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Microsporogenesis in sexual *Brachiaria* hybrids (Poaceae)

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ABSTRACT. Three sexual interspecific hybrids of *Brachiaria* (HBGC076, HBGC009, and HBGC014) resulting from crosses between *B. ruziziensis* (female genitor) and *B. decumbens* and *B. brizantha* (male genitors) produced by Embrapa Beef Cattle in the 1980s were cytologically analyzed by conventional methods for meiotic studies. The cytogenetic analysis showed the occurrence of common meiotic abnormalities among them. The most frequent abnormalities were those related to irregular chromosome segregation due to polyploidy. Other abnormalities, such as chromosome stickiness, absence of cytokinesis, irregular cytokinesis, abnormal spindle orientation, and abnormal nucleolus disintegration, were found in the three hybrids, while, chromosome disintegration was detected only in HBGC014. All the abnormalities, except for abnormal nucleolus disintegration, can cause unbalanced gamete formation, leading to pollen sterility. Multivalent chromosome association at diakinesis revealed genome affinity between the two parental species in the hybrids, suggesting some possibility for gene introgression. Presently, the *Brachiaria* breeding program has the objective of releasing, primarily, apomictic hybrids as new cultivars since they do not segregate but preserve the genetic makeup indefinitely. Besides, they result in homogeneous pastures which are easier to manage. The sexual hybrids, however, are paramount in the breeding program: they work as 'bridges' to introgress traits of interest into the apomictic genotypes. The cytogenetic analyses of these three hybrids substantiate their maintenance in the breed-

ing program due to low frequency of meiotic abnormalities, complemented by interesting agronomic traits. They may be used in crosses to generate new cultivars in the future.

Key words: *Brachiaria*, Breeding program, Microsporogenesis, Sexual hybrids

INTRODUCTION

The forage potential of *Brachiaria* was first recognized in Australia about 50 years ago. The major impact of the genus, however, was realized only in the past three decades, when some *Brachiaria* cultivars, derived from natural occurring germplasm, were widely sown in tropical America (Miles and Valle, 1996). Current estimates of the acreage covered by *Brachiaria* pastures in Brazil range from 50 to 70 million hectares. The rapid expansion of acreage did not occur without problems owing to severe limitations of the two most widely grown cultivars: *B. brizantha* cv. Marandu requires high soil fertility and good drainage, and *B. decumbens* cv. Basilisk lacks resistance to spittlebugs (Miles et al., 2004).

Pastures of *Brachiaria* allowed Brazil to become the second largest beef producer in the world in the last two decades. The high vulnerability associated with the wide monoculture of the few planted cultivars in the Brazilian savannas, however, has worried farmers. An extensive breeding program based on intra- and interspecific hybridizations is in progress at the Embrapa Beef Cattle Research Center since 1988 aiming to combine genes of interest from *B. brizantha* or *B. decumbens* using sexual *B. ruziziensis* as the bridge species. Hybridization in the genus *Brachiaria* is not easy due to ploidy differences among accessions and species, and to reproduction by aposporic apomixis. Most accessions in the genus are polyploid, mainly tetraploid (Valle and Savidan, 1996; Penteado et al., 2000; Utsunomiya et al., 2005; Mendes-Bonato et al., 2002a, 2006; Risso-Pascotto et al., 2006) and apomictic (Valle and Savidan, 1996). Sexuality is found among diploids, or occasionally tetraploids as in *B. humidicola* (Valle and Savidan, 1996). Accessions with the same ploidy level are required in hybridization to overcome the sterility barrier. *B. ruziziensis* genotypes have vigorous growth, high nutritive value and are excellent seeders, but all natural accessions are diploid (2n = 2x = 18) and behave as obligate sexual plants. On the other hand, the best accessions for hybridization of *B. brizantha* and *B. decumbens* are tetraploid and apomictic. Crosses only became possible after some sexual diploid accessions of *B. ruziziensis* were artificially tetraploidized by colchicine (Swenne et al., 1981; Gobbe et al., 1981). Since then, one diploid accession of *B. brizantha* (Pinheiro et al., 2000) and one of *B. decumbens* (Simioni C, personal communication, 2007) have also been tetraploidized and can now be used in crosses.

