

## 2n Gamete formation in the genus *Brachiaria* (Poaceae: Paniceae)

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**Abstract** Microsporogenesis of several *Brachiaria* species of the Brazilian collection at Embrapa Beef Cattle has been analyzed in detail. This paper reports abnormal cytokinesis in three accessions of three different species (*Brachiaria humidicola*,  $2n = 4x = 36$ , *Brachiaria decumbens*,  $2n = 4x = 36$ , and *Brachiaria dura*,  $2n = 6x = 54$ ). Chromosomes paired in bi-, tri-, and quadrivalents in these accessions, whereas chromosome segregation at meiosis I was characterized by exclusion of laggards as micronuclei. In a high number of meiocytes, the first sign of cytokinesis appeared only in metaphase II and did not divide the meiocyte into a dyad. Total absence of cytokinesis was also detected among meiocytes in the second division. Since in both cases the two metaphase plates were very close, they favored the rejoining of chromosome sets after anaphase II and formed a restitutional nucleus in telophase II. Second cytokinesis occurred after telophase II in most meiocytes. Monads, dyads, and triads with  $n$  or  $2n$  nuclei were observed among meiotic

products. The  $2n$  gametes observed correspond to the first division restitution (FDR). The number of affected cells in each accession was variable, but the number of microspores with restitutional nucleus, including those scored in tetrads and the released ones, did not exceed 9%. Although polyploidy is common in the genus *Brachiaria*, its origin is still unclear. Current results suggest that  $2n$  gametes may have contributed to the evolutionary history of the genus.

**Keywords** *Brachiaria* · Microsporogenesis · Abnormal cytokinesis · Restitutional nucleus ·  $2n$  gamete · Polyploidization

### Introduction

Polyploidy is a key element in the evolution of higher plants and leads toward the formation of new species. Most flowering species have evolved through one or more rounds of polyploidization, either by the doubling of their chromosome number (autopolyploidy) or by combining chromosome sets from distinct-related species (allopolyploidy). Although this behavior suggests that polyploidy must confer some selective advantage (Osborn 2004), the evolutionary success of polyploidy relies on the ability of polyploid individuals to reproduce and transmit their genes to subsequent generations (Pannell et al. 2004).

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Despite being 90 years since the discovery of polyploidy, the percentage of the angiosperm that is polyploidy is unknown. Estimates among angiosperms range from a liberal 70–80% to a conservative 30% (Bennett 2004).

Polyploidization may be asexual through somatic chromosome doubling and sexual through the formation of  $2n$  gametes. The first assumption was seriously questioned by Harlan and De Wet (1975) since it has been shown that  $2n$  gametes are widespread in plants (Harlan and De Wet 1975; Veilleux 1985; Bretagnolle and Thompson 1995). Actually,  $2n$  gametes are considered to be the dominant process involved in the origin of polyploidy in plants. The  $2n$  gametes are the result of modified meiosis affecting specific stages of micro- and megasporogenesis, which leads to the formation of restitutional nucleus. Several mechanisms involved in meiotic nuclear restitution have been reported in numerous plant species (see Ramanna 1979; Veilleux 1985; Bretagnolle and Thompson 1995).

During an extensive study on cytogenetic behavior in several *Brachiaria* species from a Brazilian collection at Embrapa Beef Cattle (Campo Grande, MS, Brazil),  $2n$  gamete was recorded in three of the six different species studied (*Brachiaria humidicola*, *Brachiaria decumbens*, and *Brachiaria dura*). The mechanism responsible for its formation is discussed in this paper.

## Materials and methods

Accessions of *Brachiaria brizantha*, *Brachiaria jubata*, *Brachiaria nigropedata*, *B. humidicola*, *B. decumbens*, and *B. dura* from the Embrapa Beef Cattle collection, were cytologically analyzed. Three accessions belonging to three species, *B. humidicola* (H047,  $2n = 4x = 36$ ), *B. decumbens* (D076,  $2n = 4x = 36$ ), and *B. dura* (Du001,  $2n = 6x = 54$ ) revealed abnormalities worth discussing and were studied in detail.

