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Meiotic behavior in apomictic *Brachiaria ruziziensis* × *B. brizantha* (Poaceae) progenies

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ABSTRACT: Hybrids combining desirable traits from divergent parents are the main objective of some *Brachiaria* (Syn. *Urochloa* P. Beauv.) breeding programs. There is great interest in the development of apomictic hybrid cultivars that combine desirable genes such as resistance to spittlebugs, high nutritive value, and tolerance to acid soils. Microsporogenesis of six apomictic progenies resulting from a tetraploid (2n = 4x = 36) cross between *B. ruziziensis* × *B. brizantha* was evaluated under light microscopy. Genetic recombination, ensured by multivalent chromosome association and crossing-over at prophase occurred in low frequency among progenies, and in one, recombination was almost nonexistent. The percentage of meiocytes with meiotic abnormalities among progenies ranged from 16.6 % to 85.6 %. Besides an observed irregular chromosome segregation typical of polyploid hybrids in these five progenies, putative meiotic mutations characterized as desynapsis and divergent spindle organization occurred in three progenies. These anomalies caused frequent fractionation of the genome into several microspores of different sizes. In *Brachiaria*, new cultivars must be apomictic to fix the genotype. However, *Brachiaria* is a pseudogamous apomict, and viable gametes are necessary to produce viable seeds. Considering meiotic behavior, only two progenies are promising for advancement in the breeding program.
Keywords: *Brachiaria*, microsporogenesis, breeding, pastures, apomixis

Introduction

The extensive use of a few *Brachiaria* cultivars in the last three decades has placed Brazil as the major beef cattle exporter and the second largest beef producer in the world. However, there is a great demand for new cultivars to diversify the millions of hectares of *Brachiaria* pastures, and these must combine desirable agronomic characteristics such as those found in the two most widely used cultivars in the country: (i) the high production, nutritive value and resistance to spittlebugs (Homoptera: Cercopidae) as found in *B. brizantha* cv. Marandu, and (ii) the tolerance to acid soils as found in *B. decumbens* cv. Basilisk (Miles et al., 2004).

Obtaining spittlebug resistance was the major incentive for initiating *Brachiaria* breeding programs in America. Since no compatible sexual genotypes have been found within *B. brizantha* or *B. decumbens*, such combination of characters is possible only through interspecific hybridization. In the 1980s, the Embrapa Beef Cattle Center initiated a program of hybridization to better exploit the genetic variability found in some *Brachiaria* accessions. Interspecific hybridization is difficult to accomplish due to differences in ploidy levels and genetic affinity (Valle and Savidan, 1996; Valle and Pagliarini, 2009). Compatibility has been found among some species of Group 5 (Renvoize et al., 1996): *B. brizantha, B. decumbens* and *B. ruziziensis,* which form an agamic complex and produce fertile hybrids (Valle and Savidan, 1996; Valle and Milles, 2001).

Brachiaria breeding is a recent endeavor. Polyploid accessions with desirable agronomic characteristics are apomictic (Valle and Savidan, 1996), and sexuality is found only in diploid accessions. Ferguson and Crowder (1974) attempted interploidy crosses to no avail and thus suggested doubling the chromosome number of sexual diploids to create a cross-compatible sexual tetraploid plant. This was accomplished a decade latter, in Belgium, when diploid accessions of *B. ruziziensis* were tetraploidized with colchicine (Gobbe et al., 1981; Swenne et al., 1981). These accessions continue to be the basis of the interspecific hybridization program both in CIAT (Miles et al., 2004) and in Brazil (Valle et al., 2009). Interspecific hybridization in *Brachiaria* was successfully accomplished between tetraploid accessions of *B. ruziziensis* × *B. brizantha* or *B. decumbens.* Attempts to cross sexual *B. decumbens* × *B. brizantha* were recently performed (Souza-Kaneshima et al., 2010). The single diploid accession of *B. brizantha* (Pinheiro et al., 2000) and some diploid accessions of *B. decumbens* (Simioni and Valle, 2009) were artificially tetraploidized by colchicine, creating new opportunities to recombine the genetic variability existing in each species.