Hybridization between tetraploid sexual accessions and apomictic *B. brizantha* or *B. decumbens* has produced progenies that segregate 1:1 for sexuality:apomixis (Valle and Savidan, 1996). The *Brachiaria* breeding program underway at Embrapa Beef Cattle seeks, preferentially, superior apomictic hybrids in which characters of interest are permanently fixed by apomixis. Sexual hybrids with high performance, however, are paramount in the program since it is on the sexual population that genes of importance need to be pyramidized and later introgressed into the apomictic elite genotypes. Superior sexual hybrids remain in the program, in an open polycross

block to generate new genetic variability by genetic recombination during meiosis. Polyploidy, in general, affects the meiotic process, compromising pollen viability. Thus, hybrids can only act as genitors in polycross blocks if they show regular meiotic behavior that ensure male and female gamete viability. This paper reports the meiotic behavior in three promising sexual hybrids between *B. ruziziensis* and *B. brizantha* or *B. decumbens* developed by Embrapa Beef Cattle.

MATERIAL AND METHODS

Three tetraploid sexual hybrids (HBGC009, HBGC014, and HBGC076) were analyzed. They were produced by controlled pollination at the Embrapa Beef Cattle Research Center (Campo Grande, MS, Brazil) and are under agronomic evaluation. They resulted from crosses using two artificially tetraploidized sexual accessions (R30 and R50) of *B. ruziziensis* and pollen of the two most important cultivars of *Brachiaria* used in the tropics: *B. brizantha* cv. Marandu and *B. decumbens* cv. Basilisk, natural tetrapolid apomictic accessions. Two hybrids (HBGC009 and HBGC014) are full-sibs. Table 1 shows the genitors in the crosses. The diploid parental accessions of the female genitor *B. ruziziensis* were collected in the African savannas and artificially tetraploidized in Belgium in the early 1980s by Swenne et al. (1981) and Gobbe et al. (1981). The male genitors are derived from natural tetraploid ecotypes, also of African origin. *B. brizantha* cv. Marandu was released in 1984 in Brazil by Embrapa derived from original accessions introduced from the Zimbabwe Grasslands Station Marandella. *B. decumbens* cv. Basilisk is derived from the Ugandan Department of Agriculture in 1930. It was introduced in Brazil in 1965.

Table 1. Hybrids and their genitors.

Hybrid	Female genitor	Male genitor	Hybrid plant number
HBGC076	*B. ruziziensis* - R50	*B. brizantha* cv. Marandu	65
HBGC009	*B. ruziziensis* - R30	*B. decumbens* cv. Basilisk	24
HBGC014	*B. ruziziensis* - R30	*B. decumbens* cv. Basilisk	31

Inflorescences of each hybrid for meiotic studies were collected from free-growing plants in the field, and fixed in a mixture of ethanol, chloroform and propionic acid (6:3:2, v/v) for 24 h; they were then transferred to alcohol and stored under refrigeration until use. Microsporocytes, prepared by squashing, were stained with 0.5% propionic carmine. Over 1500 microsporocytes were analyzed in each hybrid. Images were photographed with Kodak Imagelink - HQ, ISO 25 black and white film.

RESULTS AND DISCUSSION

The three sexual hybrids showed variable frequency of meiotic abnormalities (Table 2). In diakinesis, one (Figure 1a) to four quadrivalents were found in the meiocytes, with a predominance of one quadrivalent. Precocious chromosome migration to the poles in metaphase (Figure 1b and f), laggards in anaphase (Figure 1c and g), leading to micronuclei formation in telophase (Figure 1d and h) and in prophase II (Figure 1e) occurred in all hybrids. The fate of micronuclei varied among hybrids. In HBGC076 there was a large predominance of tetrad with micronuclei (Figure 1i) in relation to tetrads with one or more microcytes (Figure 1j to n). However, in HBGC009 there was a predominance of tetrads with microcytes.

