

# Symmetric pollen mitosis I and suppression of pollen mitosis II prevent pollen development in *Brachiaria jubata* (Gramineae)

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## Abstract

Microsporogenesis and pollen development were analyzed in a tetraploid ( $2n = 4x = 36$ ) accession of the forage grass *Brachiaria jubata* (BRA 007820) from the Embrapa Beef Cattle *Brachiaria* collection that showed partial male sterility. Microsporocytes and pollen grains were prepared by squashing and staining with 0.5% propionic carmine. The meiotic process was typical of polyploids, with precocious chromosome migration to the poles and laggards in both meiosis I and II, resulting in tetrads with micronuclei in some microspores. After callose dissolution, microspores were released into the anther locule and appeared to be normal. Although each microspore initiated its differentiation into a pollen grain, in 11.1% of them nucleus polarization was not observed, i.e., pollen mitosis I was symmetric and the typical hemispherical cell plate was not detected. After a central cytokinesis, two equal-sized cells showing equal chromatin condensation and the same nuclear shape and size were formed. Generative cells and vegetative cells could not be distinguished. These cells did not undergo the second pollen mitosis and after completion of pollen wall synthesis each gave rise to a sterile and uninucleate pollen grain. The frequency of abnormal pollen mitosis varied among flowers and also among inflorescences. All plants were equally affected. The absence of fertile sperm cells in a considerable amount of pollen grains in this accession of *B. jubata* may compromise its use in breeding and could explain, at least in part, why seed production is low when compared with the amount of flowers per raceme.

## Key words

- *Brachiaria jubata*
- Male sterility
- Microgametogenesis
- Pollen mitosis
- Sperm cell

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## Introduction

The events that culminate in the formation of the pollen grain involve an intricate and tightly controlled set of structural and molecular gene expression events in both the

gametophytic and sporophytic tissues of the anther. Firstly, pollen mother cells undergo meiosis, giving rise to a tetrad of haploid cells. The individual cells of the tetrad are released as free microspores by the action of callase, an enzyme produced by the tapetum

layer of the anther (1).

After an initial burst of growth and exine synthesis, the haploid microspores undergo cytological reorganization in preparation for a key event in pollen development, i.e., the asymmetric mitotic division (2). This mitotic division, also known as 'pollen mitosis one' (PM I), results in the formation of two dimorphic cells, the vegetative cell and the generative cell, with very different fates (1-5). The smaller generative cell produces two sperm cells (male gametes) after 'pollen mitosis two' (PM II), whereas the larger vegetative cell, which constitutes the bulk of the pollen grain, produces an elongated pollen tube (a gametophytic cell) to deliver the male gametes to the embryo sac (5). The generative/sperm cells have very little cytoplasm and sparse nuclear chromatin, suggesting that they have lower transcriptional activity (6).

Defective mutants operating at various stages of microspore development have been reported in *Arabidopsis* (7-16) and *maize* (17-20). In *Brachiaria decumbens* c.v. Basilisk, a variety extensively used for forage

in Brazil, a possible mutation affecting pollen development has been recently reported (21). In the current paper we describe an abnormal pollen mitosis division in one accession of *Brachiaria jubata*, a species of interest in the forage breeding program.

## Material and Methods

Cytogenetic studies were carried out on the BRA 007820 accession of *B. jubata* from the Embrapa Beef Cattle *Brachiaria* collection kept in Campo Grande (State of Mato Grosso do Sul, Brazil), which comprises 475 accessions of 15 species.

Inflorescences for microsporogenesis and microgametogenesis studies were collected and fixed in a mixture of 95% ethanol, chloroform and propionic acid (6:3:1) for 24 h, transferred to 70% alcohol and stored under refrigeration until use. Microsporocytes and pollen grains were prepared by squashing and staining with 0.5% propionic carmine. All meiotic phases and stages of pollen development were evaluated in inflorescences

Table 1. Frequency of abnormalities recorded in *Brachiaria jubata* pollen during microsporogenesis.

