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ANALYSIS OF BRACHIARIA FEMALE GAMETOPHYTE DEVELOPMENT USING CLEARED OVULES TECHNIQUES

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The present study detailed chronological and embryological comparison of Brachiaria apomictic and sexual female gametophyte development. Establishing differences and similarities between them and identifying cytoembryological markers during the development. Pistils were classified in four stages based on stigma feature and pistil length and now can be used to specific steps of development to analyse expression and pathways of the apomictic mode of reproduction.

INTRODUCTION

The asexual mode of plant reproduction known as apomixis gives rise to embryos genetically identical to the mother plant. Therefore, it is a natural plant cloning process, which represents a potential biotechnological tool to be exploited for genotype fixation [1]. Comparative investigation of the sexual and apomictic megasporogenesis and megaganietogenesis, as well as the fertilization process and embryo development, is imperative to improve understanding of plant reproduction and apomixis.

Brachiaria brizantha (A. Rich.) Stapf, in its diploid form (2n = 2x 18), shows a sexual mode of reproduction, while in its tetraploid form (2n = 4x 36), shows facultative aposporic apomctic mode of reproduction [2,3], thus representing an interesting model for comparative reproductive studies.

Development of embryo sac in sexual plants begins with the megaspore mother cell (MMC) differentiation followed by meiosis, leading to the formation of four megaspores. The three micropylar megaspores degenerate and the most chalazal megaspore, corresponding to the meiotic embryo sac initial cell (Ml) undergoes three mitosis and gives rise to eight-nucleated reduced embryo sac (ME) of *Polygonum*-type [4]. In most aposporic apomictic plants, MMC and megaspores are present, however, megaspores degenerate. An exception was observed in *Brachiaria decumbens*, where MMC degenerates before undergoing meiosis and megaspores are not formed [5]. Aposporous embryo sacs arise from enlarged non-reduced nucellar cells, designated apospore initials (Al) which undergo mitosis and give rise to four or eight-nucleated, non reduced aposporic embryo sacs (AE) of *Panicum* or *Hieracium*-type, respectively [6]. In *Brachiaria brizantha*, the embryo sac presents a three celled egg-apparatus and a central cell with one nucleus. The diploid egg-cell develops into embryo parthenogenetically [3].

Callose deposits are found in MMC and megaspores cell walls and have been considered an

important marker for the different modes of reproduction [1, 6, 7, 8]. Callose, a substance of low permeability, is considered a physical barrier isolating the cell from the influence of surrounding nucellus [9]. In apomictic plants, variations in callose presence and the pattern of distribution were observed in different species. Callose in MMC and megaspores was characteristic for most of diplosporic apomictic accessions, while in aposporic accessions was observed [1, 7, 8] an erratic distribution of this component. Analysis of the role of this component in different female gametophyte development behaviour is important for a better comprehension of apomixis.

First data on *Brachiaria* gametophyte development showed the temporal comparative development of male and female gametophyte of *diploid and artificially induced tetraploid B.* ruziziensis [10], followed by a similar report [11] with tetraploid *B. brizantha*, *B. decumbens* and Fl hybrids with induced tetraploid B. *ruziziensis.* New studies about gametophytic development were carried out and a reproductive calendar was stablished for *B. decumbens* [5]. These studies presented no detailed information about comparative morphology *of the sexual and apomictic development of B.* brizantha.

A single ultrastructural report giving emphasis on the origin, structural and functional aspects of apospory was recently published [1], describing details of *Panicum maximum* gametophyte development and organization.

This work describes comparative chronological and morphological analysis of female gametophyte development prior to anthesis and sexual *B.brizantha,* using light and transmisson microscopy, establishing developmental cytoembryological markers.

MATERIAL AND METHODS

Florets from isolated inflorescences of apomictic and sexual *B. brizantha*, BRA 000591 and BRA 002747 respectively, were collected between two to five days before first signs of anthesis.

Pistils were classified into stages I, II, Ill and IV, accordingly to the stigma feature and pistil length size, measured from the basal portion of the ovary to the tip of the stigma. Measurements were taken in a calibrated ocular microscale in a SVI 1 stereomicroscope, and samples were collected from different inflorescences of each stage.