Table 2. Meiotic abnormalities in the three sexual hybrids of *Brachiaria.*

Phase	Abnormalities	HBGC076		HBGC009		HBGC014	
		No. of PMCs analyzed	No. of abnormal PMCs (%)	No. of PMCs analyzed	No. of abnormal PMCs (%)	No. of PMCs analyzed	No. of abnormal PMCs (%)
Metaphase I	Precocious chromosome migration	449	63%	215	36%	231	35%
	Abnormal nucleolar disintegration		70%		14%		13%
Anaphase I	Laggard chromosomes	178	117%	97	22%	111	43%
	Abnormal nucleolar disintegration		5%		6%		11%
	Chromosome stickiness		6%		38%		6%
Telophase I	Micronuclei	231	62%	217	30%	220	12%
	Abnormal nucleolar disintegration		111%		57%		101%
	Chromosome stickiness		11%		31%		12%
Prophase II	Micronuclei	178	15%	244	58%	268	21%
	Abnormal nucleolar disintegration		60%		47%		51%
	Absence of cytokinesis		7%		17%		-
	Iregular cytokinesis		6%		22%		5%
	Chromosome stickiness		18%		-		3%
	Chromosome disintegration		-		-		9%
Metaphase II	Precocious chromosome migration	209	32%	236	20%	356	52%
	Abnormal nucleolar disintegration		34%		10%		6%
	Absence of cytokinesis		11%		-		-
	Irregular cytokinesis		-		19%		11%
	Chromosome stickiness		-		-		-

Continued on next page

Table 2. Continued.

Phase	Abnormalities	HBGC076		HBGC009		HBGC014	
		No. of PMCs analyzed	No. of abnormal PMCs (%)	No. of PMCs analyzed	No. of abnormal PMCs (%)	No. of PMCs analyzed	No. of abnormal PMCs (%)
	Abnormal spindle orientation		28%		17%		-
	Chromosome disintegration		-		-		27%
Anaphase II	Laggard chromosomes	62	20%	62	27%	59	18%
	Abnormal nucleolar disintegration		6%		9%		14%
	Abnormal spindle orientation		3%		-		-
	Absence of cytokinesis		6%		-		-
	Chromosome stickiness		-		13%		6%
	Chromosome disintegration		-		-		6%
Telophase II	Micronuclei	150	10%	196	39%	365	12%
	Abnormal nucleolar disintegration		53%		36%		35%
	Absence of cytokinesis		6%		-		-
	Irregular cytokinesis		10%		11%		6%
	Chromosome stickiness		-		21%		7%
	Chromosome disintegration		-		-		213%
Tetrad	Micronuclei	293	76%	261	46%	399	85%
	Abnormal nucleolar disintegration		65%		-		-
	Microcytes		6%		81%		38%
	Monads		2%		-		-
	Binucleated dyads		3%		-		-
	Triads		5%		-		-
	Polyads		-		52%		16%
	Chromosome disintegration		-		-		4%
Total		1750	933 (53.31%)	1528	779 (50.98%)	3762	1753 (46.59%)