The Brazilian program of interspecific hybridization in *Brachiaria* was initiated in 1988, when over 200 hybrids resulted from crosses between *B. ruziziensis* × *B. brizantha* or *B. decumbens.* Several sexual and apomictic hybrids, mainly those resulting from *B. ruziziensis* × *B. brizantha*, presented desirable traits such as resistance to spittlebugs, high nutritive value, and tolerance to acid soils. The sexual hybrids are maintained in an open polycross block, whereas the apomictic are under agronomic evaluation. In the genus *Brachiaria*, apomixis is pseudogamic, which means that viable male gametes are necessary to fertilize the secondary nucleus of the embryo sac to guarantee endosperm development to produce viable seeds (Valle and Savidan, 1996). However, polyploidy in *Brachiaria* causes abnormal meioses (Mendes-Bonato et al., 2002ab, 2006; Utsunomiya et al., 2005, Risso-Pascotto et al., 2006). Previous cytogenetic analyses of F_1 hybrids

of *B. ruziziensis* × *B. brizantha* hybrids (Risso-Pascotto et al., 2005a; Fuzinatto et al., 2007a, 2008, Adamowski et al., 2008) have revealed different types and frequencies of meiotic abnormalities, indicating that selection for meiotic stability is possible among them. We report herein an analysis of microsporogenesis in six apomictic progenies of a cross between a sexual tetraploidized accession of *B. ruziziensis* and a natural tetraploid of *B. brizantha*. Our goal was to select meiotically stable progenies for inclusion in our *Brachiaria* breeding program.

Materials and Methods

Hybrids (F_1 generation) were obtained in Campo Grande, state of Mato Grosso do Sul, Brazil (20°25′ S, 54°46′ W, 526 m a.s.l.), by crossing two accessions (R038 and B140) that exhibited desirable characters. R038 is an artificial tetraploid (2n = 4x = 36) that maintained sexuality after chromosome doubling in Belgium, and B140, the pollen donor, is a natural apomictic tetraploid (2n = 4x = 36) with high productivity and nutritive value. The latter is undergoing animal performance trials to be released as a new cultivar. Both accessions, the original diploid of *B. ruziziensis* (Zimbabwe, Africa, 20°28′ S, 55°40′ E, 520 m a.s.l) and B140 of *B. brizantha* (Rwanda, Africa, 20°26′ S, 54°90′ E, 1,030 m a.s.l.) were collected in Africa in 1984-85. Hybrids were planted in the field, and mode of reproduction was previously determined by examination of embryo sacs using interference contrast microscopy of methyl salicylate-cleared ovaries (Young et al., 1979). Among the obtained sexual and apomictic progenies, the six apomictic ones analyzed in the present paper (P05, P06, P07, P013, P014, and P020) were selected for use in our breeding program based on vigor and leafiness.

Inflorescences for meiotic studies were collected in each field-grown plant and fixed in a mixture of 95 % ethanol, chloroform and propionic acid (6:3:2) for 24 h, transferred to 70 % alcohol and stored under refrigeration until use. Microsporocytes were prepared by squashing and staining with 0.5 % propionic carmine. Meiotic abnormalities were observed in each phase of microsporogenesis. The number of cells analyzed per plant ranged from 958 to 1,717, according to the availability of inflorescences collected in each progeny. Photomicrographs were made with a Wild Leitz microscope using Kodak Imagelink – HQ, ISO 25 black and white film.

