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Abnormal meiotic behavior in *Brachiaria brizantha* (Poaceae) leading to microspore degeneration

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Abstract — The current paper reports an original abnormality recorded in one accession of *Brachiaria brizantha* (B003) of the Brazilian germplasm collection for the genus *Brachiaria* maintained at Embrapa Beef Cattle. Among 25 inflorescences analyzed, 15 presented an abnormality affecting several phases of microsporogenesis. The abnormality appeared after nucleolus disintegration at diakinesis and was recorded till the end of meiosis. In affected inflorescences, bivalents were not able to congregate in a metaphase plate, remaining scattered or sometimes grouped in the cytoplasm. They then gave rise to pycnotic nuclei of different sizes that were later rejoined into telophase nuclei allocated peripherically. Anaphases I were never seen. After the first cytokinesis, the meiocytes entered meiosis II. In prophase II, nuclei presented abnormal positions. In metaphase II, once more, metaphase plates were not observed and chromosomes were grouped into two small and pycnotic nuclei sometimes irregularly located. From telophase II, independently of nuclei position, some meiocytes underwent the second cytokinesis, but the majority of meiotic products was characterized by dyads. Independently of the origins, microspores degenerated as soon as they were released, acquiring irregular shapes, and becoming intensely stained. At the end, nuclei degenerated and microspores gave rise to an amorphous mass. Pollen grains were not found in the affected inflorescences. The importance of this abnormality for *Brachiaria* breeding is discussed.

Key words: apoptosis, *Brachiaria brizantha*, grass, microspore degeneration, microsporogenesis, spindle, sterility.

INTRODUCTION

Microsporogenesis in higher plants is an extremely complex process that produces a callose-enclosed tetrad of haploid microspores. From the many meiotic mutants described in plants and other organisms, it is obvious that meiosis is controlled by a large number of genes (Gottschalk and Kaul 1974; Baker *et al.* 1976; Golubovskaya 1979; 1989; Koduru and Rao 1981; Kaul 1988; Chaudhury *et al.* 1992; Bhatt *et al.* 2001), the knowledge of which is mainly restricted to their cytological and genetic characteristics. Only recently, however, the traditional emphasis on the cytology of meiotic mutants has given way to dissection of meiosis in model organisms such as *Arabidopsis* and maize through isolation of genes and proteins involved in the process (Bhatt *et al.* 2001; Schommer *et al*. 2003; Caryl *et al.* 2003).

Although mutant genes affecting pre-meiosis, meiosis, and post-meiosis have been reported interfering in microsporogenesis in higher plants, new mutations continue to be described. In this context, accessions of different species of the polyploidy tropical grass of the *Brachiaria* genus and interspecific hybrids have proven to be an excellent model for conventional studies of genetic control of meiosis. Spontaneous mutations causing abnormal nucleolar cycle (Risso-Pascotto *et al.* 2002), abnormal cytokinesis (Mendes-Bonato *et al*. 2002), absence of cytokinesis (Risso-Pascotto *et al.* 2003a), inability of chromosome co-orientation in meiosis II (Risso-Pascotto *et al.* 2003b), syncytes formation and cell fusions (Mendes-Bonato *et al*. 2001a, 2003), chromosome stickiness (Mendes-Bonato *et al.* 2001b), cytomixis (Utsunomyia *et al.* 2004), and abnormalities in pollen mitoses (Junqueira Filho *et a*l. 2003; Mendes-Bonato *et al*. 2004; Risso-Pascotto *et al.* 2005) have been reported in this genus. This paper reports an unique abnormality recorded in one accession of *Brachiaria brizantha* maintained in the Brazilian germplasm collection for the genus. The

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abnormality affected many steps of meiosis and culminated with microspore degeneration.

MATERIALS AND METHODS

Cytological studies were carried out on 25 accessions of *B. brizantha* from the Embrapa Beef Cattle *Brachiaria* collection grown in Campo Grande (state of Mato Grosso do Sul, Brazil) which comprises of 475 accessions in 15 species. The *B. brizantha* collection is represented by 233 accessions. Site characteristics are: climate type Aw: tropical humid savanna; average annual precipitation = 1526mm; average temperature = 22ºC; altitude 520m; latitude = 20º 28'S; longitude = 55º 40'W; soil type: Dark Red Latossol (59% sand; 8% silt; 33% clay; pH = 4.2 and poor chemical composition).

