ELENIZA DE VICTOR ADAMOWSKI

MICROSPOROGÊNESE EM *Brachiaria* (POACEAE): HÍBRIDOS E ACESSOS

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas (área de concentração – Biologia Celular) da Universidade Estadual de Maringá, para obtenção do grau de Doutor em Ciências Biológicas.

Maringá – PR

2007

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Maringá - PR

2007

Dados Internacionais de Catalogação-na-Publicação (CIP)
(Biblioteca Central - UEM, Maringá – PR., Brasil)

A199m

Adamowski, Eleniza de Victor
Microsporogênese em *Brachiaria* (Poaceae): híbridos e acessos / Eleniza de Victor Adamowski. -- Maringá : [s.n.], 2007.
30 f. : il., figs., tab.

Orientadora : Profª. Drª. Maria Suely Pagliarini.
Co-Orientadora: Drª. Cacilda Borges do Valle.
Tese (doutorado) - Universidade Estadual de Maringá. Departamento de Ciências Biológicas, 2007.

1. Biologia celular. 2. Citogenética vegetal. 3. Melhoramento genético. 4. Meiose. 5. Poliploidia. 6. Apomixia. 7. Brachiaria humidicola. 8. Híbridos. 9. Citocinese irregular. 10. Anormalidades meióticas. 11. Gramineae. I. Universidade Estadual de Maringá. Programa de Pós-Graduação em Ciências Biológicas (área de concentração - Biologia Celular). II. Título.

CDD 21.ed. - 571.6845

Aos meus filhos

Maria Fernanda

Francisco

Ao meu esposo

Fernando

Aos meus pais

Victor

Maria

Dedico

AGRADECIMENTOS

À Deus, luz na minha vida.

À Profª. Drª. Maria Suely Pagliarini, por me permitir realizar este trabalho; pela determinação e dedicação, que me soube transmitir com profissionalismo seus conhecimentos, a quem admiro muito.

À Drª. Cacilda Borges do Valle, pesquisadora da Embrapa Gado de Corte, pela co-orientação e concessão do material analisado.

Ao Programa de Pós-Graduação em Ciências Biológicas da UEM, pela oportunidade de realizar este curso.

Ao técnico da Embrapa Gado de Corte, Amado Monção, pela dedicação e ajuda em cada coleta.

Ao esposo, Fernando Borin Chiquetti, pelo apoio, incentivo, amor e compreensão.

Aos meus filhos, Maria Fernanda e Francisco pelo amor e carinho.

À minha amiga Kellen Regina Boldrini pela amizade, apoio, carinho em todos os momentos dessa jornada.

Às amigas Andréa e Neide, pela presença constante e amizade.

E, principalmente, à minha família, pelo amor incondicional e incentivo; pelo apoio em todos os momentos; e pelo esforço de cada um, que me permitiu estar aqui e vencer mais esta etapa.

APRESENTAÇÃO

Esta tese é composta por dois artigos científicos intitulados:

1. Abnormal cytokinesis in microsporogenesis of *Brachiaria humidicola* (Poaceae: Paniceae), originado a partir da análise 58 acessos de *Brachiaria humidicola*. Este artigo será submetido à revista **Genetics and Molecular Research** (aceito para publicação).

2. Meiotic behavior in three interspecific 3-way hybrids between *Brachiaria ruziziensis* and *B. brizantha* (Poaceae: Paniceae), originado a partir da análise de 3 híbridos interespecíficos. Este artigo será submetido à revista **Genetics and Molecular Biology** (Qualis A).

Microsporogênese em *Brachiaria* (Poaceae): híbridos e acessos

Eleniza de Victor Adamowski
Maria Suely Pagliarini
Cacilda Borges do Valle

RESUMO. *Brachiaria brizantha, B. decumbens, B. humidi*cola e *B. ruziziensis* são as espécies de *Brachiaria* mais exploradas comercialmente. Atualmente, o gênero *Brachiaria* é responsável por 85% das pastagens cultivadas do país, sustentando o maior rebanho do mundo. As pastagens brasileiras, todavia, estão sendo severamente degradadas por fertilização e manejo inadequad. A renovação das pastagens demanda novas cultivares. Para aumentar a variabilidade genética no gênero, um extensivo programa baseado em cruzamentos intra- e interespecíficos foi iniciado na Embrapa Gado de Corte em 1988. Inicialmente os cruzamentos foram realizados entre acessos sexuais de *B. ruziziensis* com acessos apomíticos de *B. brizantha* ou *B. decumbens*. Um grande número de híbridos foi obtido e alguns estão sob avaliação agronômica que neste gênero podem ser obtidas pela seleção direta dos genótipos superiores na coleção de germoplasma ou entre os híbridos elite produzidos pelo melhoramento genético. Destes, alguns dos sexuais superiores foram utilizados em cruzamentos gerando híbridos de segunda geração como os descritos nesta tese. O comportamento meiótico de três híbridos obtidos entre *B. ruziziensis* x *B. brizantha*, retrocruzados com *B. brizantha*. Descreve, ainda, uma anormalidade relacionada à citocinese irregular observada em um acesso de *B. humidicola*. Tanto os acessos de *B. humidicola* quanto os híbridos foram analisados pela metodologia convencional para estudos meióticos, utilizando-se carmim propiônico como corante. Dentre os 60 acessos da coleção de *B. humidicola*, um acesso (H121) apresentou uma anormalidade relacionada à citocinese nunca descrita em outro acesso do gênero. Dentre os meiócitos analisados, 10,7% passaram por citocinese precoce em metáfase I, fracionando o genoma e o citoplasma em duas ou mais partes. A citocinese esperada após a telófase I não ocorreu. Os meiócitos resultantes da primeira divisão entraram na segunda divisão, mas a segunda citocinese após a telófase II também foi anormal. Na segunda divisão, 10,9% dos meiócitos apresentaram citocinese

irregular. Entre os produtos meióticos, foram encontrados díades e micrósporos binucleados. O uso deste acesso no programa de melhoramento fica comprometido pela presença desta anormalidade. O comportamento meiótico dos três híbridos analisados revelou a presença de 2n = 33 cromossomos em dois híbridos meios-irmãos. Os cromossomos parearam-se predominantemente em multivalentes, sugerindo que recombinação genética e introgressão de genes específicos de *B. brizantha* em *B. ruziziensis* pode ser esperada. Apesar da proximidade filogenética entre as duas espécies, estes três híbridos apresentaram alta freqüência de anormalidades meióticas, principalmente aquelas relacionadas à segregação irregular de cromossomos típica de poliplóides: H34 = 69,1%, H27 = 56.1% e H17 = 44.9%. A partir de resultados acumulados através de análise citogenética em vários híbridos de *Brachiaria*, torna-se evidente que a análise citogenética é fundamental para determinar quais genótipos podem ser usados com sucesso no programa de melhoramento para obter variedades superiores. Anormalidades meióticas como as descritas nos híbridos aqui analisados comprometem seriamente a produção de sementes, uma característica chave para se adotar uma nova cultivar de *Brachiaria* para a formação de pastagens. Portanto, tais híbridos não devem ser utilizados como genitores em novos cruzamentos. Caso sejam recomendados para continuarem no programa de desenvolvimento de cultivares, há necessidade de analisar a capacidade de produção de sementes em condições de campo.