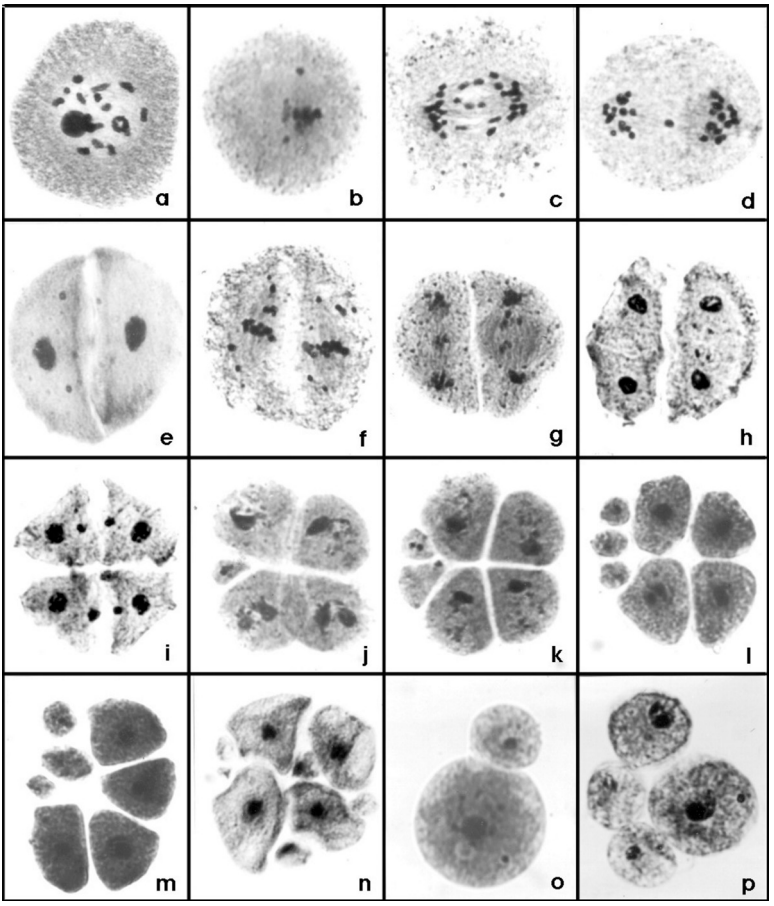

Figure 1. Meiotic abnormalities related to irregular chromosome segregation observed in the three sexual hybrids. a) Meiocyte in diakinesis showing a quadrivalent chromosome association and 16 bivalents. b) Metaphase I with precocious chromosome migration to the poles. c) Anaphase I with laggard chromosomes. d) Early telophase I with a micronucleus. e) Prophase II with micronuclei. f) Metaphase II with precocious chromosome migration to the poles. g) Anaphase II with laggard chromosomes. h) Telophase II with micronuclei. i) Tetrad with micronucleus in all microspores. j to n) Meiotic products with micronuclei eliminated as microcytes. o, p) Pollen grains of different sizes (Magnification 400X).

Irregular chromosome segregation is an abnormality typical of polyploids (Sybenga, 1992; Singh, 1993). The occurrence of various and identical genomes in the same nucleus predisposes to multiple chromosome associations, leading to irregular chromosome segregation. This type of occurrence was widely reported in different species of *Brachiaria* (Mendes-Bonato et al., 2001a, 2002a, 2006; Risso-Pascotto et al., 2003a,b, 2005a, 2006; Mendes-Vieira et al., 2005; Utsunomiya et al., 2005). The meiotic fate of micronuclei found in these hybrids, either remaining as micronuclei in the tetrads or originating microcytes, seems to be genotype specific because it varies among accessions (Mendes-Bonato et al., 2001a, 2002a,b, 2006; Junqueira Filho, 2003; Risso-Pascotto et al., 2003a,b, 2006; Utsunomiya et al., 2005). In any way, whatever its fate, the result will be the loss of chromosomes and the consequent generation of unbalanced gametes with different sizes (Figure 1o and p), leading to pollen sterility.

Other meiotic abnormalities, such as absence of the first or second cytokinesis (Figure 2a to c) or irregular cytokinesis (Figure 2d to l), were found in the three hybrids.