All accessions were previously collected in the wild African savannas in the mid-1980s by the Centro Nacional de Agricultura Tropical (CIAT, Colombia) and introduced in Brazil between 1986 and 1990. Site's characteristics in Brazil are:

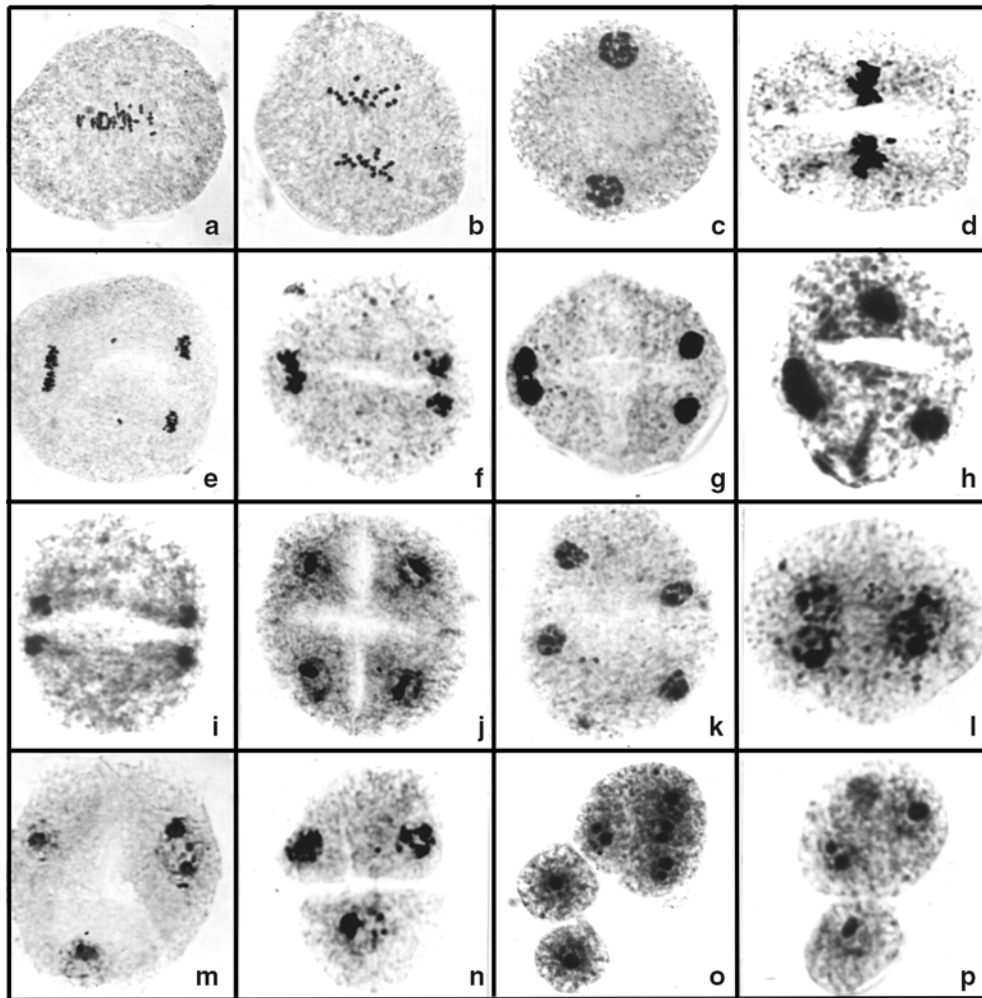
climate type Aw: tropical humid savanna; average annual precipitation = 1,526 mm; average temperature = 22°C; altitude = 520 m; latitude = 20°28'S; longitude = 55°40'W; poor Dark Red Latossol (59% sand; 8% silt; 33% clay; pH = 4.2).

Inflorescences from several plants representing each accession were collected for meiotic study and fixed in a mixture of ethanol 95%, 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. Photomicrographs were made with a Wild Leitz microscope using Kodak Imagelink—HQ, ISO 25 black and white film.