Phase	No. of cells analyzed	No. of abnormal cells (%)	Abnormalities	No. of cells
Metaphase I	155	52 (33.55)	Precocious chromosome migration Fusion	46 6
Anaphase I	172	45 (26.16)	Laggard chromosomes Fusion	43 2
Telophase I	158	15 (9.50)	Micronuclei Fusion	13 2
Prophase II	161	22 (13.66)	Micronuclei Microcytes	18 4
Metaphase II	245	63 (25.71)	Precocious chromosome migration Microcytes	54 9
Anaphase II	155	26 (16.77)	Laggard chromosomes Microcytes	24 2
Telophase II	154	13 (8.44)	Micronuclei Microcytes	8 5
Meiotic product	158	22 (13.92)	Tetrad with micronuclei Tetrad with microcytes	18 4

collected from four plants of this accession obtained by vegetative propagation. Photomicrographs were obtained with a Wild Leitz microscope using Kodak Imagelink-HQ, ISO 25 black and white film.

## Results

Cytological characterization of the accession was carried out from meiosis to pollen formation. The chromosome number scored at diakinesis revealed tetraploidy ( $2n = 4x = 36$ ). The meiotic process was typical of polyploids (Table 1), with chromosomes associating in bi-, tri-, and quadrivalents. Precocious chromosome migration to the poles, laggards and micronucleus formation were found in both meiosis I and II, resulting in tetrads with micronuclei in some microspores (Figure 1a). After callose dissolution, microspores were released into the an-

ther locule and seemed to be normal. Each microspore then initiated PM I. In most microspores, pollen mitosis developed normally, with the characteristic polarization of the nucleus and its subsequent asymmetric division (Figure 1b) into two unequal cells (Figure 1c). However, in 11.1% of the microspores, after callose dissolution (Figure 1d), the released microspores underwent symmetric pollen mitosis, i.e., the nucleus was not displaced from the cell center to one side of the cell (Figure 1e). The typical hemispherical cell plate, like that observed in normal pollen grains (Figure 1c), was not seen in these cases, and all mitotic stages from prophase to telophase occurred without polarization (Figure 1f and g). After cytokinesis, two equal-sized cells were observed as a result of symmetric division (Figure 1h). Both cells also showed equal chromatin condensation and the nuclei had