These abnormalities have been widely reported in the *Brachiaria* genus. Absence of the first or the second cytokinesis, or both, led to dyad, triad or monad formation. Dyads and triads gave rise to binucleated microspores or 2n gamete formation after restitution nucleus occurrence. Both cases have been reported for *Brachiaria* (Risso-Pascotto et al., 2003a, 2006). In the sexual hybrids under analysis, restitutional nucleus did not occur and dyads were always binucleated. Abnormal cytokinesis leading to cellularization, as observed in several meiocytes (Figure 2), was also widely reported in other *Brachiaria* species (Mendes-Bonato et al., 2002b; Risso-Pascotto et al., 2003a, 2005b,c; Utsunomiya et al., 2005). Irregular cytokinesis is caused by irregular chromosome segregation in the first division caused by the spread of the genome in the cytoplasm. According to Brown and Lemmon (1992), the metaphase plate position determines the plane of cytokinesis and the number of cells that will be formed. The result of cellularization is the formation of polyads with different number and sizes of microspores and microcytes (Figure 2m to p), fractionating the genome into small parts and causing pollen sterility. Multiple spindles were also recorded in those meiocytes where the first cytokinesis did not occur.

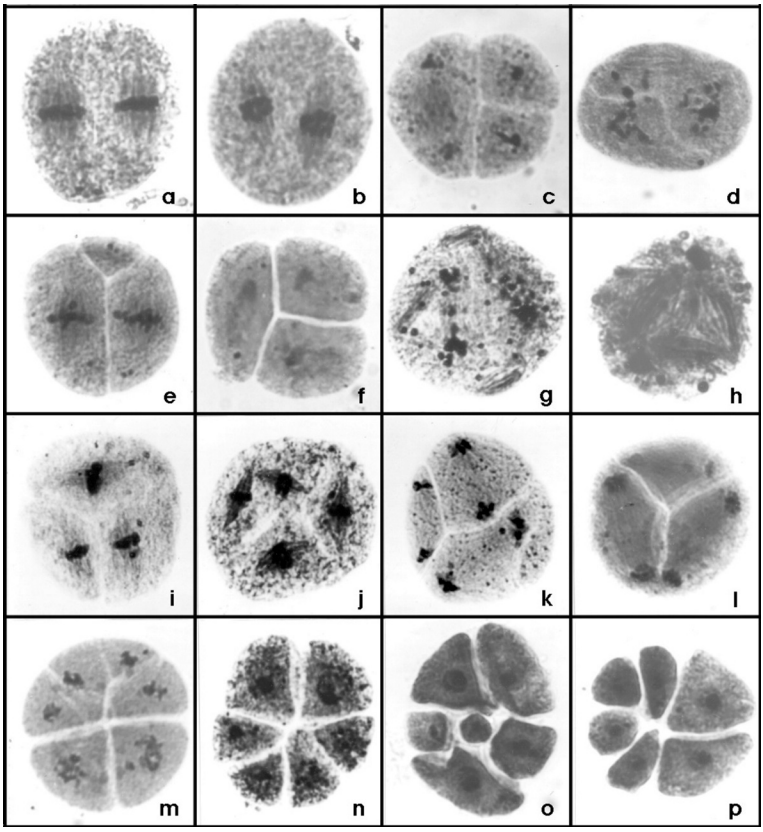

Figure 2. Meiotic abnormalities related to abnormal cytokinesis observed in the three hybrids. a to c) Absence of first and second cytokinesis observed in HBGC076. Metaphases I with normal (a) and perpendicular (b) spindle with absence of the first cytokinesis, and telophase II (c) with absence of the second cytokinesis in one cell forming a triad. d) Prophase II with irregular pattern of cytokinesis. e, f) Metaphase II with irregular cytokinesis dividing the meiocyte into three unequal (e) and equal (f) cells. g, h) Meiocytes with multiple and multipolar spindles. i, j) Metaphases II with three (i) and four (j) multipolar spindles and irregular cytokinesis. k, l) Early (k) and late telophase II with irregular cytokinesis. m, n) Early (m) and late (n) tetrad with irregular cytokinesis. o, p) Hexads resulting from irregular cytokinesis (Magnification 400X).