## Results and discussion

The Brazilian *Brachiaria* collection comprises of 475 accessions of 15 species, of which nearly half from the species of greater economic importance has been cytologically analyzed till now. The analyzed accessions belong to *B. brizantha* (Mendes-Bonato et al. 2002), *B. jubata* (Mendes-Bonato et al. 2006), *B. nigropedata* (Utsunomiya et al. 2005), *B. humidicola* (K. R. Boldrini unpublished data), *B. decumbens* (A. B. Mendes-Bonato unpublished data), and *B. dura* (C. Risso-Pascotto unpublished data). Among these, three accessions belonging to distinct species, *B. humidicola* (H047,  $2n = 4x = 36$ ), *B. decumbens* (D076,  $2n = 4x = 36$ ) and *B. dura* (Du001,  $2n = 6x = 54$ ), revealed total absence or an abnormal type of cytokinesis following the first or the second meiotic division. As all accessions were polyploid, characteristic polyploidy abnormalities related to irregular chromosome segregation were also found among meiocytes. However, meiosis proceeded normally in its course till telophase I (Fig. 1a–c).

*Brachiaria* is a genus of African origin widely used as forage grass in the humid and sub-humid tropical regions, especially in Brazil. As a monocotyledonous species, it shows successive cytokinesis, or rather, the first cytokinesis occurs after telophase I, dividing the cell into a dyad, followed by the second cytokinesis after telophase II,



**Fig. 1** Aspects of abnormal cytokinesis and restitutional nucleus formation in *Brachiaria*. Regular meiosis I: metaphase I (**a**), anaphase I (**b**), and telophase I (**c**). (**d**) Metaphase II with incomplete cytokinesis. The proximity of the two metaphase plates may be observed. (**e**) Anaphase II with convergent spindles in a cell with total absence of cytokinesis. Chromosomes rejoining in one pole is shown. (**f**) Early telophase II with a restitutional nucleus. (**g**) Telophase II with the formation of a restitutional

nucleus. (**h**) Telophase II with the restitutional nucleus sharing the cytoplasm with  $1n$  nucleus. (**i–j**) Different aspects of telophase II in relation to cytokinesis. (**k–l**) Telophase II with total absence of cytokinesis. (**m–n**) Triads with one restitutional nucleus in one microspore. (**o**) Tetranucleated monad and two normal  $n$  microspores. (**p**) Binucleated microspore and a normal one (magnification 400 $\times$ )

giving rise to microspore tetrads. In the accessions under study, a large number of meiocytes (Table 1) displayed the first sign of cytokinesis in metaphase II (Fig. 1d), albeit partial, and it did not divide the meiocyte into a dyad. During prophase II, cytokinesis was not evident in most cells. In metaphase II, anaphase II, and telophase II, the number of meiocytes with partial cytokinesis was much higher than those lacking it. In

both cases, the two metaphase plates were very close (Fig. 1d) favoring the rejoining of chromosome sets after anaphase II (Fig. 1e, f). Consequently, a restitutional nucleus in telophase II was formed (Fig. 1g, h). The above phenomenon was prevalent and restitutional nucleus formation was increased by moderate convergence of spindles, i.e., tripolar spindles (Fig. 1e). Depending on its position, the restitutional nucleus occupied either