Sixty pistils of each stage were collected and fixed in formalinacetic acid-alcohol (FAA; 95% ethanol:water:40% formalin:glacial acetic acid, 40:14:3:3 v/v), for 24 h at room temperature. They were subsequently stored in 70% ethanol for 24 h and then incubated in pure lactic acid for 24 h. Finally, they were transferred to Herr's clearing solution [12]. Whole- cleared pistils were mounted in clearing solution and examined with Normasky DIC in a Zeiss Axiophot microscope.

Twenty isolated pistils of each stage of both accessions were fixed in 2,5% glutaraldehyde and 4% paraformaldehyde in 0.05M cacodylate buffer (pH 7.2) for 2h at room temperature, post-fixed in 1% 0s04 in 0.1 M cacodylate buffer (pH 7.2) containing 5 mM $CaCl_2$ and 1.6 % FeCK for 30 mm at room temperature. Samples were rinsed in 0.1 M cacodylate buffer and distilled water and stained with 5% aqueous uranyl acetate for 12h at 4°C. Specimens were dehydrated in a crescent acetone series and embedded in Spurr resin [13]. Longitudinal semi- thin and corresponding ultra-thin sections were obtained either in Sorvall MBT-2 or Leica ultramicrotome, using a Drukker diamond knife. Semi-thin sections were stained with toluidine blue and ultra-thin sections post-stained with uranyl acetate. Zeiss Axiophot and TEM 109 microscopes were used, respectively, for observations.

Fifty isolated pistils at stage I and fifty at stage II of both stages, stored in 70% ethanol after FAA fixation, were stained with a 0.005% solution of aniline blue in 0.15 M K_2HPO_4 [2]. Ovules were gently released from ovaries and examined with DIC and UV epifluorescent Zeiss Axiophot microscope.

RESULTS AND DISCUSSION

Stigma appearance of sexual and apomictic plants was very similar, however, sexual pistils were 20% and 30% larger than apomictic pistils at stage I and II. Pistils were classified into stages I, II, Ill and IV, accordingly to the stigma feature and pistil length size (Fig. 1), measured from the basal portion of the ovary to the tip of the stigma. Measurements were taken in a calibrated ocular microscale in a SVI 1 stereomicroscope, and samples were collected from different inflorescences of each stage, for both accessions.

Stage I. Primordial stigma. Apomictic pistil length: 0.29 mm ± 0.07 (n = 26). Sexual pistil length: 0.48 mm ± 0.04 (n = 41). Ovules at this stage, in both

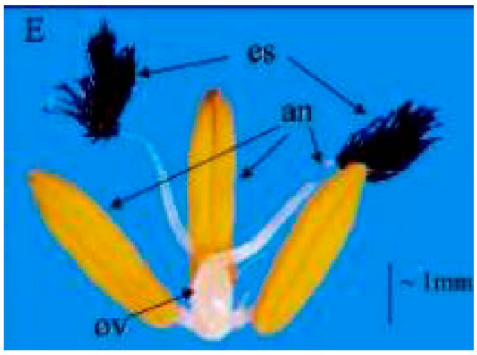

Fig.1. A florets of B. brizantha: pistil and anthers. ov – ovary, an – anther, es – stigma

accessions, were characterized by the presence of nucellar cells or nucellar cells surrounding the megaspore mother cell (MMC) (Fig.2). In sexual plants, a higher percentage of ovules with MMC was noticed when compared to the apomictic plants. Ovules were at atropic position, partially surrounded by inner and outer integuments at primordium state. Apomictic plants showed a higher number of aborted ovules than sexual plants.

Stage II. Elongated primordial stilodium. Apomictic pistil length: 0.96 mm ± 0.12 (n = 29). Sexual pistil length: 1.38 mm ± 0.07 (n = 49). In both accessions the presence of the initial cells of the embryo sacs were detected: initial cells of the aposporus *Panicum*-type embryo sac (Al) and the initial cell of the meiotic embryo sac (Ml), the remaining chalazal megaspore. Other events, corresponding to megasporogenesis and megagametogenesis, were also observed. In apomictic plants, the majority of the pistils showed MMC or megaspores while in sexual plants, the majority of the pistils showed megaspores, mainly at the degenerating state, indicating a faster sexual pistil development in these early stages I and II. Similar number of aborted ovules were detected in both accessions. The transition of the ovules to the anatrope position was still taking place in both accessions.