Chromosome stickiness was also recorded in the three hybrids (Figure 3a to d). This abnormality clumps the chromatin, impairing chromosome segregation in both meiotic divisions, and sometimes after the completion of meiosis. It has been widely reported in the genus *Brachiaria* (Mendes-Bonato et al., 2001a,b; Utsunomiya et al., 2005; Risso-Pascotto et al., 2005a). Although described in several genera of plants for more than a century, adequate explanations of its origin are still lacking. Pollen grains produced by cells with meiotic stickiness are generally non-viable because they are genetically unbalanced due to chromosome fragmentation (Golubovskaya, 1979, 1989).

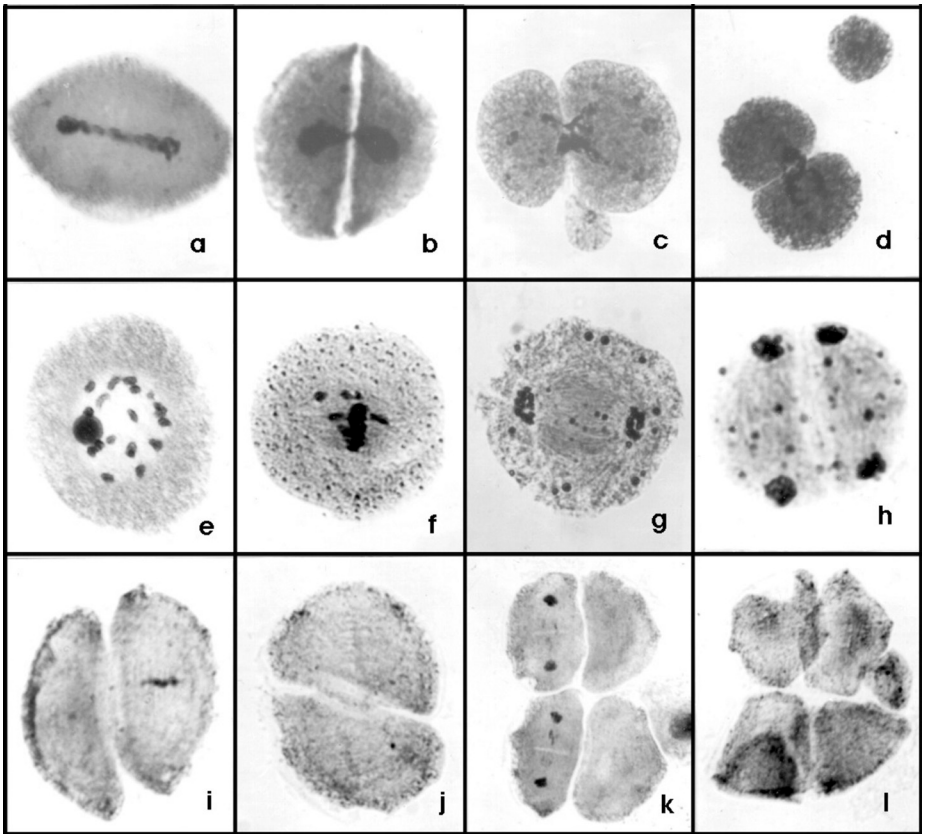

Figure 3. Other meiotic abnormalities observed in the sexual hybrids. a to d) Chromosome stickiness detected in the three hybrids: a) telophase I showing a thick bridge; b) prophase II with a thin bridge connecting the cells; c, d) released microspores connected by thick bridge (observe also micronuclei and a lateral microcyte in c). e to h) Nucleolus behavior observed in the three hybrids: e) diakinesis with a normal nucleolus; f) metaphase I with abnormal nucleolus disintegration into small nucleolar fragments; g, h) telophase I (g) and II (h) with large nucleolar fragments. i to l) Aspects of chromosome disintegration in the second division in HBGC014: i) metaphase II with chromosomes only in one cell; j) metaphase II showing chromosome disintegration in both cells. k) Two telophases II with chromosome disintegration in one cell; l) tetrad of micropores, with a microcyte, showing total absence of chromatin (Magnification 400X).