Microsporogenesis in *Brachiaria* (Poaceae): hybrids and accessions

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Maria Suely Pagliarini
Cacilda Borges do Valle

ABSTRACT. *Brachiaria brizantha, B. decumbens, B. humidicola,* and *B. ruziziensis* are the most commercially exploited brachiariagrasses. Nowadays, *Brachiaria* alone accounts for 85% of the cultivated pastures in Brazil, sustaining the largest commercial herd in the world. Brazilian pastures are severely degraded because of inadequate fertilization and mismanagement. Renovation of pastures and intensification of production practices demand new cultivars. To increase genetic variability in the genus, an extensive program based on intra- and interspecies hybridization was undertaken at the Embrapa Beef Cattle Center in 1988. At first, hybridizations were between sexual *B. ruziziensis* and *apomictic B. brizantha* or *B. decumbens*. A great number of hybrids were obtained and some are under agronomic evaluations. A few superior sexual hybrids were crossed to apomictics to produce second generation hybrids such as the ones studied in this thesis. The meiotic behavior of three 3-way hybrids obtained between *B. ruziziensis* and *B. brizantha* and also an abnormality related to irregular cytokinesis recorded in one accession of *B. humidicola* are described. Microsporogenesis was evaluated in the *Brachiaria humidicola* collection at Embrapa Beef Cattle Center represented by 60 accessions. One accession (H121) presented an abnormal pattern of cytokinesis never reported in any other accession of the genus. Among meiocytes analyzed, 10.7% underwent precocious and multiple cytokinesis in metaphase I, fractionating the genome and the cytoplasm into two or more parts. The expected cytokinesis after telophase I did not occur. The abnormal meiocytes from the first division entered the second division but the second cytokinesis after telophase II was also abnormal. In the second division, 10.9% presented abnormal, incomplete or total absence of cytokinesis. Among meiotic products, dyads and binucleated microspores were recorded. The use of this accession in the breeding program is compromised. The meiotic behavior of three 3-way interspecific promising hybrids (H17, H27, and H34) was also evaluated. Two half-sib hybrids (H27 and H34) presented an aneuploid chromosome number (2n = 4x = 33) whereas hybrid H17 was a tetraploid (2n = 4x = 36), as expected. Chromosome paired predominantly as

multivalents suggesting that genetic recombination and introgression of specific target genes from *B. brizantha* into *B. ruziziensis* can be expected. Despite the phylogenetic proximity among these two species, these three hybrids presented a high frequency of meiotic abnormalities, mainly those related to irregular chromosome segregation, typical of polyploids: H34 = 69.1%; H27 = 56.1%; and H17 = 44.9%. From accumulated results obtained through cytological studies in *Brachiaria* hybrids, it is evident that cytogenetical analysis is of prime importance in determining which genotypes can continue in the process of cultivar development and which can be successfully used in the breeding for superior forage performance. Hybrids with serious meiotic abnormalities such as these reported here can however, seriously compromise hybridization and/or seed production, a key trait in assuring adoption of a new apomictic cultivar of *Brachiaria* for pasture formation. These hybrids should not be used as progenitors in further crosses. In the event of continuing in the process of cultivar development, they should be evaluated for seed production under regular field conditions.

Abnormal cytokinesis in microsporogenesis of *Brachiaria humidicola* (Poaceae: Paniceae)

Abnormal cytokinesis in microsporogenesis of *Brachiaria humidicola* (Poaceae: Paniceae)

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Abstract. Microsporogenesis was evaluated in the *Brachiaria humidicola* collection at Embrapa Beef Cattle Center represented by 60 accessions. One accession (H121) presented an abnormal pattern of cytokinesis never reported in any other accession of the genus. A total of 1757 meiocytes was analyzed. Among them, 10.7% underwent precocious and multiple cytokinesis in metaphase I, fractionating the genome and the cytoplasm into two or more parts. The expected cytokinesis after telophase I did not occur. The abnormal meiocytes from the first division entered the second division but the second cytokinesis after telophase II was also abnormal. Among the 857 meiocytes analyzed in the second division, 10.9% presented abnormal, incomplete or total absence of cytokinesis. Among meiotic products, dyads and binucleated microspores were recorded. The use of this accession in the breeding program is compromised and implications of this meiotic behavior are discussed.

Keywords: *Brachiaria humidicola*, breeding program, cytokinesis, forage grass, microsporogenesis

Introduction

Microsporogenesis is an ideal process for studying meiotic mutations affecting the different controlled steps involved in meiotic reduction of diploid mother cells to form haploid microspores. The pattern of divisions during microsporogenesis is highly controlled and yields a predictable end product (Staiger and Cande, 1990).

A large variety of mutants that disrupt meiosis at various stages has been reported in different plant species (Gottschalk and Kaul, 1974, 1980 a b; Baker et al., 1976, Golubovskaya, 1979, 1989). In *Brachiaria*, a genus of the forage grasses most cultivated worldwide in the tropics, recent cytological studies have indicated several putative meiotic mutations in the gene pool of the genus (Mendes-Bonato et al., 2001, 2003, 2004, 2006 a b; Risso-Pascotto et al., 2002, 2003 a b, 2005; Junqueira Filho et al., 2003; Mendes-Vieira et al., 2005).

After two rounds of chromosome segregation (karyokinesis) and one simultaneous or two successive cytoplasmic divisions (cytokinesis), the final product of male meiosis in flowering plants emerges as a tetrad of haploid microspores in a callose wall. Cytokinesis is accomplished by a typical phragmoplast which is initiated in the spindle midzone during the late anaphase and early telophase. The array of parallel phragmoplast microtubules propagates centrifugally and cytokinesis is completed before the next division (Staiger and Cande, 1991).

Several mutants are known to alter the normal progression of meiosis and can be correlated with defects in microtubule distribution. One of such mutant, *dv*, was reported in maize (Staiger and Cande, 1990). In the genus *Brachiaria*, some putative mutations have been reported affecting the pattern of cytokinesis, mainly in *B. humidicola* (Boldrini et al., 2006; Gallo et al., 2007; Calisto et al., 2007). This paper reports a new pattern of cytokinesis in one accession of this species.