Stage III. Elongated stylodium with hairy stigmas. Apomictic pistil length: 2.32 mm ± 0.06 (n =32). Sexual pistil length: 2.36 mm ± 0.04 (n = 38). Besides other cytoembriological events, embryo sacs presence characterizes this stage. The majority of ovules showed three of five immature embryo sacs. Ovules with two type of the embryo sacs (one of the *Panicum*-type and the other with more than four nuclei) were observed in apomictic plants Fig. 2 F).

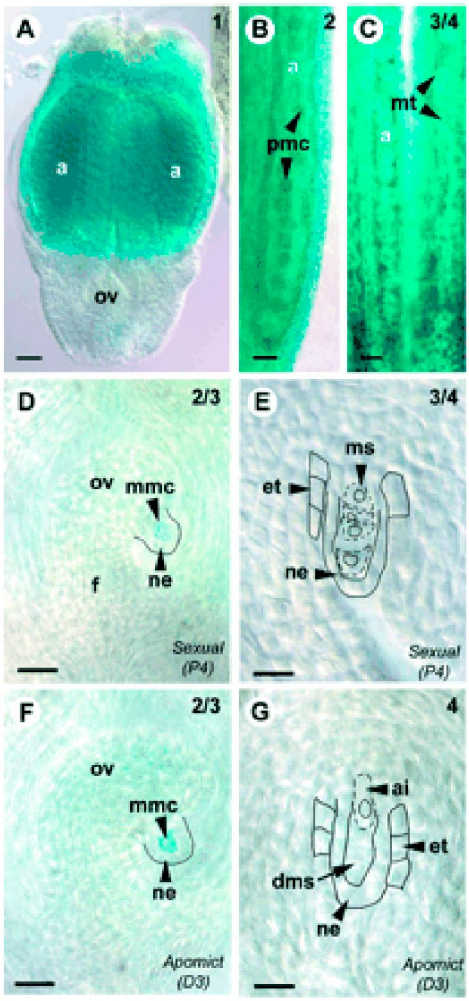

Fig.2. Brachiaria apomictic and mode of reproduction. (A) Early floret from apomictic BRA 000591 containing a small ovule (ov) and immature anthers. (B) Enlargement of an anther from apomictic BRA 000591 containing pollen mother cells (PMC). (C) Anthers from sexual BRA 002747 containing microspore tetrads (MT). (D) Ovule from sexual BRA 002747 containing a megaspore mother cell (MMC). (E) Ovule from sexual sexual BRA 002747 showing four megaspores (MS), outlined with dashed lines, surrounded by the nucellar epidermis and the developing endothelium (ET), outlined with solid lines. No staining is detected in the indicated structures. (F) Ovule from apomictic BRA 000591, showing the corresponding stage of apomictic development to (D), containing a MMC. (G) Ovule from apomictic BRA 000591, showing the corresponding stage of apomictic development to (E), containing an expanding aposporous

In sexual plants, the majority of the pistils showed a single immature embryo sac, with more than four nuclei (fig. 2. D). Ovules were at the nuclellus. Again, an increased number of aborted ovules were observed in apomictic plants.

Stage IV. Red plumed stigmas. Apomictic pistil length: 3.79 mm ± 0.09 (n = 36). Sexual pistil length:

3.63 mm ± 0.08 (n = 39). In both accessions, only developed embryo sacs could be observed within all pistils. No MMC megaspores were identified. In apomictic plants, the percentage of ovaries showing multiple embryo sacs was lager than ovaries showing a single embryo sac. In addition, the percentage of ovaries in apomictic plants were two types of embryo sac within the same ovule was reduced when compared to stage III (Fig.2 G). Sexual plants showed only a single *Polygonum*-type embryo sac (Fig. 2 E). The number of aborted ovules was high in apomictic and sexual plants.

Stigmas in both accessions of Brachiaria brizantha showed similar feature. However, pistil longer length and the presence of first development cytoembriological markers indicated an earlier development of the sexual gametophytes compared to the apomictic at stages I and II. At later stages, development was chronologically similar for both accessions.

Only nucellar cells were observed surrounding MMC, as reported for *Panicum maximum* [1], differing from previous observations that found aposporous embryo sac initial cells (Al) associated to the apomictic *Brachiaria* MMC in cleared ovules [5, 11]. Therefore, stage I does not present specific morphological traits particular to apomixis. Morphological similarity was noted in nucellar cells and MMC of sexual and apomictic accessions. An uniform and active cellular aspect of nucellar cells in young ovules was characterized by the frequent presence of reserve structures, many organelles involved with high cellular activity as well as frequent cell communication throughout plasmodesmata.

