microspore mother cells were also detected in two plants derived from the D30 accession (cD30-1 e cD30-8).

The percentage of polyploidy induction varies among different works. The protocols tested by Pinheiro et al. (2000) to duplicate the chromosome numbers of diploid *B. brizantha* using *in vitro* culture demonstrated that efficiency depended on the concentration and time of exposure to colchicine. Of the 75 explants treated, 44 plants were recovered of which 18 were duplicated. The mode of reproduction was sexual in six of the induced tetraploids, and meiotic behavior was stable with prevalence of bivalents in meiosis (Araújo et al. 2005). Swenne et al. (1981) duplicated chromosomes of sexual diploid accessions

of *B. ruziziensis* ($2n=2x=18$) with colchicine applied in three day old seedlings, with immersion of the entire plantlet in colchicine solutions at different concentrations. Of the 189 plants regenerated, 135 remained diploid; 19 resulted in mixoploid and 35 were tetraploidized (18.5% of the plants). In *Panicum maximum*, three sexual autotetraploids (0.45% of plants recovered) were produced by treating germinating diploid seeds with 0.1% colchicine for 4 hours (Nakagawa and Hanna 1992).

Analysis of mode of reproduction

Rates of sexuality varied from 8.0% (on plant cH4-8) to 70.3% (on plant cD24-45) on clarified ovaries (Table 2). No aposporic sacs were observed, thus confirming sexual reproduction on the polyploidized plants. The presence of aposporic sacs indicates reproduction by apomixis whereas embryo-sacs of the *Polygonum* type and total absence of aposporic sacs indicate exclusive sexual reproduction (Valle and Savidan 1996).

The D24 diploid which gave rise to the cD24-2, cD27-27 and cD24-45 tetraploids and the B105 x D4 diploid hybrid which gave rise to the cH4-8 and cH4-100 tetraploids both reproduce sexually (Table 2), with 52 and 25% of the ovaries displaying meiotic sacs, respectively. The others displayed shrivel, abnormal, sterile ovaries and/or had unidentifiable embryo sacs. The presence of high rates of sterile embryo sacs in the B105 x D4 (71.6%) diploid hybrid proved that hybrid plants present major problems of ill-formed embryo-sacs.

In the cH4 – 8 and cH4 – 100 polyploidized plants high rates of sterility, abnormalities or unidentifiable embryo sacs reach 92 % and 88%, respectively (Table 2) which could greatly impair hybridization and seeds set. This is not uncommon for recently polyploidized plants (Araújo et al. 2005). Despite these problems, all induced tetraploids maintained sexual reproduction as their original genotypes.

According to Valle et al. (1994), apomixis in *Brachiaria* are correlated with polyploidy and under simple genetic control with apomixis dominant over sexuality. Duplication of chromosomes therefore was not expected to bring about apomixis since this allele is not present in sexual plants (Araújo et al. 2005).

Hybridization

A total of 2528 flowers were pollinated but only 14 seeds were produced or 0.55% of success (Table 3). These results reflect the difficulty in producing viable seeds under artificial pollination right after duplication of chromosomes. Chromosome abnormalities are expected and could result in a high rate of sterility such as observed in embryo-sacs of the duplicated plants (Table 2).

Pollen viability was also assessed on the apomictic genitors to verify if that could be impairing hybridization and values of 65.2% (*B. brizantha* cv. Marandu) and 73.8% (*B. decumbens* cv. Basilisk) were determined (Simioni and Valle, unpublished data), thus problems were most probably on the side of the female gamete, as confirmed by high rates of sterile, shrivel and abnormal ovaries observed in the embryo-sacs of the duplicated plants (Table 2).

Other factors such as bagging conditions and climatic conditions could also have interfered in the rate of success of seed fill. Also, even considering the

rather limited number of plants involved in the crosses, it was possible to conclude that the next crossing generation will require much more pollinated flowers to ensure that some fertile progeny will be produce.

Lutts et al. (1991, 1994) produced viable hybrids in artificial crosses between artificially tetraploidized *B. ruziziensis* and natural tetraploid accessions of *B. brizantha* ($2n=4x=36$) and *B. decumbens* ($2n=4x=36$). Seed germination reached 80%. Plant mortality was 41.9% (*B. ruziziensis* x *B. decumbens*) and 56.6% (*B. ruziziensis* x *B. brizantha*). Hybrids resulting from these crosses amounted to 31.4% and 8.7%, respectively.