Materials and Methods

Accessions of *Brachiaria humidicola* from the Embrapa Beef Cattle *Brachiaria* germplasm collection (Campo Grande, state of Mato Grosso do Sul, Brazil) collected in the wild African savannas in the mid 1980s by CIAT (Colombia) were cytologically analyzed. Site characteristics of the plots in the field in Embrapa Beef Cattle Research Center in Brazil were: climate type Aw: tropical humid savanna; average annual precipitation = 1526 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 for meiotic study were collected in plots with 16 plants and fixed in a mixture of ethanol 95%, chloroform and propionic acid (6:3:2) for 24 hours, 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 *Brachiaria humidicola* collection at Embrapa Beef Cattle Center is represented by 60 accessions. Among them, fifty eight accessions have had the meiotic behavior evaluated. One accession (H121) presented an abnormal pattern of cytokinesis never reported in other accession of the genus. A total of 900 meiocytes in the first division

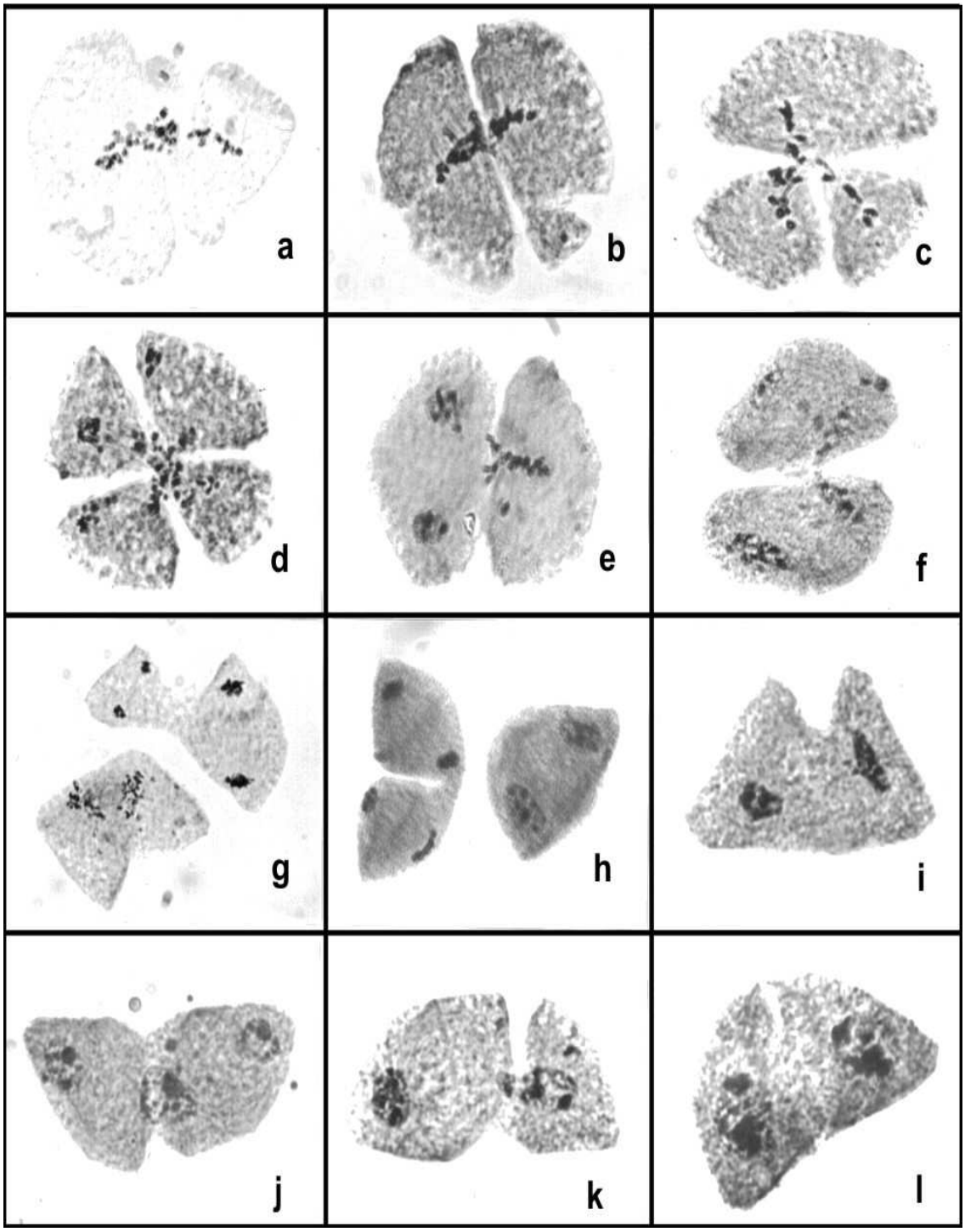

Figure 1. Aspects of abnormal cytokinesis in the accession H121. a to c) Microsporocytes in metaphase I divided by precocious cytokinesis. Notice that micronuclei are isolated as microcytes. d) Metaphase I with the genome fractionated into four cells. e, f) Microsporocyte that underwent precocious cytokinesis and showing asynchrony between cells. g, h) Microsporocytes in the second division resulting from precocious cytokinesis in the first division. i to l) Different aspects of abnormal cytokinesis in binucleated microspores. (Magnification 400X)

was analyzed. Among them, 96 meiocytes (10.7%) underwent precocious and multiple cytokinesis in metaphase I, fractionating the genome and the cytoplasm into two or more parts (Fig. 1 a to f). The expected cytokinesis after telophase I did not occur, thus abnormal meiocytes from the first division entered the second division (Fig. 1 g, h). The second cytokinesis after telophase I in these meiocytes was also abnormal. Among the 857 meiocytes analyzed in meiosis II, 94 (10.9%) presented abnormal incomplete (Fig. 1 i to l) or total absence of cytokinesis. Among meiotic products, dyads and released binucleated microspores were recorded.

The present accession of *B. humidicola* has 2n = 54 chromosomes and the meiotic behavior in normal meiocytes suggested that it is a polyploidy accession (2n = 9x = 54), probably derived from x = 6. Chromosome number countings in the *B. humidicola* collection at Embrapa Beef Cattle Center revealed accessions with 2n = 36, 42, and 54 chromosomes (Boldrini, unpublished data). Among the accessions with 2n = 54 chromosomes, another accession (H022) also presented an abnormal pattern of cytokinesis (Calisto et al., 2007) similar to the abnormality reported in H121. However, in H022 the abnormal cytokinesis occurred only in meiocytes where chromosome associations were disrupted at the end of diakinesis, separating the chromosomes by desynapsis; the 54 univalents were aligned forming a wide metaphase plate. In H121, the normal cytokinesis after telophase I was also absent and the second cytokinesis after telophase II occurred normally. In H121, cytological evidence related to precocious cytokinesis was not found.

