Brachiaria brizantha is a forage grass that has several apomictic accessions. B. brizantha cv. Marandu is a natural tetraploid aposporous apomict widely cultivated in Brazil. Pseudogamy was detected in this species by observation that seed set is suppressed in plants that have had the stigmas excised from the flowers. The egg cell develops parthenogenetically in the apomictic plants, meaning that fertilisation is necessary for the formation of the endosperm. A thorough knowledge of all the events of seed formation in natural apomictic plants is essential for a complete understanding of this mode of reproduction. In this paper, we show direct evidence of pseudogamy in B. brizantha through the cytological analysis of polar nucleus fertilisation and the determination of triploid level of the endosperm tissue. The development of the male gametophyte gives rise to a reduced tri-celled pollen, the viability of which varies throughout the year, reaching 88% in the peak of the flowering period. Discharge of the male gamete takes place around 10 h after pollination and monospermy is the predominant system observed. Precocious embryony was also observed in these plants; embryos arise from egg cells. Endosperm development followed the free nucleus model and was associated with the presence of an embryo. Cellarisation and reserve uptake occurred 2 days after pollination (DAP) and mature endosperm was observed 8 DAP. The triploid level of the endosperm in the apomictic accession confirmed the 2:1 maternal:paternal ratio of genome contribution in the tissue.

**Keywords** Pseudogamy · Parthenogenesis · Endosperm · Pollen · Seed

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**Introduction**

Many genera of the Poaceae family are economically important forage grasses and produce seeds through apomixis, an asexual mode of reproduction (Nogler 1984; Hanna and Bashaw 1987). An example is the genus Brachiaria, a native of Africa that is widely cultivated in South America. The agronomic and nutritional qualities of these grasses have made them the major pasture plants in Brazil, where estimates of areas of Brachiaria plantation range from 30 to 70 million ha (Miles et al. 1996).

Analysis of 275 different natural accessions of B. brizantha revealed just a single diploid (2n=2x=18) sexual accession, BRA 002747 (Miles and Valle 1991; Valle and Savidan 1996). The other accessions were tetraploid (2n=4x=36) facultative apomicts (Miles and Valle 1991; Valle and Savidan 1996). The apomicts in Brachiaria present unreduced embryo sacs with a four-nucleated structure of the Panicum type while sexual plants have a Polygonum-type embryo sac. The mode of reproduction of the different accessions is inferred by the percentage of each type of embryo sac found using clearing techniques. In B. brizantha, the range of sexuality varied from 0 to 74% (Valle and Savidan 1996), cv. Marandu showing 98% of Panicum-type embryo sac (Valle and Savidan 1996; Araujo et al. 2000). The Panicum-type embryo sac in B. brizantha is induced in nucellar cells, characterising apomixis as apospory (Gobbe et al. 1981; Dusi and Willemse 1999; Araujo et al. 2000).

Pseudogamy in angiosperms implies that pollination is necessary for endosperm formation; the polar nuclei must be fertilised while the egg cell develops parthenogenetically (Asker and Jerling 1992). Ngendahayo demonstrated that apomixis is pseudogamous in Brachiaria species by showing the requirement of pollination for seed development (Ngendahayo 1988). In spite of the agronomic importance of these species, and the interest in apomixis, data on the reproductive development and caryopsis formation of Brachiaria species are limited. General aspects of male and female gametophyte development have been described for B. brizantha, B. dec-
umbens and F1 hybrids derived from crosses with the artificially induced tetraploid B. ruziziensis, and a developmental calendar was established (Ndikumana 1985; Lutts et al. 1994). Later, Naumova et al. (1999), Dusi and Willemse (1999) and Araujo et al. (2000) detailed female gametophyte development in apomictic and sexual B. decumbens and B. brizantha. The aposporous initials in B. decumbens arise associated with the megaspore mother cell (Dusi and Willemse 1999) while in B. brizantha they are associated with the reduced megaspores (Araujo et al. 2000). In B. setigera, analyses of embryo and endosperm outcome suggested that polylembrony was due to the formation of embryos arising from egg cell, synergid and endosperm cells (Muniyamma 1977). Although these reports describe important steps of apomictic reproductive development, no data on the fertilisation of the polar nucleus have yet been presented.