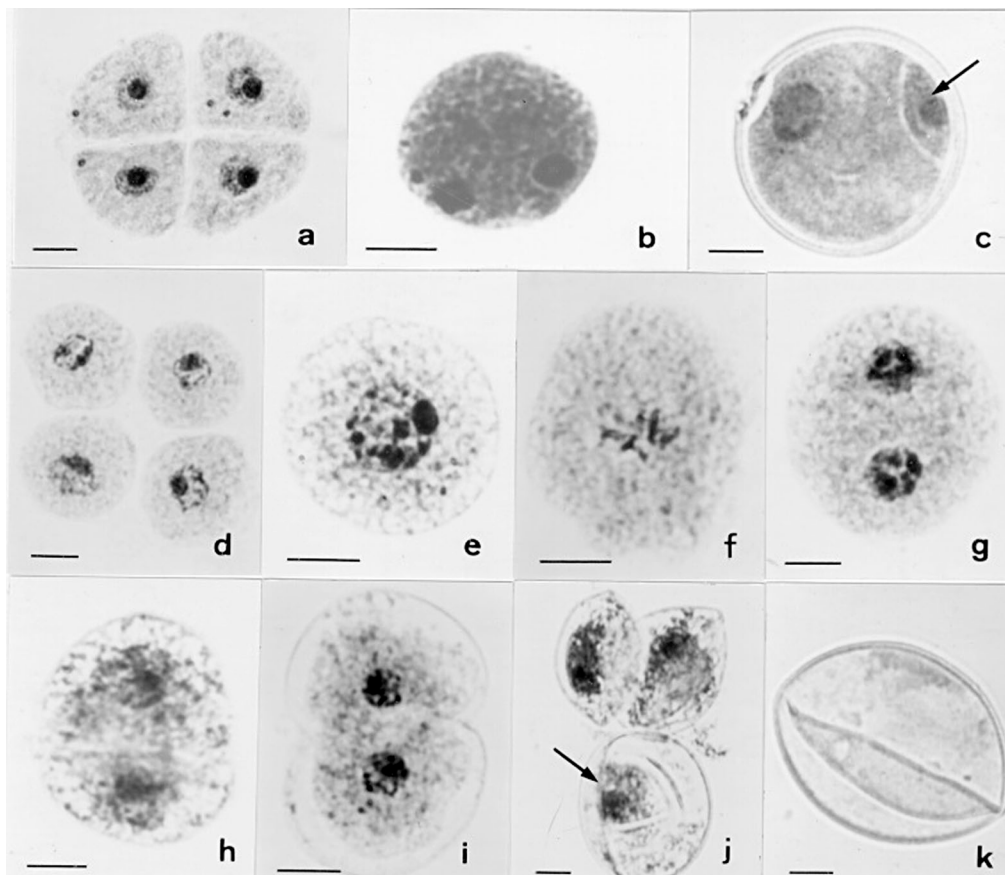


Figure 1. Pollen development in *Brachiaria jubata* (BRA 007820). *a*, Normal tetrad with several micronuclei in microspores. *b*, Normal pollen mitosis (PM I): telophase. Note the polarized nucleus position. *c*, Pollen grain after a normal and asymmetric PM I. Observe the typical hemispherical cell plate between the generative (arrow) and the vegetative cells. *d*, Tetrad after callose dissolution. *e*, Abnormal released microspore without nuclear polarization. *f*, *g*, Symmetric PM I: metaphase (*f*) and telophase (*g*). Observe the lack of displacement of cell division from the cell center. In telophase (*g*), note that both nuclei are of equal sizes and chromatin is equally condensed. *h*, Cytokinesis after telophase dividing the microspore into two equal-sized cells. *i*, Two uninucleate sister pollen grains. *j*, Two sister pollen grains flanked by a normal one (arrow). Note that the normal pollen grain is double the size of those resulting from symmetric PM I. *k*, Sterile pollen grain. (Scale bars = 0.5  $\mu$ m).

the same spherical shape and were of equal sizes (Figure 1h), i.e., the generative nucleus and vegetative nucleus could not be differentiated. After this stage, each cell developed into a pollen grain (Figure 1i). These cells did not undergo PM II and completed the synthesis of the pollen wall (Figure 1j). Each pollen grain was half the size of a normal pollen grain (Figure 1j). The frequency of abnormal pollen mitosis varied among flowers and also among inflorescences. All plants were equally affected. Sterile pollen grains resulted from this abnormal pollen development (Figure 1k). Pollen sterility, however, was much higher due to abnormal chromosome segregation caused by polyploidy.

## Discussion

The pollen grain is released from the anther when it consists of just generative and vegetative cells in approximately 70% of plant families, and therefore PM II occurs while the pollen tube grows through the female pistil. In other plant families, including the Gramineae, this second mitotic division occurs before the pollen is shed from the plant (1). Therefore, in the genus *Brachiaria*, normal pollen production should display this mitosis before pollen shedding from the anther. In the accession of *B. jubata* under analysis, abnormal pollen mitosis (PM I) impaired the generative and vegetative cell differentiation, and the absence of PM II prevented sperm cell formation in over 10% of the microspores, causing pollen sterility.

Pollen development and male gametogenesis are critically dependent upon cell polarization which contributes to a highly asymmetric cell division (PM I). In normal microspores, the nucleus is displaced from the cell center to one side of its long axis at the G<sub>1</sub> phase of the microspore cell cycle. Displacement continues throughout the mitotic cycle (22) and the nucleus polarization is maintained by the microtubule cytoskel-

eton (4). In the present accession, displacement of the nucleus did not occur in abnormal microspores. A similar behavior was also reported to occur naturally in *B. decumbens* (21), whereas the symmetric divisions observed in *Tulipa gesneriana* (23), tobacco (24), and *Arabidopsis* (15) were induced.

Asymmetric cell division relies on the formation of a typical hemispherical cell plate, in which spindle orientation is very important for normal differentiation of the generative and vegetative cells (25). An asymmetric, cone-shaped spindle perpendicular to the plasma membrane appears in this division (26,27). The generative pole lies adjacent to the plasma membrane and the vegetative pole is located in the inner cytoplasm of the microspore. In the present study, a hemispherical cell plate was not recorded in the first pollen mitosis of this *B. jubata* accession, as also reported by Junqueira Filho et al. (21) for a *B. decumbens* accession. Tanaka (4) postulated that microtubules are involved in nuclear displacement and in the maintenance of the nuclear position of the hemispherical phragmoplast, and both are prerequisites for asymmetric cell division in microspores. Based on this assumption, we suggest that the abnormal plants found in both *Brachiaria* species display anomalies in microtubule organization since nuclear displacement did not occur, nor was the hemispherical cell plate formed.