Inflorescences for meiotic studies were collected and fixed in a mixture of ethanol 95%, chloroform and propionic acid (6:3:2) for 24 hours; they were then transferred to 70% alcohol and stored under refrigeration until use. Microsporocytes were prepared by squashing and then staining with 0.5% propionic carmine. All meiotic phases were evaluated in more than 1000 microsporocytes per accession. Chromosome counting was done in microsporocytes at diakinesis. Photomicrographs were taken using a Kodak Imagelink – HQ, ISO 25 black and white film.

RESULTS

During cytological analysis of the 25 accessions of *B. brizantha*, one accession (B003) presented an abnormality never recorded previously for this genus. Particular attention and careful cytological analysis of meiotic behavior was done in 24 inflorescences. Pollen mother cells were tetraploid (2n=4x=36), with chromosomes pairing mainly as bivalents (Fig 1a). Only a few multivalent associations were found at diakinesis. Nine inflorescences presented only abnormalities related to polyploidy, i.e., precocious chromosome migration to the poles, laggards, and micronuclei in both meiotic divisions, leading to micronuclei in the tetrads (Table 1). In the remaining 15 inflorescences, however, abnormalities appeared after nucleolus disintegration at diakinesis, and from then on until the end of meiosis (Table 2). In these inflorescences, bivalents and multivalents were not able to congregate in the metaphase plate, remaining scattered or in groups in the cytoplasm (Fig 1b, c). Then, isolated bivalents, or the grouped ones, gave rise to pycnotic nuclei of different sizes (Fig 1d, e). These were later rejoined into telophase nuclei with apparently the same sizes and allocated peripherically (Fig 1f, g). Anaphases I were never seen. The first cytokinesis occurred normally (Fig 1g, h) and the meiocytes entered meiosis II. In prophase II, pycnotic nuclei were not observed; normal size nuclei with normal chromatin decondensation were found (Fig 1h, i). However, nuclei remained sideways, with both nuclei allocated in the same side (Fig 1h) or

Table 1 — Meiotic anomalies observed in 9 inflorescences of accession B003 of *B. brizantha* explained by polyploidy.

Phases	No. of analyzed cells	Abnormalities - No. of cells (%)
Metaphase I	160	Precocious chromosome migration 25 (15.60)
Anaphase I	155	Laggard chromosomes 108 (69.68)
Telophase I	146	Micronuclei 48 (32.88)
Prophase II	150	Micronuclei 42 (28.0)
Metaphase II	147	Precocious chromosome migration and micronuclei 62 (42.18)
Anaphase II	112	Laggard chromosomes 62 (55.36)
Telophase II	142	Micronuclei 52 (36.62)
Tetrad	182	Micronuclei 74 (40.66)

Table 2 — Meiotic abnormalities observed in 15 inflorescences of accession B003 of *B. brizantha* related to the anomaly under description.

Phases	No. of analyzed cells	Abnormalities - No. of cells (%)
Metaphase I	235	Absence of metaphase plate 235 (100.0)
Telophase I	241	Normal nuclei positioning 82 (34.02) Abnormal nuclei positioning 159 (65.98)
Prophase II	281	Nuclei positioned in opposite sides 34 (12.10) Both nuclei in the same side 47 (16.73) Central nuclei 200 (71.17)
Metaphase II	250	Absence of metaphase plate 250 (100.0)
Telophase II	311	Normal nuclei positioning 65 (20.90) Central nuclei 103 (33.12) Abnormal nuclei positioning 143 (45.99)
Tetrad	1304	Tetrads 22 (1.69) Triads 26 (1.99) Dyads 130 (9.97) Degenerated microspores 1126 (86.35)