In another accession of *B. humidicola* (H003) with 2n = 7x = 42, also probably derived from x = 6, one more abnormal pattern of cytokinesis was recorded (Boldrini et al., 2006). In this accession, the first cytokinesis occurred after telophase II, generating dyads with binucleated microspores, which after release from the callose wall, initiated

the second cytokinesis by invagination, giving rise to four normal microspores. In the H121 and in H022 (Calisto et al., 2007), both with 2n = 54 chromosomes, cytokinesis was initiated marginally in metaphase I and migrated to the center, similar to an invagination process. Incomplete cytokinesis leading to 2n gamete formation was recorded in another accession of *B. humidicola* (H047), 2n = 36 chromosomes (Gallo et al., 2007). In this accession, the first cytokinesis was initiated in the center of the cell after telophase I but did not get to the borders, allowing the rejoining of nucleus after prophase II. The number of microsporocytes affected by abnormal cytokinesis recorded in all above cited accessions of *B. humidicola* was always small. In accession H022, with a similar abnormality, the percentage of affected cells was 16,8% (Calisto et al., 2007).

A large number of genes, generally dominant, which are stage, site- and time-specific are involved in the control of meiosis (Gottschalk and Kaul, 1974, 1980 a b; Baker et al., 1976, Golubovskaya, 1979, 1989). Among genes acting in the meiotic process, those responsible for the partitioning of the cytoplasm after nuclear division play a very important role in the formation of viable gametes. The timing of cytokinesis varies among angiosperms. In most monocotyledons, cytokinesis is successive, i.e, one partitioning of the cytoplasm occurs after telophase I and another after telophase II, so that there is a distinct dyad stage. In most dicots, however, it is simultaneous and occurs after telophase II (Peirson et al., 1996). In higher plants, cytokinesis during mitosis is a genetically controlled multistep process. At least three cellular components play important roles in its occurrence: (i) the Golgi apparatus produces secretory vesicles and synthesizes the cell wall polysaccharides; (ii) Golgi-derived vesicles fuse to form a cell plate: and (iii) the cytoskeleton required for phragmoplast formation and expansion controls the cell division planes. Other cellular components, including the endoplasmic

reticulum, intermediate filaments, calmodulin and myosin may also play important roles in cytokinesis (Staelin and Hepler, 1996). During mitosis in higher plants, a cortical ring of microtubules, called 'the preprophase band', marks the site where the cell plate will be formed, determining the division pattern. Meiosis, on the other hand, lacks the preprophase band, although the division pattern seems to be accurately controlled. Cytokinesis-defective mutants have been characterized in several species of higher plants during mitosis and meiosis (Beadle, 1932; Peirson et al., 1996; Hulskamp et al., 1997; Nickle and Meinke, 1998; Boldrini et al., 2006; Calisto et al., 2007).

Absence of cytokinesis was recorded in other *Brachiaria* species (Risso-Pascotto et al. 2003 a, Utsunomiya et al., 2005). In these cases, the failure of cytokinesis occurred after telophase I or telophase II, generating balanced 2n gametes. In H121 and H022, the cytokinesis occurred precociously in metaphase I. It is suggested that the genetic control for cytokinesis in these meiocytes was activated very early and not synchronized with karyokinesis, generating unbalanced gametes. The cytological analysis in the *B. humidicola* collection suggests that this species is more propense to abnormal cytokinesis than the other *Brachiaria* species previously analyzed (Mendes-Bonato et al., 2002, 2006 a; Utsunomiya et al., 2005). Several putative meiotic mutations have been described in the genus *Brachiaria* (Mendes-Bonato et al., 2001, 2003, 2004, 2006 b; Risso-Pascotto et al., 2002, 2003 a, 2005, Mendes-Vieira et al., 2005) and also in the post-meiotic process (Junqueira Filho et al., 2003; Mendes-Bonato et al., 2004) that could represent putative mutations, suggesting that these genes were incorporated in the gene pool of the genus during its evolutionary process.

Some promising apomictic accessions of *B. humidicola* are under careful agronomic and grazing evaluation in hopes of selecting new cultivars. This species is well adapted to poorly drained and infertile acid soils (Keller-Grein et al, 1996), for

which very few options are available, thus the urgent demand for improved cultivars. The occurrence of precocious cytokinesis detected in this accession affects pollen viability and unbalanced gametes are generated by partitioning of the genome in metaphase I. Other accessions of this species, may be better progenitors in intra- and interspecific hybridization as pollen donors. However, the amount of meiocytes affected by precocious cytokinesis in H121 (about of 10%) may not be enough to discard the accession from the breeding program. Other abnormalities due to polyploidy (2n = 9x = 54) was also detected: precocious chromosome migration to the poles in metaphase I (55.7%) and metaphase II (55.6%), and laggards in anaphase I (82.5%) and anaphase II (58.7%), which generated micronuclei in telophase I (44.9%) and telophase II (11.4%).

Polyploidy in *Brachiaria* is correlated with apomixis, which bypasses meiosis in the megagametogenesis process: the embryo-sac is formed by parthenogenesis of a somatic cell thus the embryo is maternal, but for seed development, the secondary nuclei of the embryo sac need to be fertilized by a male gamete - pseudogamy. Accessions with high frequency of meiotic abnormalities due polyploidy which severely impairs pollen viability need to be discarded early in the breeding program to avoid passing on defective genes to the progenies. The on-going hybridization program in *Brachiaria*, until now, involves intra- and interspecific crosses only between tetraploid (2n = 4x = 36) genotypes derived from x = 9. The 2n = 54 chromosome pool is still impervious to breeding due to lack of compatible sexual source for crossing. Interploid crosses have been unsuccessful in this genus (Hacker, 1988). Therefore, accession H121 cannot be used in crosses due to its high level of ploidy, but also due to its abnormal cytokinesis and difference in its basic chromosome number, x = 6.

Acknowledgments

Authors are grateful to UNIPASTO for financial support.

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Meiotic behavior in three interspecific 3-way hybrids between *Brachiaria ruziziensis* and *B. brizantha* (Poaceae: Paniceae)

Meiotic behavior in three interspecific 3-way hybrids between *Brachiaria ruziziensis* and *B. brizantha* (Poaceae: Paniceae)

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Summary. The meiotic behavior of three 3-way interspecific promising hybrids (H17, H27, and H34) was evaluated. These hybrids resulted from crosses between (*B. ruziziensis* x *B. brizantha*) backcrossed to *B. brizantha*. Two half-sib hybrids (H27 and H34) presented an aneuploid chromosome number (2n = 4x = 33) whereas hybrid H17 was a tetraploid (2n = 4x = 36), as expected. Chromosome paired predominantly as multivalents suggesting that genetic recombination and introgression of specific target genes from *B. brizantha* into *B. ruziziensis* can be expected. Arrangement of parental genomes in distinct metaphase plates was observed in H27 and H34, which have different male genitors. Hybrids H17 and H34 have the same male genitor but did not display this abnormality. In H17, abnormalities were more frequent from anaphase II, when many laggard chromosomes appeared, suggesting that each genome presented a different genetic control for meiotic phase timing. Despite the phylogenetic proximity among these two species, these three hybrids presented a high frequency of meiotic abnormalities, mainly those related to irregular chromosome segregation, typical of polyploids: H34 = 69.1%; H27 = 79.0%; and H17 = 44.9%). From accumulated results obtained through cytological studies in *Brachiaria* hybrids, it is evident that cytogenetical analysis is of prime importance in determining which genotypes can continue in the process of cultivar development and which can be successfully used in the breeding for superior forage performance. Hybrids with interesting agronomic traits such as these reported can however, seriously compromise hybridization and/or seed production, a key trait in assuring adoption of a new apomictic cultivar of *Brachiaria* for pasture formation.