Results and Discussion

In the progenies here analyzed, bivalents predominated (Figure 1A), especially in P020, in which multivalents were rare. A maximum of three quadrivalents were observed in the other progenies. In plant breeding, the success of gene introgression via sexual hybridization depends on the phylogenetic relationships between species, opportunities for genetic recombination, and stability of the introgressed genes (Marfil et al., 2006). Among domesticated plants, cytological analyses are usually performed to evaluate the meiotic process in experimental hybrids. In *Brachiaria*, some studies were conducted in sexual and apomictic hybrids resulting from crosses between *B. ruziziensis* × *B. brizantha* or *B. decumbens* (Risso-Pascotto et al., 2005a; Fuzinatto et al, 2007a; Adamowski et al., 2008; Felismino et al., 2010). Although these species belong to the same taxomonic group (Renvoize et al., 1996), the frequency of genetic recombination varies among them. A high frequency of multivalent chromosome association was found in three-way hybrids (Adamowski et al., 2008). One to three quadrivalents were found in the F_1 generations (Risso-Pascotto et al., 2005a; Fuzinatto et al., 2007a). The degree of genetic divergence in the hybrids is provided by chromosome pairing between the two genomes (Sundberg and Glimelius, 1991). A high pairing affinity of chromosomes indicates that interchange between gene pools of the genitors is possible (Zwierzykowski et al., 1999). Thus, a gene introgression can be expected among these progenies. Although the hybrids analyzed in the genus *Brachiaria* resulted from *B. ruziziensis* × *B. brizantha*, genetic affinity in the genus *Brachiaria* has shown to be genome-specific (Risso-Pascotto et al., 2005a; Fuzinatto et al., 2007a; Adamowski et al., 2008; Felismino et al., 2010).

The degree of genetic divergence between genitors in a hybrid can be estimated not only by chromosome pairing, but also by the frequency of meiotic abnormalities (Rieseberg et al., 2000). In the six progenies, the mean percentage of abnormalities ranged from 16.6 % to 85.6 % (Table 1). Except for P020, all progenies presented irregular chromosome segregation, a typical abnormality of polyploids and widely reported in tetraploid *Brachiaria* hybrids (Risso-Pascotto et al., 2005a; Fuzinatto et al., 2007a; Adamowski et al., 2008; Felismino et al., 2010). Irregular chromosome segregation was characterized by precocious chromosome migration to the poles (Figure 1B), laggards (Figure 1C), and micronuclei (Figure 1D) in both meiotic divisions. Micronuclei remained in the microspores (Figure 1E, F) or were isolated in microcytes after abnormal cytokinesis (Figure 1E). In such cases, unbalanced microspores would be formed. The absence of irregular chromosome segregation in P020, coupled with the low frequency of the observed quadrivalent formation, suggest that the segregant parental genomes had low affinity, and the chromosomes associated preferentially as bivalents.

Two other meiotic abnormalities were recorded in some progenies: divergent spindles (P05, P07, and P020) and desynapsis (P05). Several putative meiotic mutations have been reported among *Brachiaria* accessions (Mendes-Bonato et al., 2002b, 2003, 2007; Risso-Pascotto et al., 2003, 2005b; Mendes-Vieira et al., 2005; Boldrini et al. 2006; Gallo et al., 2007; Adamowski et al., 2007; Calisto et al., 2008) and in hybrids (Mendes-Bonato et al., 2004, 2006b; Risso-Pascotto et al., 2005a; Fuzinatto et al., 2007ab; Adamowski et al., 2008; Felismino et al., 2008, 2010). These putative mutations are frequently be-

Table 1 – Meiotic abnormalities and percentage of abnormal cells in apomictic progeny of *Brachiaria* hybrids.

Phase	Abnormalities	P05		P06		P07		P013		P014		P020	
		No. of cells	Abn** cells	No. of cells	Abn. cells	No. of cells	Abn. cells	No. of cells	Abn. cells	No. of cells	Abn. cells	No. of cells	Abn. cells
			%		%		%		%		%		%
MI*	Precocious migration		13.5		18.5		14.3		24.2		26.2		-
	Desynapsis		10.9		-		-		-		-		-
	Divergent spindle		28.5		-		22.6		-		-		13.6
	Multiple spindle	193	17.6	168	-	168	-	182	-	237	-	206	-
AI	Laggards		23.8		34.3		29.9		54.3		27.7		-
	Divergent spindle		32.1		-		17.1		-		-		11.8
	Multiple spindle	84	26.2	70	-	117	-	173	-	289	-	68	-
TI	Micronuclei	186	84.4	215	13.0	198	39.9	176	39.8	198	26.8	211	17.6
PII	Micronuclei		72.4		13.6		4.9		29.3		14.2		-
	Abnormal cytokinesis	76	-	162	-	184	58.1	123	-	190	-	152	67.1
MII	Precocious migration	245	2.9		-		-		42.5		14.3		-
	Multiple spindle		87.8		-		-		-		-		-
	Abnormal cytokinesis		-	81	-	131	87.8	40	-	259	-	120	64.2
AII	Laggards		5.9		20.0		-		44.4		22.3		-
	Multiple spindle		77.9		-		-		-		-		-
	Abnormal cytokinesis	68	-	45	-	13	7.7	18	-	94	-	19	21.1
TII	Laggards		3.18		9.9		-		45.2		26.2		-
	Multiple spindle		91.7		-		-		-		-		-
	Abnormal cytokinesis	157	-	81	-	92	-	42	-	183	-	121	58.7
Tetr	Micronuclei		5.9		6.5		9.9		55.7		28.5		10.4
	Microcytes		3.3		4.3		21.6		37.1		9.7		6.7
	Polyads	454	80.0	185	13.5	352	36.1	70	45.7	267	9.0	137	10.4
Total		1463	85.6	1007	16.6	1255	53.1	824	46.7	1717	26.4	1034	33.2