The results shown here demonstrate the importance of pollination in the apomictic B. brizantha cv. Marandu and describe fertilisation events and morphological details of the first steps of caryopsis formation. This contributes to a better understanding of seed development in an aposporous apomict.

Material and methods

Plant material

Plants of B. brizantha (A. Rich) Stapf cv. Marandu (accession BRA 00591) tetraploid apomict (2n=4x=36) were vegetatively propagated at Embrapa Genetic Resources and Biotechnology, Brasilia-DF, Brazil and maintained in an experimental field.

Analysis of floral biology

Floral biology aspects were analysed through field observations during the period February to July, 1998 and 1999.

To estimate cv. Marandu stigma receptiveness, flowers collected at 1 h intervals from 2–3 h prior to anthesis until 48 h after anthesis were immersed in 3% hydrogen peroxide solution (Kearns and Inouye 1993) and the peroxidase activity in living stigma tissue was confirmed through observation of bubble formation.

Analysis of male gametophytes

Anthers were classified according to their length in stages I (anthers of 0.7 mm in length) and II (0.78 mm), corresponding to early and late microsporogenesis, respectively, and stages III (2.6 mm) and IV (3.1 mm) corresponding to early and late microgametogenesis, respectively. Approximately ten anthers of each stage were collected and fixed in 2% glutaraldehyde, 2% paraformaldehyde in 0.05 M cacodylate buffer pH 7.0, dehydrated with acetone, and embedded in Spurr's resin (Spurr 1969). Sections 3–4 µm thick were stained with toluidine blue and observed using a Zeiss-Axiophot light microscope. Callus detection was performed according to Martin (1959). Pollen viability was evaluated monthly through the analysis of 400 pollen grains, randomly collected from ten different plants at anthesis. Pollen was monthly harvested using the same plants. Pollen grains were observed after anther fixation with 70% ethanol and subsequent staining with acetic carmine (Fukui and Nakayama 1996). Germination, growth and discharge of male gametes were observed in cv. Marandu until controlled self-pollination. Approximately 20 pollinated pistils were collected at anthesis and another 20 at every hour after pollination (HAP) for 12 h. After fixation in 70% ethanol, clearing using 9 N NaOH for 8–10 min at 60 °C and staining with 0.1 M K2PO4 pH 7.0 containing 0.1% aniline blue (Martin 1959), pollen tube growth was observed under UV light using a Zeiss-Axiophot microscope.

Analysis of caryopsis development

Morphological analysis of embryo and endosperm development in cv. Marandu was observed by sectioning pistils collected at anthesis, hourly post-anthesis and daily for 9 days after pollination (DAP). After fixation and dehydration of the samples as described in the section on analysis of male gametophytes, some samples were embedded either in Paraplast or JB4 (JB4 Embedding kit; Polysciences, Warrington, Fla.). Sections of Paraplast- and JB4-embedded samples of 9 µm and 5 µm thick, respectively, were stained with basic fuchsin/Astra blue. Sections were analysed using a Zeiss-Axiophot light microscope. Many (502) caryopsis of cv. Marandu were observed to determine polylembrony frequency.

Endosperm cytogenetic analysis

Caryopsis collected 9 DAP were incubated in 1% bromonaphthalene for 2 h and acetic acid: ethanol (1:3 v/v) for 24 h at room temperature, hydrolysed in 1 N HCl solution for 11 min at 60 °C and stained with Feulgen to determine the chromosome number in the endosperm cells of cv. Marandu.

Results

B. brizantha cv. Marandu has a terminal inflorescence with 2–6 racemes, containing 20–36 spikelets each. The racemes mature from the apex to the bottom of the inflorescence. Within the raceme, central spikelets are the first to open. It takes 6–7 days for all spikelets of the raceme to open. Each spikelet has two flowers: a staminate and a hermaphrodite containing two lodicules, three stamens and a single pistil with bifid style and hairy, red-coloured, stigmas. The ovary is unilocular and has one anatropous ovule. The flowering period of cv. Marandu at the experimental field in Brasilia, DF (Brazil) initiated in February and extended into July in 1998 and 1999. The flowering peak occurred during March/April when day-length was around 12 h, with humidity of 70% and temperatures between 17 and 30 °C. Cv. Marandu anthesis occurred between 7.30 a.m. and