Key words: *B. brizantha*, *B. ruziziensis*, backcross hybrids, genome affinity, meiotic abnormalities

Introduction

Brachiaria, a pantropical genus containing about 100 species mainly from the African continent, is found in a wide range of habitats from semi-desert to swamps (González and Morton, 2005). While grasses from this genus have been continuously exploited by local pastoralists for millennia, the interest in species of this genus as sown and managed forage only began in the 1960s. This occurred first on a limited scale in humid, coastal, tropical Australia, followed by tropical South Africa, and later in Brazil in the early 1970s (Miles *et al*., 2004). Currently, the genus *Brachiaria* is the most widely used forage grass in the South American savannas due to its physiological tolerance to low-fertility acid soils of the tropics (Rao *et al*., 1996).

Brachiaria brizantha (A. Rich) Stapf (palisadegrass), *B. decumbens* Stapf (signalgrass), *B. humidicola* (Rendle) Schweick. (koroniviagrass), and *B. ruziziensis* Germain & Evrard (ruzigrass) are the most commercially exploited brachiariagrasses. Their economic importance is greatest in tropical America, where extensive adoption over the past three decades has had a revolutionary impact on the productivity of vast areas of previously underused, marginal soils (Miles *et al*., 2004). Nowadays, *Brachiaria* alone accounts for 85% of the cultivated pastures in Brazil (Miles and Valle, 1996), covering over 50 millions hectares and sustaining the largest commercial herd in the world – about 205 million heads (IBGE, 2004).

Two cultivars, *B. decumbens* cv. Basilisk and *B. brizantha* cv. Marandu are undoubtedly the most widely grown species not only in the Brazilian savannas but throughout the tropics. This rapid expansion of their acreage did not occur without

problems: both cultivars have significant limitations. The first one lacks resistance to a ubiquitous family of sucking insects, the spittlebugs (Homoptera: Cercopidae); the second, while resistant, requires higher soil fertility and does not tolerate waterlogged soils (Miles *et al*., 1996; Barbosa, 2006). Brazilian pastures are severely degraded because of inadequate fertilization and mismanagement. Renovation of pastures and intensification of production practices demand new cultivars. Eight cultivars are presently commercialized by a dynamic seed industry, dominated by Brazilian companies, and seven of these cultivars are direct selections from naturally occurring germplasm collected in Africa (Miles *et al.*, 2004). All the cultivars are polyploid (2n = 4x = 36) and apomictic which held back the initiation of brachiariagrass breeding programs until suitable sexual germplasm was developed in the mid- 1980s (Valle and Savidan, 1996).

To increase genetic variability in the genus, an extensive program based on intra- and interspecies hybridization was undertaken at the Embrapa Beef Cattle Center in 1988 with the objective of determining the inheritance of apomixis and thus manipulating this character for the development of new improved hybrids (Valle and Savidan, 1996). At first, hybridizations were between sexual *B. ruziziensis* and apomictic *B. brizantha* or *B. decumbens*. A great number of hybrids was obtained and are under agronomic evaluations. Some interesting sexual hybrids were selected to be crossed to some superior ecotypes of the paternal species. The *B. ruziziensis/B. decumbens/B. brizantha* complex provides a wealth of genetic variation for the introgression of derived genes of interest, such as for spittlebugs resistance and nutritive value, among others. This paper describes the meiotic behavior of three 3-way hybrids obtained between *B. ruziziensis* and *B. brizantha* and compares types and frequencies of

abnormalities which may affect pollen viability and seed production, thus impairing use as cultivars or genitors in the breeding program.

Material and Methods

Cytological studies were carried out on three 3-way interspecific hybrids. The original female genitor in these hybrids was an artificially tetraploidized sexual accession of *B. ruziziensis* (R50, 2n = 4x = 36), which was crossed to apomictic *B. brizantha* cv. Marandu (M), (2n = 4x = 36). One F_1 sexual hybrid obtained, S13 (R50 x M/27) was then pollinated by *B. brizantha*-B132 to produce hybrid H27, and by *B. brizantha*-B166 to produce both H17 and H34. Thus the hybrids H17 and H34 are full sibs and half sibs to hybrid H27.

The hybrids were produced by controlled pollination in the greenhouse at Embrapa Beef Cattle Center (Campo Grande, State of Mato Grosso do Sul, Brazil). These three hybrids have excellent phenotypes from the forage standpoint and are under small plot agronomical evaluation.

Inflorescences for meiotic studies were collected from individual plants under free growth in the field and fixed in a mixture of ethanol 95%, chloroform and propionic acid (6:3:2 v/v) during 24 hours and stored under refrigeration until use. Microsporocytes were prepared by squashing and stained with 0.5% propionic carmine. More than 2100 pollen mother cells (PMCs) were analyzed in each hybrid. Images were photographed with Kodak Imagelink – HQ, ISO 25 black and white film.

Results and Discussion

Conventional cytological analyses revealed one hybrid (H17) with 2n = 4x = 36 chromosomes as its genitors and the two others from the cross between sexual F_1 hybrid S13 and two different apomicitic *B. brizantha* (H27 and H34) with an abnormal chromosome number, 2n = 4x = 33 (Fig. 1 a, b). The original female progenitor in the three hybrids was an obligate sexual accession (R50) artificially tetraploidized with colchicine in Belgium (Swenne *et al.*, 1981; Gobbe *et al*., 1981). These materials have allowed apomixis to be exploited in the breeding of *Brachiaria*. In the second generation of crosses, the sexual S13 hybrid was crossed to two different apomictic ecotypes of *B. brizantha*, chosen for superior performance in agronomic trials: B166 and B132.