MMC in both ovules is easily recognized by its spatial position, shape and large volume. Ultrastructurally, *B. brizantha* MMC shows many cellular differentiation features which are also observed in transition states to gametophyte in other plants [1], with an increased number of membrane-structures such as plastids and membrane figures, usually related to new organelle biogenesis and lytic activity [1, 14]. It is similar to that described for *Panicum* [1], Zea [15] and *Capsella* [16, 17]. Although ultrastructural analysis has indicated that the larger thickness of the MMC cell walls was eventually associated to an irregular electron-lucent wall component which could correspond to callose, *the observation that the majority of B. brizantha* apomictic MMC wall did not fluoresce after anilin blue treatment, suggests that callose presence in MMC can not be used as a criterion of apospory in *B. brizantha.*

The complete tetrad degeneration and the Al outset in apomictic plants and the Ml development in the sexual plants are present in stage II, therefore, this is the most important stage for differentiation of sexual and apomictic gametophytes of *B. brizantha*, although other cytoembriological events are also present.

Tetrad presence was observed in the apomictic accession, although not very frequently in the viable state as a consequence of megaspore short life. Therefore, MMC meiosis occurs in this accessions, diferently from apomictic B. decumbens, where only in few cases the presence of dyad, triad and tetrad in were observed as a consequence of MMC meiosis *absence [5]. In addition, the majority of the B.* brizantha apomictic plants showed simultaneous degeneration of the four megaspores, differing from previous reports [1, 18] on apomictic gametophytes, including *B. brizantha* [19], where a functional chalazal megaspore was observed with the other three degenerating megaspores.

Similar to *P. maximum* [1], Al are large vacuolated cells showing ultrastructural signals of cellular activation, such as many vacuoles, vesicles, plastids and degenerating structures in *B. brizantha.*

Report describing the existence of aposporous embryo sac in cleared ovules of sexual accession of *B. decumbens* was recently published [20]. Although our results obtained with light microscopy (clearing techniques and semi-thin sections) suggested the probable incipient Al outset, no ultrastructural evidence confirming Al development nor the presence of aposporous embryo sac in mature pistils confirm that these cells do not possess potential to develop *and give rise to aposporous embryo sacs in B.* brizantha sexual accession in the growth condition *here established. Another report using B. decumbens* cleared ovules [5] showed the exclusive presence of aposporous embryo sacs in the apomictic accession, in agreemnet with our results.

Immature and mature embryo sacs can be recognized at stages III and IV. No degenerated megaspores could be recognized together with immature embryo sacs in *B. brizantha* as described for *B. ruziziensis* [10]. These embryo sacs contained an active cytoplasm with low interaction with neighbouring cells. The larger thickness of their cell

walls may correspond to callose deposits, as similarly described in *Torenia fournieri* [21].

As it is well accepted [6], many nucellar cells can differentiate into Al in apomictic ovules. Although many more nucellar cells showed activation signals in apomictic *B. brizantha*, only one to six nucellar cells culminated in Al. Our analysis indicated that the majority of mature ovules contained multiple embryo sacs, similar to previous observations in other *Brachiaria* species [11]. However, at the end of the maturation, it was noted a preferential survival of the embryo sac situated at the micropylar pole, a position where, accordingly to previous data [22], efficient nutrient uptake can occur.

The *B. brizantha* all *Panicum*-type embryo sac showed one egg-cell, one polar nucleus and two synergids, differently from previous work [11], where a single synergid, one egg-cell and two polar nuclei *were observed in 10% of the embryo sacs of B.* brizantha. Sexual plants showed a single embryo sac, always at the micropyle pole, containing 8-16 nuclei, similar to other observations for this species [11].

Although our data points to many differences on the gametophyte development and embryo sac organization in *Brachiaria* species, it has been already suggested [11] that these differences can be observed in different species of the same genus, members of the same species from different origins, and between accessions grown in different conditions.