These artificial tetraploid plants need to be stabilized to eliminate meiotic abnormalities as well as defective embryo-sacs which compromise fertility and seed set. The strategy could be to allow open-pollination among these plants in a crossing block and so that the seeds produced exhibit less sterility. Selection pressure applied for seed production and against pollen sterility was done for red clover in order to obtain new polyploids and avoid mixoploids which impaired reproduction (Simioni et al. 2006).

Even considering the low percentage of polyploid induction and the very low success in the crosses with the apomictic cultivars, the results here presented open new possibilities in *Brachiaria* breeding. The tetraploids once stabilized, will be invaluable in accomplishing intraspecific crosses never before attempted both in *B. decumbens*, and in *B. brizantha*, using the H4 hybrid in this case. Hybrids of these duplicated plants crossed to natural apomicts should result in a progeny segregation for sexual and apomictic plants in a 1:1 proportion, as previously reported for interspecific hybrids of *Brachiaria* (Valle and Savidan 1996). Sexual hybrids may be used in further crosses in the breeding program, whereas the apomictic ones should be tested

Table 2. Number and percentage of meiotic (S), shriveled (Sh), abnormal (Ab), sterile (St) and unidentifiable (U) embryo sacs on the induced tetraploids and on the three original diploid plants of *Brachiaria*

Plant	S	Sh	Ab	St	U	Mode of reproduction	Number of analyzed ovaries
cD24-2	21 (42%)	2 (4%)	1 (2%)	8 (16%)	18 (36%)	sexual	50
cD24-27	8 (22.2%)	5 (13.9%)	1 (2.8%)	-	22 (61.1%)	sexual	36
cD24-45	26 (70.3%)	-	-	1 (2.7%)	10 (27.0%)	sexual	37
cH4-8	4 (8%)	5 (10%)	5 (10%)	34 (68%)	2 (4%)	sexual	50
cH4-100	6 (12%)	5 (10%)	1 (2%)	38 (76%)	-	sexual	50
B105x D4(H4) - Original 2x	15 (25%)	1 (1.7%)	1 (1.7%)	43 (71.6%)	-	sexual	60
D24(Original 2x)	26 (52%)	5 (10%)	2 (4%)	11 (22%)	3 (6%)	sexual	50

against existing cultivars. The ones 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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Table 3. Seed production, germination and plantlet survival rate of the crosses involving sexual tetraploid plants x natural apomictic cultivars of *Brachiaria*

Mother plant	Pollen donor	Number of pollinated flowers	Number of seeds produced	Germination (%)	Number of surviving plantlets
cH4-8	<i>B. brizantha</i> cv. Marandu	466	-	-	-
cH4-100	<i>B. brizantha</i> cv. Marandu	246	-	-	-
cD24-2	<i>B. decumbens</i> cv. Basilisk	728	08	1 (12.5%)	0
cD24-27	<i>B. decumbens</i> cv. Basilisk	470	02	1 (50%)	0
cD24-45	<i>B. decumbens</i> cv. Basilisk	618	04	1 (25%)	1
Total		2528	14	3 (25%)	1

Duplicação cromossômica em *Brachiaria* (A. Rich) Stapf viabiliza cruzamentos intraespecíficos

RESUMO - *Brachiaria decumbens* cv. Basilisk é a gramínea forrageira mais utilizada nas pastagens tropicais. O melhoramento para produzir cultivares melhoradas tem sido difícil devido à falta de ecótipos sexuais compatíveis. Este trabalho registra o sucesso da duplicação cromossômica somática de plantas diplóides de reprodução sexual de *B. decumbens* e de um híbrido entre *B. decumbens* e *B. brizantha*, que foram cruzadas com os acessos tetraplóides apomíticos destas espécies. A poliploidização foi induzida em explantes cultivados *in vitro* em um meio suplementado com colchicina à 0,01% por 48h, transferidos para o mesmo meio sem colchicina até a regeneração dos brotos. Cinco plantas de reprodução sexual e tetraplóides (3,9% das plantas analisadas) foram obtidas. Cruzamentos com cultivares apomíticas produziram 14 sementes. Os novos tetraplóides sexuais gerados são únicos e representam uma grande inovação no melhoramento de *B. decumbens* por permitir cruzamentos intraespecíficos para obtenção de híbridos superiores.

Palavras-chave: Autopoliploides, *Brachiaria*, duplicação cromossômica, melhoramento genético, cruzamentos intraespecíficos.

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