**Table 1** Frequency of meiotic abnormalities in the three accessions analyzed

Phase	Abnormalities	Accession					
		H047		D076		Du001	
		Analyzed cells	Number of cells affected (%)	Analyzed cells	Number of cells affected (%)	Analyzed cells	Number of cells affected (%)
Prophase II	Total absence of cytokinesis	366	227 (62.0)	150	85 (56.7)	146	1 (0.7)
	Partial cytokinesis		36 (9.8)		0		80 (54.8)
Metaphase II	Total absence of cytokinesis	721	22 (3.1)	199	21 (10.6)	244	6 (2.5)
	Partial cytokinesis		83 (11.5)		23 (11.6)		134 (54.9)
Anaphase II	Total absence of cytokinesis	177	2 (1.1)	140	11 (7.9)	147	3 (2.0)
	Partial cytokinesis		87 (49.2)		34 (24.3)		69 (46.9)
Telophase II	Tripolar spindle		21 (11.9)		22 (15.7)		0
	Total absence of cytokinesis	564	19 (3.4)	215	13 (6.1)	206	3 (1.46)
Tetrad	Partial cytokinesis		210 (37.2)		35 (16.3)		124 (60.2)
	Restitutional nucleus		43 (7.6)		36 (16.7)		12 (5.8)
	Partial cytokinesis	519	132 (25.4)	1235	146 (11.8)	166	24 (14.5)
	Monad trinucleated		15 (2.9)		23 (1.9)		0
	Dyad binucleated		13 (2.5)		81 (6.6)		0
	Triad		86 (6.6)		0		4 (2.4)
Microspores	Restitutional nucleus in dyad or triad		45 (8.7)		0		7 (4.2)
	Trinucleated	676	22 (3.3)	620	14 (2.3)	539	0
	Restitutional nucleus		3 (0.4)		13 (2.1)		7 (1.3)
	Binucleated		104 (15.4)		60 (9.7)		60 (11.1)
	Tetranucleated		0		7 (1.3)		0

one cell (Fig. 1p) or shared the cytoplasm with another  $n$  nucleus (Fig. 1h). After telophase II, the start of the second cytokinesis occurred although the first cytokinesis was either not yet complete (Fig. 1h, j) or totally absent (Fig. 1k, l). As a consequence, the number of tetrads with incomplete cytokinesis was high, and monads, dyads, and triads with  $n$  or  $2n$  nuclei were recorded among meiotic products. Abnormal microspores with different combinations of  $n$  and  $2n$  nuclei were observed (Fig. 1m–p). Similar mechanism was reported in *Hierochloë odorata*, a rhizomatous perennial grass (Ferris et al. 1992). The number of affected cells in each accession was variable, but the number of microspores with restitutional nuclei, including those scored in tetrads and the released ones, did not exceed 9%. The trend to form  $2n$  gametes in plants is highly variable, and it varies among individuals within a single taxonomic group or even among flowers of an individual plant (Bretagnolle and Thompson 1995).

Meiotic nuclear restitution may be caused by different mechanisms, including semiheterotypic division, pseudohomotypic division, mitotised meiosis, first division restitution (FDR), second division restitution (SDR), premature cytokinesis 1 and 2, and pre- and post-meiotic doubling of chromosomes (Ramanna 1979; Veilleux 1985; Bretagnolle and Thompson 1995). Most mechanisms have been described for dicotyledons with scanty studies and revealed  $2n$  gamete formation in monocots (Pagliarini et al. 1999; Lim et al. 2001; Barba-Gonzalez et al. 2004). In monocots it is very important to determine how events of chromosomal division and cytokinesis occur to yield restitutional nucleus. Although  $2n$  gametes may result from several different meiotic abnormalities, two types of  $2n$  gametes are the product of one of two basic processes and depend on the mode of nuclear restitution: FDR and SDR. The  $2n$  gametes in the present accessions resulted from FDR. In spite of correct chromosome pairing and homologous segregation at anaphase

I, the homologous chromosomes were rejoined in the second division. Heterozygosity level in  $2n$  gametes is influenced by the timing of nuclear restitution. Although FDR generally conserves heterozygosity of proximal segments, it reduces that of distal segments by half (Veilleux 1985; Bretagnolle and Thompson 1995). According to Lim et al. (2001), an important feature of restitutional meiosis with successive cytokinesis, such as that observed in the present *Brachiaria* accessions, is the manner in which chromosomal division and cytokinesis events occur. Cytological observations in these accessions clearly indicated that meiosis had been modified: chromosomes paired near normally in prophase I, formed bi-, tri-, and quadrivalents and then segregated simultaneously in anaphase I, displaying certain abnormal segregation due to their polyploid condition. As genetic recombination occurred, FDR nuclei gave rise to  $2n$  gametes with recombinant chromosomes. Recombination is one of the most important events during meiosis for introgression. Most of the evidence for using  $2n$  gametes in breeding programs has been focused on their use in autopolyploids or their polysomic polyploids (Mariani and Tavoletti 1992; Carputo et al. 2000). In these cases, the importance of FDR gametes lies in the transference of heterosis and intact parental gene combinations to sexual polyploids. The  $2n$  gametes allow breeders to broaden the genetic bases of cultivated species.