In tropical America, the main objective of the *Brachiaria* breeding is to use the sexuality of the tetraploid ruzigrass to release the genetic diversity locked in the natural tetraploid apomictics, signalgrass and palisadegrass, to produce novel apomictic hybrid cultivars (Valle and Savidan, 1996; Miles *et al.*, 2004). Results of experimental hybridization in Brazil showed that sexual and apomictic plants occur in approximately equal proportions among hybrids, indicating a monogenic inheritance of apomixes (Miles and Valle, 1996; Savidan, 2000). Superior apomictic hybrids are desirable in the breeding program since their traits are fixed by apomixis, and homogeneous improved permanent pastures can, thus, be established. On the other hand, superior sexual hybrids to be used as female genitors in crosses are needed to broaden the genetic base and to introgress more desirable genes into the pool.

All the original progenitors in these hybrids were tetraploid (2n = 4x =36). It was not possible to detect with conventional staining from which genitor the aneuploidy (2n =

4x = 33) arose in the two hybrids but apparently it is related to the S13 mother plant since aneuploid hybrids had different male genitors. Considering that: (i) female gametes are more tolerant to aneuploidy than male gametes (Sybenga, 1992; Singh, 1993); (ii) severe meiotic abnormalities were found in tetraploidized accessions of *B. ruziziensis* (R50) used as female genitor in the original cross, seriously compromising pollen viability (Risso-Pascotto *et al*., 2005 a); and (iii) in a previous study, the genome of *B. ruziziensis* was delayed during meiosis in relation to the genome of *B. brizantha* (Risso-Pascotto *et al*., 2004), we suggest that ruzigrass R50 and later S13 was probably the genitor that provided the aneuploid gamete, despite the fact that hybrid H17 did not display it.

In diakinesis, the three hybrids presented a high number of multivalent chromosome pairing (Fig. 1 c), showing that the species are closely related and that gene introgression is possible among them through meiotic recombination. Chromosome pairing in hybrids is used as a method of assessing genomic relationships between species (Alonso and Kimber, 1981). In addition, it also provides an important starting point in alien introgression programs (Gale and Miller, 1997). Based on morphological characters, Renvoize *et al*. (1996) showed that *B. brizantha*, *B. ruziziensis*, and *B. decumbens* belong to the same taxonomic group. The phylogenetic proximity among them was reinforced by molecular markers (Suárez, 1994). The meiotic pairing of chromosomes in the hybrids suggests that *B. ruziziensis* and *B. brizantha* form a coherent gene pool or agamic complex, once ploidy barriers are overcome (Lutts *et al*., 1991).

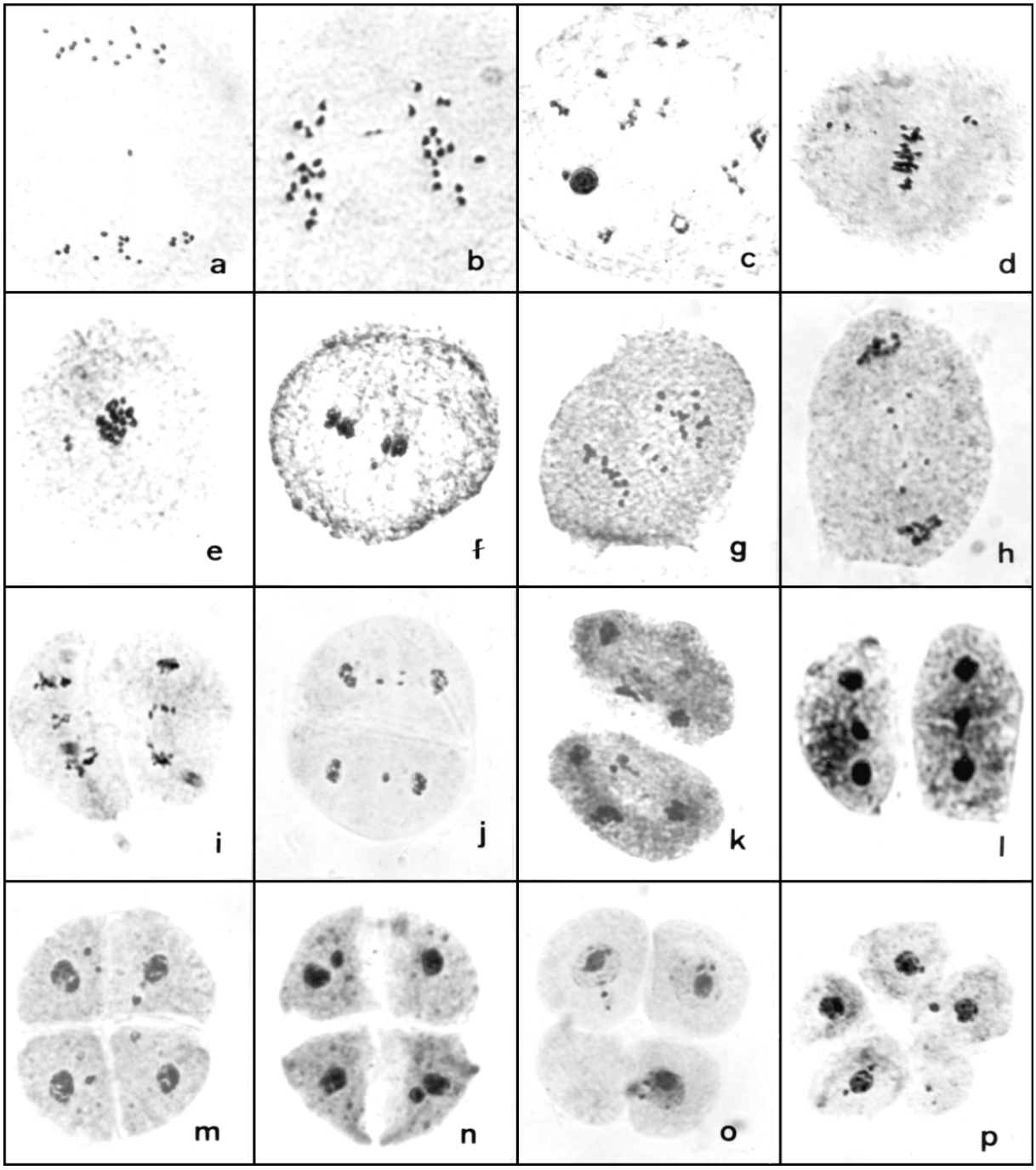

Figure 1. Some aspects of meiotic behavior in hybrids H34 and H27 of *Brachiaria* with 2n = 4x = 33 chromosomes. a, b) Two anaphases I with 16 chromosomes in each pole and a laggard. In b the laggard is undergoing sister-chromatid segregation. c) Diakinesis with several multivalents. d) Metaphase I with precocious chromosome migration to the poles. e) Polar view of metaphase I with a non-congregated bivalent. f) Metaphase I with two distinct plates. g) Anaphase I with three laggard chromosomes. h) Early telophase I with small micronuclei. i) Anaphase II with laggard chromosomes. j) Telophase II with small micronuclei. k, l) Telophase II with large micronuclei. m, n) Tetrads with small and large micronuclei, respectively. o) Tetrad with an anucleated microspore. p) Tetrad with micronuclei and microcyte. (400x).