Four and eight nucleated embryo sacs within the same ovary were observed in 3-5% of the apomictic accession, indicating its maturation, the frequency of these ovaries decreased, indicating the *Panicum*-type embryo sac prevalence over the eight-nucleated embryo sac. It should also be taken into account that an eight-nucleated embryo sac can be observed in aposporous *Hieracium*-type embryo sac as well as in the diplosporic embryo sac, suggesting two types of apomixis rather than facultative apomixis. Such results were observed in *Paspalum minus* [23], *where diplosporous Taraxacum*-type and *Panicum* aposporous embryo sacs coexist within the same ovary. Detailed cytogenetic analysis of the MMC meiotic process, embryo sac cells ploidy analysis and observation of off-type frequency must be conducted to identify the origin of the eight-nucleated embryo ***sac in*** *B. brizantha.*

The present analysis of the apomictic *B.* brizantha embryo sac indicated an active ultrastructural aspect of the egg-cell prior to anthesis, in accordance to recent data comparing initial zigotic *and parthenogenetic pathways in Triticum aestivum* [24] and *Pennisetum ciliare* [25], indicating mainly temporal differences in the sexual and apomictic activation of egg-cell and synergids. The high level of metabolism prior to fertilization suggests that the apomictic egg-cell is an active cell, differently from the metabolically inactive sexual egg-cell described in Zea [26, 31] *Gossypium* [27] and *Capsella* [28], barley [29], pearl millet [30] pointing to one possible mechanism to avoid fertilization, apomictic egg-cell precocious activation. Observations did not indicate the presence of *B. brizantha* embryo before anthesis, indicating that embryo outset occurs after pollination.

Cell walls surrounding the egg-cell, central cell and synergids in this species is not complete, which would not impede, at that time, the sperm cell entrance, as suggested by Savidan [32], who raised the egg-cell wall completion hypothesis to explain how egg-cell avoids fertilization.

Apomixis in *B. brizantha* follows the aposporous pattern, where megaspores develop and simultaneously degenerate, initial cells of aposporous embryo sacs (Al), ultrastructrually characterized as an active cells, are identified associated to viable or degenerating tetrad. The Panicum-type embryo sac found in this accession presents one egg-cell, two synergids with an active ultrastructural aspect and a single polar nucleus thin the central cell. These cells present incomplete cell wall at this stage, which would not function as a barrier for male gamete fertilization of the egg-cell. Two types of embryo sacs (four and eight- nucleated) found within apomictic ovaries suggest the facultative apomixis or even two types of apomictic embryo sacs: *Hieracium* or diplosporic type. Other studies are necessary to confirm the origin of this eight-nucleated embryo sac. The prevalence of the four-nucleated embryo sac over the eight-nucleated during development was noticed. Some differences were observed within different accessions of this species, among the genera and with other *Poaceae.* Although the first steps of the apomictic female gametophyte development are chronologically delayed compared to the sexual, morphologically, they are similar, showing alike nucellar cells and MMC, except by the exclusive presence of callose in the sexual MMC. With the classification here used to describe pistils (based on

stigma feature and pistil length) megasporogenesis events were identified in stages I and II while stages Ill and IV correspond to megagametogenesis in both accessions. For futher apomictic molecular analysis, stage II, where first specific apomictic citoembryological events are present, is the most interesting to compare different apomictic and sexual expression.

The present study concerned detailed chronological and morphological developmental comparisons of apomictic and sexual B. brizantha, establishing ultrastructural and chronological differences and similarities between them and identifying cytoembryological markers of the female gametophyte development, what can be used to select specific stages to analyse apomictic expression and pathways.

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Резюме

Проведено хронологическое и эмбриологическое сравнение развития женского гаметофита у *Brachiaria brizantha* с апомиктичным и с половым способами репродукции. Показаны различия и сходства в их развитии и выявлены цитоэмбриологические маркеры. Установлены четыре стадии развития пестиков на основании особенности рыльца, длины завязи, что позволит ускоренно отбирать растения с апомиктичным размножением.

Резюме

Brachiaria brizantha-ның аналық гаметофитінің апомиктік және жыныстық жолдарымен көбеюі хронологиялық және эмбриологиялық тұрғыдан салыстырмалы түрде зерттелінді. Олардың даму ерекшеліктерімен ұқсастығы цитоэмбриологиялық маркерлер арқылы көрсетілді. Түйіннің дамуының төрт сатысы аналық ауыздың ерекшелігі, тұқым бүршіктің ұзындығы арқылы анықталды. Соның нәтижесінде апомикті жолмен көбейетін өсімдіктерді жылдам таңдауға болады.