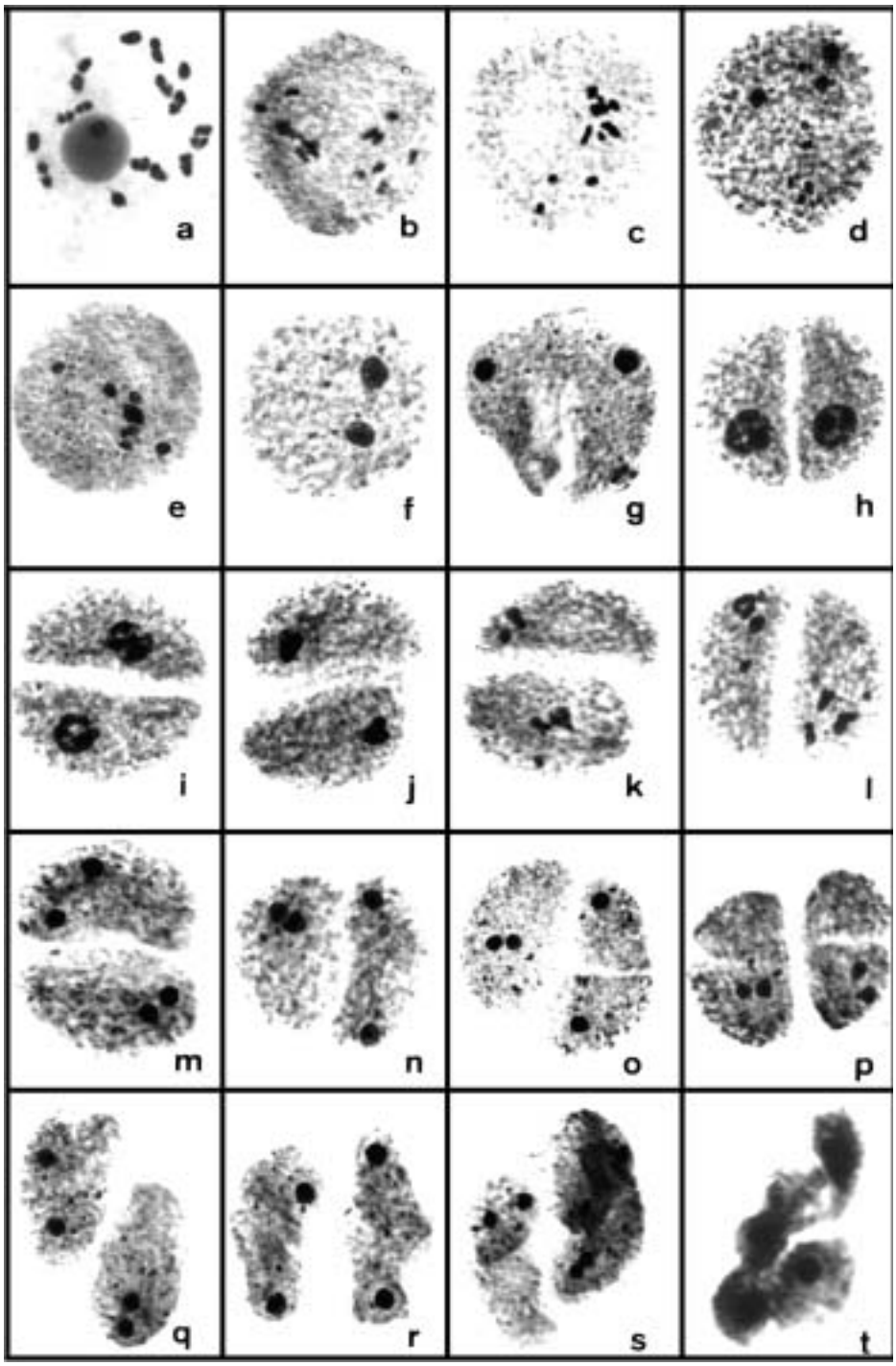

Fig. 1 — Some aspects of meiotic behavior in inflorescences affected by the abnormality. a) A typical diakinesis with 18 bivalents. b, c) Metaphases I with absence of equatorial plate and chromosomes scattered in the cytoplasm. d, e) Stage subsequent to metaphase I showing several pycnotic micronuclei of different sizes. f, g) Telophase I with pycnotic nuclei allocated peripherically. In g observe the beginning of cytokinesis. h, i) Prophase II exhibiting nuclei with normal chromatin condensation and presenting abnormal localization. j) Early metaphase II with nuclei allocated in opposite poles in the cells. k, l) Metaphase II without equatorial plate. Observe pycnosis in l. m, n) Telophase II with both pycnotic nuclei presenting abnormal positionin the cells. o) A triad formed by absence of cytokinesis in the cell with both telophase nuclei located in the middle of the cell. p) A tetrad showing two anucleate microspores and two binucleate ones formed by abnormal nuclei positioning. q, r) Binucleate microspores resulted from dyads starting the degenerative process. Observe the abnormal shapes of both cells. s,t) Microspores in advanced stage of degeneration. Observe the intense staining and the chromatin degeneration. (400 X).