According to Rieseberg *et al*. (2000), the degree of differentiation between hybridizing taxa can be estimated not only by analyses of chromosome pairing behavior, but also by estimating and analyzing meiotic abnormalities. Despite this phylogenetic proximity among species, the three hybrids presented a high frequency of meiotic abnormalities (Table 1). Although some abnormalities were common among hybrids, others occurred solely or in two of the hybrids. Abnormalities recorded ranged from precocious chromosome migration to the poles in metaphase I and II (Fig. 1 d), non-congressed bivalents at metaphase plate (Fig. 1 e), genomes arranged in two distinct metaphase plates (Fig. 1 f), some laggard chromosomes (Fig. 1 g, i), or an entire laggard genome at anaphase I and II, small micronuclei (Fig. 1 h, j) or large micronuclei at telophase I and II (Fig. 1 k, l), and tetrads (Fig. 1 m, n). Tetrads with micronuclei in microcytes (Fig. 1 o), with anucleate microsporores (Fig. 1 o), or with microcytes (Fig. 1 p) were also recorded among meiotic products. Precocious chromosome migration to the poles, laggard chromosomes, and micronuclei have been frequently reported among polyploid accessions of *Brachiaria* (Mendes-Bonato *et al*., 2001 a, 2002 a b, 2006 a; Utsunomiya *et al*., 2005) and also among hybrids (Risso-Pascotto *et al*., 2005 b) from the collection of Embrapa Beef Cattle.

The occurrence of genomes arranged in two metaphase plates was previously reported in a hybrid between *B. ruziziensis* and *B. brizantha* (Mendes-Bonato *et al*., 2006 b). The arrangement of parental genomes in distinct metaphase plates suggests that these genomes are not able to congregate in a single plate, reflecting differences in spindle organization among species. In this analysis, a large number of meiocytes with genomes arranged into two metaphase plates in metaphase I was observed for the two aneuploid hybrids. Laggard genomes in anaphase I and II were also observed in high frequencies. Asynchrony during meiosis of both parental genomes were reported before

Table 1. Meiotic abnormalities in the three 3-way hybrids of *Brachiaria*

Phases	Abnormalities	H34 S13 x B166/17 2n = 4x = 33		H27 S13 x B132/8 2n = 4x = 33		H17 S13 x B166/1 2n = 4x = 36	
		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	**519**	46 (8.9)	**427**	46 (10.8)	**396**	15 (3.8)
	Non-congregated bivalents		43 (8.3)		-		-
	Two metaphase plates		152 (29.3)		85 (19.9)		6 (1.5)
	Cytomictic channels		95 (18.3)		15 (3.5)		-
Anaphase I	Few laggard chromosomes	**367**	53 (14.4)	**183**	38 (20.8)	**142**	27 (19.0)
	Laggard genome		246 (67.0)		78 (42.6)		-
	Cytomictic channels		20 (5.5)		-		-
	Chromosome stickiness		-		-		5 (3.5)
Telophase I	Small micronuclei	**349**	78 (22.3)	**456**	83 (18.2)	**251**	22 (8.8)
	Large micronuclei		84 (24.1)		22 (4.8)		2 (0.8)
	Cytomictic channels		23 (6.6)		1 (0.2)		-
	Irregular shape of nucleus		46 (13.2)		30 (6.6)		-
	Chromosome stickiness		-				7 (2.8)
Prophase II	Small micronuclei	**278**	44 (15.8)	**262**	83 (31.7)	**405**	35 (8.6)
	Large micronuclei		35 (12.6)		-		-
	Irregular shape of nucleus		101 (36.3)		2 (0.8)		-
	Chromosome stickiness		-		-		23 (5.7)
Metaphase II	Precocious chromosome migration	**296**	70 (23.7)	**444**	34 (7.7)	**283**	24 (8.5)
	Irregular metaphase plate		6 (2.0)		6 (1.4)		-
	Irregular cytokinesis		-				4 (1.4)
	Chromosome stickiness		-				10 (3.5)
Anaphase II	Irregular spindles	**337**	8 (2.4)	**376**	1 (0.3)	**356**	-
	Laggard genome		190 (56.4)		268 (71.3)		-
	Laggard chromosomes		81 (24.0)		84 (22.3)		303 (85.1)
	Irregular cytokinesis						4 (1.1)
Telophase II	Small micronuclei	**308**	48 (15.6)	**341**	106 (31.1)	**259**	173 (66.8)
	Large micronuclei		153 (49.7)		52 (15.2)		-
	Irregular shape of nucleus		1 (0.3)		144 (42.2)		-
	Irregular cytokinesis						4 (1.5)
Tetrad	Small micronuclei	**540**	193 (35.7)	**534**	458 (85.8)	**755**	601 (79.6)
	Large micronuclei		210 (38.9)		49 (9.2)		-
	Micronuclei and microcytes		43 (8.0)		12 (2.3)		12 (1.6)
Total		**2994**	2069 (69.1%)	**2148**	1697 (79.00%)	**2847**	1277 (44.85%)

in a triploid hybrid (2n = 3x = 27) between *B. ruziziensis* (2n = 2x = 18) and *B. brizantha* (2n = 4x = 36), where the female genome (ruzigrass) always remained behind in relation to the male genitor (palisadegrass) and ended up being eliminated in microspores of the tetrad (Risso-Pascotto *et al*., 2004). However, the meiotic behavior of two other tetraploid hybrids previously analyzed between these species did not show chromosome elimination, although other meiotic abnormalities, including mainly those related to irregular chromosome segregation were recorded in high frequency, compromising pollen viability (Risso-Pascotto *et al*., 2005 b).

Among the three triple hybrids analyzed, the meiotic behavior of H17 (2n = 4x = 36) was distinct from the other two. H17 presented a quite regular meiosis I (Table 1) with a few meiocytes with irregular chromosome segregation. In this hybrid, an explosion of meiotic irregularities arose from anaphase II, when a large number of meiocytes presented laggard chromosomes (Fig. 2 a), but not involving an entire genome as found in the two other hybrids. This is an interesting aspect, because it shows that the meiotic behavior in hybrids is not only genotype-dependent but probably dependent on specific compatibility between genitors. H17 and H34 are full sibs and while in H34 one genome remained as laggard in anaphase I and II, and was eliminated into several large micronuclei at the end of both meiotic divisions, in H17 chromosome elimination occurred from anaphase II, and the few eliminated chromosomes gave rise to small micronuclei (Fig. 2 b, c). This behavior suggests that each genome presented a different genetic control for meiotic phase timing. In H17, this difference between the parental genomes appeared only in anaphase II.