Abnormal nucleolus disintegration was also detected in these hybrids (Figure 3). Nucleolus behavior was normal in prophase I until diakinesis, when it was visualized as a dense sphere (Figure 3e). After this phase, it was disorganized into several micronucleoli in metaphase I (Figure 3f) which were progressively rejoined from anaphase I to telophase I, forming a few and dense small nucleoli dispersed in the cytoplasm (Figure 3g). In

the second division, the nucleolus behavior was the same (Figure 3h) and micronucleoli remained outside the nucleus in the microspores of the tetrad. Abnormal nucleolus disintegration was recorded in one accession of *B. decumbens* (Risso-Pascotto et al., 2002), but with a striking difference: in that accession, in telophases I and II, the fragments of the nucleolus were rejoined into one normal nucleolus inside the nucleus. The nucleolus is a nuclear structure involved in the synthesis and processing of rRNA and thus, fundamental to the synthesis of protein. In the present hybrids, as the fragments did not rejoin into one single nucleolus in the nucleus after completion of meiosis, microspore viability and pollen fertility could be severely affected.

In one of the hybrids (HBGC014), an abnormality never reported before, either in *Brachiaria* or other plant species, was recorded: chromosomes of one or of both cells disintegrated (Figure 3i to l) in the second division. The cause of this phenomenon was not known, but it certainly affected pollen viability. In the genus *Brachiaria*, a number of mutants have been described affecting different steps of meiosis (Junqueira Filho et al., 2003; Mendes-Bonato et al., 2001b, 2004; Risso-Pascotto et al., 2003b, 2005b,c). In this case, chromosome disintegration could represent a new putative mutation affecting meiosis.

The transfer of genes between species of the same genus has played an important role in crop improvement (Goodman et al., 1987). The *Brachiaria* breeding program aims to introgress some desirable genes of *B. brizantha* and *B. decumbens* into *B. ruziziensis* and vice versa. The success of hybridization depends on genome compatibility. The degree of genetic divergence in the hybrid is provided by chromosome pairing between the two genomes (Sundberg and Glimelius, 1991). Gene transfer is assisted by homologous or homeologous recombination at meiosis. A high pairing affinity of chromosomes indicates that interchange between gene pools of the two genitors is possible (Zwierzykowski et al., 1999). The presence of one to four quadrivalents in the present sexual hybrids suggests that gene introgression from *B. brizantha* and *B. decumbens* into *B. ruziziensis* can be expected. Similar results were reported by Risso-Pascotto et al. (2005a).

For a hybrid to become a cultivar, it is not enough to ensure gene introgression. *Brachiaria* cultivars are successful not only for producing large amounts of quality forage and good animal performance, but also because of their abundant seed production, which ensures adoption and widespread utilization. Thus, a regular meiosis is indispensable to ensure pollen fertility and guarantee proper development of the endosperm, since apomixis in the genus *Brachiaria* is pseudogamic (Valle and Savidan, 1996).

According to Rieseberg et al. (2000), the degree of genetic divergence between genitor species in a hybrid can be estimated not only by chromosome pairing, but also by the frequency of meiotic abnormalities. In the three sexual hybrids, the percentage of meiotic abnormalities was 53.31% in HBGC076, 50.98% in HBGC009, and 46.59% in HBGC014. Therefore, there is considerable genetic divergence between the three species but not so much as to prevent hybridization. Considering that half of the gametes in these three sexual hybrids could be fertile and that cycles of selection in the crossing block tend to improve seed set by selecting against meiotic abnormalities, these hybrids can be kept in the polycross block at Embrapa Beef Cattle. They have been selected for good agronomic traits such as leafiness, vigor, dry matter production of good nutritive value to animals and should contribute with their genetic makeup to future cultivars.

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