*MI: metphase I; AI: anaphase I; TI: telophase I; PII: Prophase II; MII: metaphase II; AII: anaphase II; TI : telophase II; Tet: tetrad. **Abn: Abnormal.

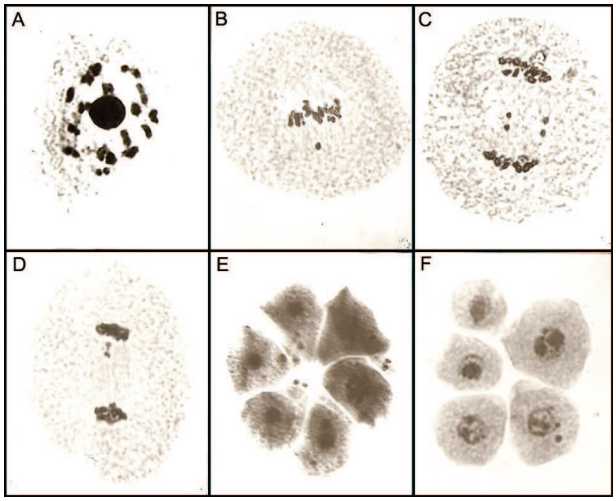

Figure 1 – Aspects of irregular chromosome segregation observed in the *Brachiaria* hybrid progenies. A) Diakinesis with 18 bivalents. B) Metaphase I with precocious chromosome migration to the pole. C) Anaphase I with laggards. D) Early telophase I with micronuclei. E) Polyad with microcytes. F) Pentad with micronucleus in one microspore.

ing expressed in different genotypes of *Brachiaria* suggesting that these genes are present in the gene pool of this genus. One of these genes is the 'divergent spindle' (*dv*), reported in another *Brachiaria* hybrid resulting from a different cross between *B. ruziziensis* × *B. brizantha* (Mendes-Bonato et al, 2006b; Felismino et al., 2008). This gene affects spindle organization such that chromosomes do not converge to focused poles. In the mutants, bivalents are distantly spread over a large metaphase plate, and they fail to converge.

Depending on the distance between chromosomes at the poles, telophase I nuclei are elongated or the chromosomes are grouped into several micronuclei of different sizes in each pole (Figure 2A, B). In the presence of micronuclei, an abnormal cytokinesis occurs dividing the cytoplasm into three, four or more cells, according to the micronuclei number (Figure 2B, C, D). In each cell, the second division progresses normally (Figure 2C, D, E), generating polyads (Figure 2F). The percentage of cells expressing the divergent spindle phenotype was variable among progenies. Variations in penetrance and expressivity of *dv* were reported in maize and *Brachiaria* (Golubovskaya and Mashnenkov, 1981; Staiger and Cande, 1990; Shamina et al., 2000; Mendes-Bonato et al., 2006b; Felismino et al., 2008). This gene leads to genome fractionation, causing pollen sterility.