In normal plants, the vegetative and generative cells have different structures and developmental fates (4,15,28). While the large vegetative cell accumulates an abundance of stored metabolites required for rapid pollen tube extension, the smaller cell is enclosed by vegetative cell cytoplasm and contains relatively few organelles and stored metabolites. A peculiar feature of the abnormal microspores of *B. jubata*, similar to the abnormal microspores of *B. decumbens* reported by Junqueira Filho et al. (21), was the lack of differentiation between the vegetative and the generative cell. Both were of

equal sizes. According to Tanaka (4), when the asymmetric cell division is inhibited or altered into a symmetric division, differentiation of the generative cell is prevented, and a cell-within-a-cell is never formed thereafter. This might indicate that there is an intimate relationship between asymmetric cell division and the subsequent fates of the two daughter cells. In *B. jubata*, the lack of differentiation between the two daughter cells resulting from the first pollen mitosis compromised the pollen fate. After an evident cytokinesis among them, PM II did not occur, and each cell immediately differentiated into pollen grains. Thus, two half-sized pollen grains with a single nucleus were originated from each microspore. In contrast, in the abnormal microspore of *B. decumbens* reported by Junqueira Filho et al. (21), the two daughter cells underwent another symmetric pollen mitosis after symmetric PM I, the result being four uninucleate and sterile pollen grains at the end of PM II. The occurrence of PM II in *B. decumbens* (21) is the main differential characteristic between the two *Brachiaria* species.

Another characteristic of PM I for both *Brachiaria* species is the similar chromatin condensation in both resulting cells, i.e., there is no difference between the nuclei. In normal asymmetric PM I division, the nuclei of the generative cell and the vegetative cell respectively contain diffuse and highly condensed chromatin, visible just after PM I. Such striking difference in the structure of chromatin is assumed to be associated with the difference in gene activity between the two types of nuclei, the generative cell nucleus being less transcriptionally active than the vegetative one (29). The absence of chromatin differentiation between nuclei resulting from PM I in *B. jubata* suggests that both are equally active.

The production of a functional pollen grain involves a complex series of regulated processes and requires the expression of a large number of sporophytic and gameto-

phytic genes (20). First pollen mitosis appears to be a critical point for gametophytic development (1), because a specific set of genes is activated at this time. Mutations that affect the nuclear division pattern at the gametophytic stage have been scarcely registered (15,16,20,30,31). An abnormality similar to that found in the present accession of *B. jubata* causing symmetric PM I and suppressing PM II has not been described in other species. This abnormality was not regularly expressed in the microsporocytes of all flowers or inflorescences examined. Only 11.1% of the microspores presented abnormal pollen mitosis. An incompletely penetrant phenotype resulting in equal, unequal and partial divisions at PM I has been reported in the gametophytic mutation *gemini pollen1* of *Arabidopsis thaliana* (15), and in *B. decumbens* (21), both disrupting microspore polarity, division asymmetry and pollen cell fate.

For double fertilization in angiosperms, two sperm cells are necessary. One sperm cell fuses with the egg cell to form the embryo and the other fuses with the central cells to form the endosperm. Abnormalities that cause male sterility in plants are considered to be of great interest in breeding programs because they might prevent self-pollination of sexual genitors but also cause lack of seed set in genotypes with important breeding traits. The *Brachiaria* breeding program aims at producing hybrids by intra- and interspecific crosses using sexual accessions or hybrids as mother plants and apomictic accessions as pollen parents. Polyploid accessions of *Brachiaria* are pseudogamous (apomictic), which 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 absence of fertile sperm cells in a considerable amount of pollen grains in this apomictic accession of *B. jubata*, caused by symmetric pollen mitosis (PM I), added to the unbalanced gametes



generated by irregular chromosome segregation during meiosis due to polyploidy, may compromise its use in breeding and could explain, at least in part, why seed production is low when compared with the amount of flowers per raceme.

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