allocated in opposite sides of the meiocyte (Fig 1i, j). After nuclear envelope disintegration, chromosomes were dispersed in the place occupied by the nuclei (Fig 1j, k, l) but, once more, metaphase plates were not observed. Despite the absence of typical anaphases, chromosomes were grouped into two small and pycnotic nuclei, allocated regularly or together in one side of the cell (Fig 1m, n), or remaining in the center of the cell (Fig 1o). From telophase II, independently of nuclei position, some meiocytes underwent the second cytokinesis (Fig 1o, p). When the nuclei were allocated in the center of the cell, cytokinesis did not occur (Fig 1o), and when both nuclei were in the same side, cytokinesis occurred normally, generating two binucleate cells and two anucleate ones (Fig 1p). Meiotic products were not always characterized by tetrads; the great majority was represented by dyads where the second cytokinesis failed to occur (Fig 1q, r). Independently of being a dyad, a triad, or a tetrad, the meiotic product entered degeneration as soon as microspores were released from the callose wall (Fig 1r, s). At this point, microspores acquired irregular shapes (Fig 1r, s), and became intensely stained (Fig 1s, t). At the end, nuclei degenerated and microspores gave rise to an amorphous mass (Fig 1t). Pollen grains were not found in these inflorescences.

DISCUSSION

Recent studies on accessions of different *Brachiaria* species and interspecific hybrids artificially produced between them have revealed a great amount of meiotic abnormalities, some of them never before reported in other plant species. This instability in the meiotic process could be explained, at least in part, by the mode of reproduction and ploidy level predominant in this genus. The majority of accessions are polyploid, mainly tetraploid, and polyploidy is closely associated with apomixis (Valle and Savidan 1996). Sexuality is generally found among diploid accessions.

Despite the great number of mutant genes described as affecting different steps of meiosis in higher plants, some inclusive for the genus *Brachiaria*, it is surprising how frequently new mutations can still be identified. The abnormality recorded in the present accession of *B. brizantha* arose spontaneously and may be considered a putative mutation. It was never reported in any other plant species. This abnormality involved a series of successive and, apparently independent events. Chromosomes were not able to congregate in the metaphase plate, remaining scattered in the cytoplasm. Absence or defective spindle fibers, or defects in kinetochore that impair spindle fiber attachment could be responsible for such abnormality.

Extensive chromosome reorganization and the formation of a single and transient spindle during mitosis and meiosis are essential for chromosome segregation. Due to the lack of centrosomes, mitotic and meiotic plant spindles are initiated by the chromosomes (Baskin and Cande 1990; Waters and Salmon 1997). The spindles assemble around the mass of chromosomes and initially appear as poorly organized, often multipolar, structures (Yu *et al.* 1999). Bipolar meiotic spindles emerge at mid-prometaphase in both meiosis I and meiosis II (Staiger and Cande 1990). The progressive self-organization of the focused bipolar spindle probably occurs through the combined effects of microtubule bundling and specific motor activities (Waters and Salmon 1997). At least 14 proven or putative proteins have been identified in plants, and some of them (the outer kinetochore proteins) are transient and only necessary for chromosome segregation (Jiang *et al.* 2003). Taking into account the molecular complexity involved in chromosome segregation in meiosis, it is possible that the *Brachiaria* accession under analysis underwent an alteration in some locus controlling a specific step of this process. As other cytological characteristics were expressed in subsequent meiotic stages, it is suggested that the gene involved in the character has a pleiotropic effect, causing pycnosis, abnormal nuclei localization, and finally, cell degeneration. A combination of various cytological defects was also reported in a meiotic mutant of *Arabidopsis thaliana* (Ross *et al.* 1997).