The other meiotic abnormality (desynapsis) was recorded only in P05. This abnormality was reported in one accession of *B. humidicola* (Calisto et al., 2008) and was correlated with abnormal cytokinesis. In P05, the expres-

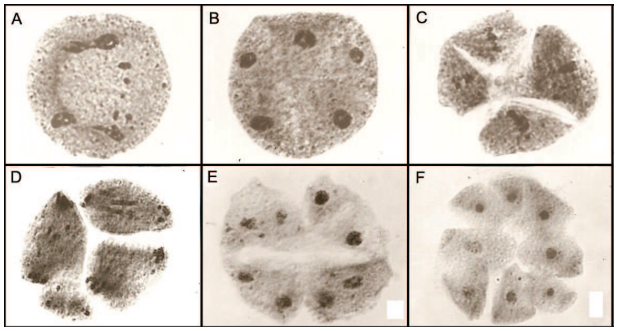

Figure 2 – Aspects of divergent spindle expression in the *Brachiaria* hybrid progenies. A) Telophase I with several micronuclei. B) Telophase I with five nuclei. C) Metaphase II after abnormal cytokinesis. D) Anaphase II. E) Telophase II. F) Octad of microspores.

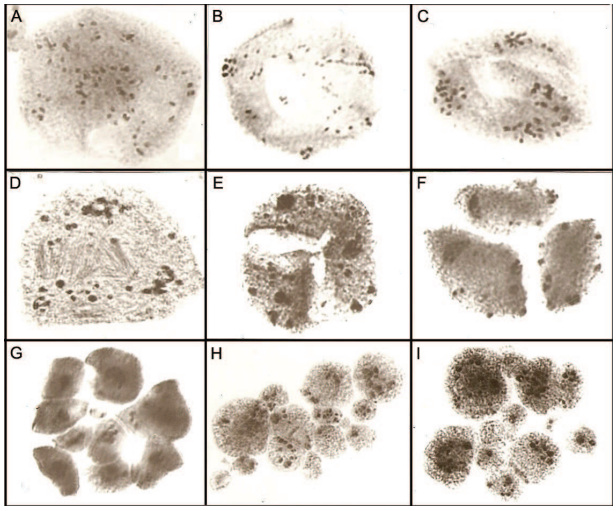

Figure 3 – Aspects of desynapsis in the progeny P05. A, B) Microsporocyte with 72 chromatids. C) Early telophase I with some groups of chromatids. D) Telophase I with several micronuclei of different sizes. E, F) Meiocytes in the second division with several micronuclei. G) Polyad of microspores. H, I) Pollen grains of different sizes resulted from the abnormal division.

sivity was different from the former. Chromosomes were associated in bi- or quadrivalents in diakinesis, but did not congregate at the metaphase plate. Chromosomes and chromatids separated precociously, so that 72 units could be counted (Figure 3A, B). Anaphase I did not occur, and the chromatids were grouped (Figure 3C) into several micronuclei of different sizes (Figure 3D). After this phase, abnormal cytokinesis occurred, dividing the cytoplasm into several cells with micronuclei (Figure 3E, F). After a new abnormal cytokinesis, polyads with unequal microspores (Figure 3G) generated meiotic products with pollen grains of different sizes (Figure 3H, I). Desynapsis is frequently reported in higher plants (Koduru and Rao, 1981). A similar phenotype was reported in *Vaccinium darrowi* (Qu and Vorsa, 1999). The direct consequences of mutations affecting synapsis are the production of abnormal gametes which are generally unable to function because of the unbalanced number of chromosomes.

Irregular chromosome segregation, desynapsis, and divergent spindles (leading to multiple spindle formation and abnormal cytokinesis) caused, to varying degrees among progenies, genome fractionation and pollen inviability. In the *Brachiaria* hybridization program, desirable combinations of agronomic characters and high seed set are required to meet the Brazilian demand for extensive pasture establishment, pasture renovation and export. Apomictic hybrids with putative mutations, such as divergent spindle and desynapsis, must be discarded from the breeding program to avoid compromising future generations. From our cytological analyses, P06 and P014, with a mean of 16.6 % and 26.4 % of cells with meiotic abnormalities, respectively, should remain in the breeding program. In these lines, abnormalities are generally limited to a low frequency of irregular chromosome segregation. Hence, genetic recombination of desirable genes is expected. Our cytogenetic studies of interspecific hybrids attest to their value in breeding programs. Time and effort can be saved when meiotic abnormalities are discovered early, and genotypes with potential cytogenetic problems are discarded.

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