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Microsporogenesis, viability and morphology of pollen grain in accessions of *Cynodon* L. C. Rich. (Poaceae)

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ABSTRACT

Plants of the genus *Cynodon* were considered as weed for a long period. However, this scenario has changed when it was observed that many species could be used in forage farming and for the recovery of degraded areas. Due to the high expansive potential, *Cynodon* spp. became an option for producers and aroused interest for breeding programs, besides requiring further studies on genetic and cytogenetic variability. Research on meiosis in *Cynodon* are scarce since the inflorescences are small and the standardization of the stages of spikelet collections is difficult. The aim of the present study was to evaluate the microsporogenesis, viability and morphology of the pollen grains of two accessions of *Cynodon dactylon var. dactylon* (L.) Pers. (2n = 4x = 36, bermudagrass) and two accessions of *Cynodon nlemfuensis var. nlemfuensis* Vanderyst (2n = 2x = 18, stargrass). The slides were prepared by squash technique and stained with 2% carmine propionic. For the viability analyses of pollen grains, 2% propionic carmine and Alexander's stain were used. The measurements and the morphology of the pollen grains were defined from analyses with the acetolysis technique and scanning electron microscopy. The meiosis of the four accessions/species was regular. The staining tests showed variations in the viability rate of the pollen grain between the diploid and tetraploid species, being higher among the tetraploids. The pollen grains measured 21.1 and 28.8 μm on average for both species and were classified as oblate spheroidal, monocolpates and showed no ornamentation, such as spicules and other attachments with non-roughened, micro reticulated exine.

1. Introduction

The genus *Cynodon* L. C. Rich. belongs to the Poaceae family and their species show significant economic value due to their use as forage for animal feeding, erosion protection and sports field coverage (Neiva et al., 1999; Taliaferro et al., 2004; Wu and Taliaferro, 2009). The plants from this genus are grouped in Bermuda grasses containing stolons and rhizomes, and in Star grasses, showing only stolons, leaves and stems larger than those of Bermuda grasses (Harlan, 1970; Andrade et al., 2009).

They are perennial grasses from tropical and subtropical climates, with wide distribution in Africa, but also present in South America (Caro and Sánchez, 1969; Harlan et al., 1970; Filgueiras and Valls, 2015). *Cynodon dactylon* (L.) Pers. belongs to the Bermuda grass group and is the most important species of the genus due to its prevalence, wide geographic distribution and its morphological variants, such as var. *afghanicus*; var. *aridus*; var. *dactylon*; var. *coursii*; var. *elegans* and var. *polevansii*. In the *dactylon* variety, three races are found: tropical, temperate and seleucidus (Wu and Taliaferro, 2009). *Cynodon nlemfuensis* Vanderyst is the most promising of the star grass group since it has favorable characteristics as forage and is genetically related to *C. dactylon* (Wu and Taliaferro, 2009).

Until the mid-1940s, *Cynodon* plants were considered as weeds due to their high adaptability, growth, and establishment in the environment. Still in 1950, farmers tried to control it by several methods, such as weeding, animal control and chemical control (Horowitz, 1996). However, according to Wu and Taliaferro (2009), important characteristics observed in this genus have aroused the interest in developing cultivars for use in forage farming, mainly due to the resistance to cold, adaptation to the temperate climate and grazing efficiency.

When considering the high expansive potential, *Cynodon* spp. has become an option for agricultural producers (Neiva et al., 1999), since grazing is a low-cost source of feed (Primavesi et al., 2004). In Brazil, plants develop satisfactorily even in soils with low fertility (Sousa et al., 1998).

Despite the importance, the numbers of species to the genus is still controversial, since there are studies reporting the occurrence of 20 species (Caro and Sánchez, 1969); 9 species and 10 varieties (Harlan et al., 1970); 10 species (Clayton et al., 2009) and, more recently, 13 species ("The Plant List, 2016"). In addition, cytogenetic studies for this

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group are still scarce, particularly summarizing to chromosome counts and determining ploidy levels, ranging from diploid to hexaploid and 16 to 54 chromosomes (Avdulow, 1931; Taliaferro et al., 2004; Dhaliwal and Gupta, 2011), and basic chromosome number x = 9 (Avdulow, 1931; Forbes and Burton, 1963) and x = 8 (Dhaliwal and Gupta, 2011).

Studies of meiosis for the genus are also scarce and some very old (Forbes and Burton, 1963; Gupta and Srivastava, 1970; Hanna and Burton, 1977; Brilman et al., 1982; Dhaliwal and Gupta, 2011). This type of study becomes an important ally to understand and follow the whole process for the formation of gametes and confirm the basic number of chromosomes. In addition, the monitoring of post-meiotic events, which result in the formation of pollen grain, is also fundamental, since they allow advancing in knowledge applied to taxonomy, palynology and breeding programs.

Based on the above, the aim of the present study was to evaluate the microsporogenesis, viability, and morphology of the pollen grains of four accessions of *Cynodon*.

2. Material and methods

2.1. Plant material

The evaluations were performed in four accessions of *Cynodon* (Table 1), provided by Embrapa Gado de Leite, Juiz de Fora, Minas Gerais State, Brazil, which are originated from the active germplasm bank of the United States Department of Agriculture-USDA, Tifton, GA, USA.