A mutation involving absence of spindle fibers and causing inability of bivalent congression at the metaphase plate was reported in maize (Taschetto and Pagliarini 1993). The abnormality recorded in the present accession of *B. brizantha* and that reported in maize were very similar in their behavior just after diakinesis, however, very different in the subsequent stages. In maize, the scattered bivalents gave rise to several normal micronuclei, whereas in the accession B003, micronuclei were pycnotic and rejoined latter into two telophase nuclei. Anaphase I was not recorded in *Brachiaria* or maize. In the latter, after micronuclei formation, cytokinesis occurred giving rise to polyads, and microspores of different sizes developed into abnormal and sterile pollen grains. In *Brachiaria*, however, although abnormal, meiosis

progressed to the end, and microspores degenerated before pollen differentiation, causing total sterility. Pycnosis is an abnormality generally caused by chromosome stickiness. Sticky chromosomes were reported during microsporogenesis in one accession of *B. brizantha* (MENDES-BONATO *et al.* 2001b), but not detected in the present accession. Another feature typical of the B003 accession was the position of telophase nuclei in both meiotic divisions, deeply characterizing this abnormality.

The events leading to cell degeneration in accession B003 of *B. brizantha* revealed some morphological characteristics typical of programmed cell death (PCD), or apoptosis, observed in plants and animals. These include chromatin condensation and fragmentation, which acquire electron dense aspect often described as pycnotic; the cytoplasm also condenses and both nuclear and cellular outlines appear lobed (BEERS 1997; LEE and CHEN 2002). At the biochemical level, apoptosis results in fragmentation of nuclear DNA, probably catalyzed by a calcium-dependent nuclease (WYLLIE *et al.* 1980). In plants, PCD has been observed to occur during many stages of plant reproduction (DONG *et al.* 1998; WANG *et al.* 1999), including the degeneration of haploid megaspores (BEERS 1997). In the fern *Marsilea*, the megaspore death involving chromatin pycnosis led BELL (1996) to propose that the death was apoptotic. An extensive report was presented by BEERS (1997) about the death of synergids. However, none was found in literature about the death of microspores, reproductive structures meant to live, not to die. The microspore degeneration observed in the present accession of *B. brizantha* with morphological characteristics of PCD could represent a mutation during microsporogenesis affecting different steps of both meiotic divisions and leading to cell death. This accession may represent an important tool for screening genes involved in apoptosis during meiosis.

An understanding of the meiotic process is pivotal to furthering research on reproduction, fertility, genetics and breeding, and in plants, has implications for crop production (ARMSTRONG and JONES 2003). The production of viable pollen involves a complex series of developmentally regulated processes, involving the differentiation and degeneration of specialized tissues, and the coordinated expression of thousands of sporophytic and gametophytic genes (PEIRSON *et al.* 1996). Male-sterile mutants have been widely reported in higher plants (KAUL 1988; PEIRSON *et al.* 1996; JIN *et al.* 1997; HE and MASCARENHAS 1998) and have represented an important breeding tool for species of economic value. Male sterility could be caused by genes acting at meiosis or post-meiosis. In the latter cases, microspore degeneration prior to pollen development, such as observed in the present accession, can be explained by abnormal tapetal development (JIN *et al.* 1997). Further studies could help to elucidate the causes of microspore degeneration in this accession.

In *Brachiaria*, a genus of forage grass of African origin that has exhibited good adaptation to the tropical savannas, especially in Brazil, the breeding program aims at intra- and interspecific hybridization (VALLE and SAVIDAN 1996). However, as apomixis severely complicates the crossing, hybridization is performed only by using the few sexual accessions as mother plants and apomictics as pollen parents. Because the sexual progenitors are allogamous and emasculation is difficult to accomplish, a male-sterile mutant might improve the efficiency of the program by avoiding self-pollination of sexual genitors. If, however, the mutant is causing serious sterility it should be reconsidered in the selection of potential genitors because apomictic accessions of *Brachiaria* are also pseudogamous. This means that the central-cell nucleus needs to be fertilized for endosperm and healthy seed development, even though the egg cell is not fertilized in an aposporous embryo sac. The present abnormality/putative mutation causing microspore degeneration totally prevented pollen development in affected inflorescences. For a cultivar to be successfully adopted, large amounts of seed need to be available. Therefore a normal meiotic process is required in hybrids or accessions released as cultivars. The pasture area covered by *Brachiaria* today exceeds 40 million hectares in Brazil and a new cultivar could potentially impact cattle production in this area if enough good seed is provided over several years.

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