Other meiotic abnormalities were recorded among the three hybrids. One of them is the irregular shape of the telophase nucleus observed in the half-sib hybrids H34 and H27. In these, the delayed ascension of one genome to the poles, gave rise to abnormal

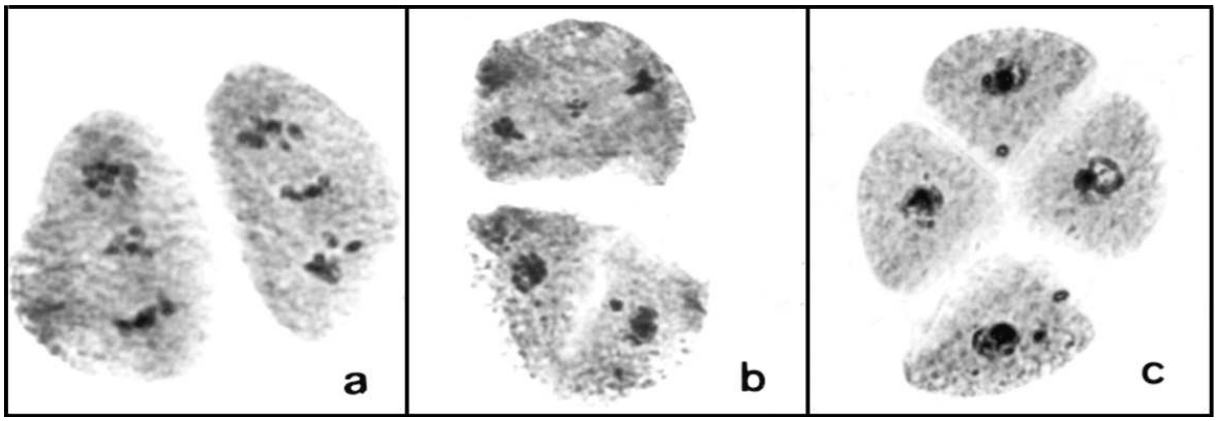

Figure 2. Some aspects of meiotic behavior in the hybrid H17 of *Brachiaria* with 2n = 4x = 36 chromosomes. a) Late anaphase II with several laggard chromosomes. b) Telophase II with small micronuclei. c) Tetrad with small micronuclei.

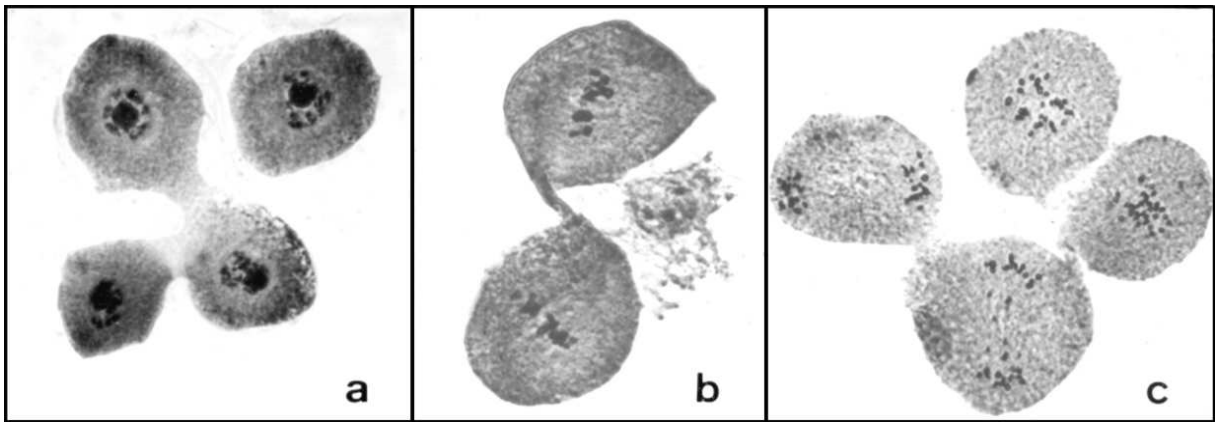

Figure 3. Some aspects of cytomictic channels observed in the hybrids with 2n = 4x = 33 chromosomes: thin and thick channels connecting the meiocytes in a, b, and c.

nuclei. The laggard genome was not always in time to be included in the nucleus, to originate the expected spherical telophase nucleus. Abnormal nucleus shape in both meiotic divisions was recently reported in two pentaploid accessions of *B. brizantha,* also presenting a laggard genome (Mendes *et al*., 2006). Irregular cytokinesis dividing the meiocytes into abnormal microsporocyte sizes, or microcytes, recorded in the three hybrids is very common in *Brachiaria* microsporogenesis and was reported in *B. brizantha* (Mendes-Bonato *et al*., 2002 a), *B. decumbens* (Mendes-Bonato *et al*., 2002 b) and *B. nigropedata* (Utsunomiya *et al*., 2005). Cytomictic channels connecting meiocytes in all phases of the first division occurred in H 34 and H 27 (Fig. 3 a to c). Cytomictic channels are indispensable for cytomixis, i.e., for the chromosome transfer among meiocytes. Although true cytomixis has been reported in *B. nigropedata* (Utsunomiya *et al*., 2004), there was no evidence of chromosome transfer among meiocytes in the present hybrids. Chromosome stickiness was observed in some meiocytes of the H17 hybrid. This abnormality has been largely reported among species and accessions of the *Brachiaria* genus (Mendes-Bonato *et al*., 2001 a, b; Risso-Pascotto *et al*., 2005 b; Utsunomiya *et al*., 2005).

The results obtained from the cytological analysis of these three triple hybrids revealed that meiotic behavior varies among genotypes, and seems to be both genotype-specific. The three hybrids were differentially affected by meiotic abnormalities in the number of affected cells (H34 = 69.1%; H27 = 79.0%; and H17 = 44.9%) and also in types of abnormalities. Knowledge accumulated from analyses of meiotic behavior in *Brachiaria* hybrids by our group have shown that *B. ruziziensis*, the unique obligate sexual species in this agamic complex has incompatibilities in sharing the same cellular space with *B. brizantha* when used as female genitor in crosses. Fortunately, this behavior seems to be genotype-specific, since not all hybrids produced compromising

abnormalities. Apomictic hybrids depend on central cell fertilization by viable pollen to develop the endosperm, assure seed set, proper seed germination, and pasture establishment. Thus, microsporogenesis needs to be fairly normal. For sexual hybrids to be used in future crosses, meiosis has to proceed normally so as not to carry defects further into progenies. Thus, promising hybrids produced in the *Brachiaria* breeding program must be selected based on their meiotic behavior besides presenting good agronomic characteristics.

Acknowledgments

Authors are grateful to UNIPASTO for financial support.

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