2.2. Evaluation of meiosis and pollen grain viability

The spikelets were collected between 9 a.m. and 4 p.m., with temperatures varying between 25 °C and 35 °C and fixed in solution of ethyl alcohol solution: acetic acid: propionic acid (6:3:2) stored in microtubes at −4 °C. For meiotic analysis, the spikelets were excised under a stereoscopic microscope to extract the anthers, which were cut and macerated on the slide with a drop of 45% acetic acid. Subsequently, the slides were prepared by squash technique (Guerra and Souza, 2002) and stained with 2% propionic carmine.

Prior to the fixation step, the size of the spikelets was measured using a caliper, and subsequently the size of each spikelet/slides was related to the phases of meiosis observed in each slides assessed.

For pollen viability, the procedure was similar to the meiosis, but two dyes were used: 2% propionic carmine and 2% Alexander's stain. A total of 200 grains of pollen/slide and five slides per dye were evaluated. For staining with propionic carmine, the pollen grains that showed highly stained red cytoplasm were considered as viable and the unstained as unviable. For Alexander's stain, purple-stained grains were considered as viable and weakly green-stained as unviable.

The slides were evaluated on a light microscope (Carl Zeiss, AxioLabA1), equipped with a microcamera (AxioCam ICc1), for capturing the images. Scott-Knott statistical test ($p < 0.05$) was applied using the SISVAR software (Ferreira, 2003) in pollen viability analyzes.

Table 1
Identification of the species, code/accession and chromosome number of the evaluated plants.

Species	Code	Accession	Chromosome number[a]
Cynodon dactylon var. dactylon	EGL 9	PI 224141-29	2n = 4x = 36
Cynodon dactylon var. *dactylon*	EGL 10	PI 29117102 PL 18	2n = 4x = 36
Cynodon nlemfuensis var. nlemfuensis	ERX 7	[b]	2n = 2x = 18
Cynodon nlemfuensis var. *nlemfuensis*	ERX 21	[b]	2n = 2x = 18

[a] Chiavegatto et al., 2016.
[b] Plants originated from breeding program, developed by Embrapa Dairy Cattle.

2.3. Evaluation of the pollen grain through acetolysis technique

The employed technique followed the recommendations of Erdtman (1960) and modified according to Melhem et al. (2003). The collected spikelets were macerated using a needle in a microtube containing 45% acetic acid and centrifuged for 10 min at 2500 rpm. Subsequently, the supernatant was removed and replaced with distilled water, centrifuged again for 10 min at the same speed and discarded at the end. Then, 1 mL of acetolysis solution (1 sulfuric acid: 9 pure acetic anhydride) was placed in a water bath at 87 °C for 2 min. The material was then centrifuged twice, removing the supernatant after each centrifugation. After the last centrifugation, the supernatant was replaced with 50% glycerinated water. The material was stored in a microtube at 10 °C for 24 h until the preparation time of the slide.

The glycerinated water was discarded and the material was placed in slides with portions of glycerinated gelatin (100 mL of distilled water, 100 mL of glycerin, 17 g of colorless gelatin and 1 g of phenol) subjected to heating in a heater plate and covered with coverslips.

The slides were evaluated on a light microscope (Carl Zeiss, AxioLabA1), equipped with a microcamera (AxioCam ICc1), for capturing the images. For each accession, five slides were evaluated and the measurements of the polar axis (P), equatorial axis (E) and exine thickness were performed in 10 pollen grains per slide using ImageJ software version 1.44 (Research Services Branch, U.S. National Institutes of Health, Bethesda, MD, USA.). Pollen grains were classified according to Erdtman (1943). Subsequently, the data were submitted to the Scott-Knott test ($p < 0.05$) using the SISVAR software (Ferreira, 2003).

2.4. Analysis of pollen grain ultrastructure through scanning electron microscopy

To observe the surface of the pollen grain, the samples were fixed in Karnovsky solution, until the moment of analyses. Samples were washed in 0.05 M cacodylate buffer (three times - 10 min each) and post fixed in 1% osmium tetroxide for 4 h at room temperature. Then, they were progressive dehydrated in acetone (25%, 50%, 75%, 90% and 100% - 3 times for 10 min), subjected to the critical desiccation point of CO_2 in BAL-TEC equipment, CPD-030, fixed in metallic bracket with silver glue and covered with metallic gold (10 nm) in BAL-TEC apparatus, SCD-050. The anthers and pollen grains were observed and electromicrographed on a scanning electron microscope LEO-EVO 40, XVP. Pollen grains were classified according to the number of openings (Erdtman, 1943).

3. Results

The meiosis analysis initially requires the definition of collection times of the floral bud and the definition of size and morphology that are ideal for the observation of meiocytes in different stages. Regarding the choice of the spikelet (Fig. 1), a gradient related to size, morphology, and staining was observed. In the smaller ones, about 1 mm and showing lighter staining, the initial stages of prophase I were found (Fig. 1A); in the spikelets of intermediate size, between 1.5 and 2 mm, the stages between metaphase I and metaphase II were found (Fig. 1B), and the larger ones, from 2.5 mm to 3 mm and darker staining, showed meiocytes between the stages of anaphase II to tetrad (Fig. 1C) and pollen grains, respectively (Fig. 1D).

Tetraploidy (2n = 4× = 36) was confirmed for both accessions of *C. dactylon* var. *dactylon* (EGL 9 and EGL 10), with diplotene and diakinesis showing univalent, bivalent and tetravalent configurations (Fig. 2C-D), being one bivalent connected to the nucleolus and rarely two or three (Fig. 2D-E). In these accessions were observed