Although the genetic determination of  $2n$  pollen production has been studied in detail, the genetic control of  $2n$  egg formation is still poorly understood. Bretagnolle and Thompson (1995) presented an extensive list of genes responsible for this feature in several plant species, in which monogenic recessive status of mutant alleles is largely predominant. Although the genetic base of abnormal cytokinesis found in these accessions is not known, the fact that accessions of three different *Brachiaria* species have precisely the same abnormality, strongly suggests that this characteristic is genetically controlled. The  $2n$  gametes resulting from total absence either of the first or the second cytokinesis have been reported in *B. brizantha* (Risso-Pascotto et al. 2003), but in much lower frequency. Some exhaustive reviews have shown the influence of seasonal and envi-

ronmental factors, such as high and low temperature, on  $2n$  gamete production (see Bretagnolle and Thompson 1995).

In the genus *Brachiaria*, polyploidy is of common occurrence (Valle and Savidan 1996). Studies performed by flow cytometry on 435 accessions, belonging to 13 species, revealed that only 13% are diploids. The ploidy level ranged from  $2n = 4x$  to  $2n = 7x$ , with predominance (58.13%) of tetraploidy (Penteado et al. 2000). The origin of polyploidy in the genus is not well known. Taking into account the meiotic pairing and the meiotic behavior of several accessions of different *Brachiaria* species studied by these authors, there is evidence of autopolyploidy and segmental allopolyploidy (Mendes-Bonato et al. 2002, 2006; Utsunomiya et al. 2005), and even of true allopolyploidy (Mendes-Vieira et al. 2006). At any rate,  $2n$  gametes may have contributed to the evolutionary history of the genus. In fact, recent studies of natural polyploidy complexes have shown that the production of  $2n$  gametes may have played a role in the creation of new polyploids by unilateral or bilateral sexual polyploidization (see Bretagnolle and Thompson 1995).

The forage potential of *Brachiaria* species was acknowledged in Brazil about 40 years ago. However, the importance of the genus was felt only in the past three decades when two to three *Brachiaria* cultivars were extensively sown in tropical America (Miles et al. 1996, 2004). *B. decumbens* cv. Basilisk (D) and *B. brizantha* cv. Marandu (B) undoubtedly make up the most extensively planted cultivars, covering over 50 million hectares of poor and acid soils of central Brazil and Latin America. To increase genetic variability in the genus, a dynamic breeding program based on intra- and interspecies hybridization is underway at the Embrapa Beef Cattle Center since 1988. Hybridization in the genus *Brachiaria* is rather difficult mainly owing to ploidy differences among accessions and species, and to reproduction by apomixis (Valle and Savidan 1996). Most accessions in promising species are tetraploid and apomictic. Thus, several tetraploid interspecific hybrids involving different accessions of (D) and (B) were synthesized by the use of artificially tetraploidized sexual accessions of *Brachiaria ruziziensis* as female genitors. The success of such



program depends heavily on the high-pollen fertility of the apomictic accessions used as the male parent. These crosses obviously involve  $2n$  gametes generated by regular chromosome segregation of both tetraploid genitors. Since  $2n$  gametes from restitutional nucleus have never been explored in the genus for breeding purposes, current results open new exciting possibilities and further exploration for *Brachiaria* breeding. In the case of *B. humidicola*, where several agronomically superior accessions are hexaploid, with  $2n = 6x = 54$ , the pollination of a normal sexual tetraploid megagametophyte ( $n = 18$ ) with  $2n$  gametes from the apomictic H047 can produce sexual hexaploids amenable to cross with the untouched apomictic hexaploid pool in this species. This would finally allow breeding at this ploidy level since no sexual hexaploids have been found in this germplasm. The commercial cultivar of a *B. humidicola* is a hexaploid and a very important forage grass in the tropics due to its good adaptation to acid and poorly drained soils. The  $2n$  gametes may ultimately allow the diversity in this germplasm pool to be used as a major breakthrough in the breeding of *B. humidicola*.

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