

Full Length Research Paper

Reproduction mode and apospory expressivity of selected hybrids of *Paspalum notatum* Flüggé

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Received 28 January, 2021; Accepted 22 March, 2021

***Paspalum notatum* Flüggé (bahiagrass) is the most important forage grass in the native grasslands of southern Brazil; the native germplasm is tetraploid and apomictic. Breeding to produce cultivars has been possible due to chromosome doubling of sexual diploid accessions, allowing hybridizations with apomictic genotypes, generating progenies with variability for agronomic traits and segregating for reproduction mode. The aim of this study was to determine the reproduction mode of selected hybrids derived from intraspecific crosses through cytoembryological analysis and to evaluate the level of apospory expressivity. Eighty one tetraploid hybrids corresponding to 21 families of sexual and apomictic parents were generated and 28 most productive plants based on their superior agronomic performance in the field were evaluated. Sixteen hybrids were reproduced sexually and 12 of them were highly apomictic or facultative apomictic. Sexual hybrids may be used for further crosses in the breeding program while highly apomictic plants produce uniform progeny and become candidates of new cultivars to promote pasture diversification.**

Key words: Aposporous apomixis, bahiagrass, intraspecific crosses, plant breeding.

INTRODUCTION

Genetic improvement of apomictic forage grasses has an important role in the economy of beef cattle production systems in the tropics and subtropics. Among warm-season apomictic forage grasses, the genus with the greatest economic and social value are *Brachiaria*, *Panicum*, *Cenchrus*, and *Paspalum* (Jank et al., 2014).

Paspalum notatum Flüggé (bahiagrass) is an important forage species in the grasslands in South America and is used as forage due to its yield, quality, and persistence.

Around the world, it is also used as turf and forage (Gates et al., 2004). It is an important provider to native pasture in southern Brazil and also can be used as pioneering species to reduce the negative effects of soil erosion and degradation (Barbosa et al., 2019).

Aposporous apomixis, asexual reproduction through seeds, is a common reproductive mode in polyploid (tetraploid) ($2n = 4x = 40$) biotypes of bahiagrass, while the natural sexually reproducing plants are diploid ($2n =$

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$2x = 20$) (Miles, 2007). The reproductive barriers between tetraploids apomictic plants and sexual diploids are a clear obstacle for breeding programs (Zilli et al., 2018). Besides that, the apomixis affects the plant diversity among species and populations (Acuña et al., 2019). By doubling the chromosome number of sexual diploid accessions, sexual tetraploids were produced (Forbes and Burton, 1961; Quarin et al., 2001, 2003; Quesenberry et al., 2010; Weiler et al., 2015). The feasibility of crosses between sexual $4x$, used as female parents, and apomictic $4x$, used as pollen donors, have been reported widely in the literature (Acuña et al., 2009; 2011; Zilli et al., 2015; Weiler et al., 2018). So, it's possible to produce populations segregating for apomixis and hybrids that display heterosis for agronomic traits of interest (Acuña et al., 2009, 2011; Ortiz et al., 2020), for example forage yield and cold tolerance as already reported in *P. notatum* (Acuña et al., 2011; Zilli et al., 2015; Weiler et al., 2018; Barbosa et al., 2019).

The selected genotypes, with superior agronomic characteristics fixed by apomictic reproduction at the tetraploid level in the breeding programs can be considered potential new cultivars (Acuña et al., 2009; Acuña et al., 2011; Jank et al., 2014; Zilli et al., 2015; Novo et al., 2020).

Inheritance and expressivity of apomixis in *Paspalum* is the consequence of a complex genetic and/or epigenetic control of the trait (Podio et al., 2014). Sexuality is a recessive character and apomixis is conditioned by a single dominant Mendelian factor which is the most widely accepted genetic model for the inheritance of apomixis in grasses (Acuña et al., 2011; Martínez et al., 2001; Ozias-Akins and Van Dijk, 2007; Stein et al., 2004). However, the full expression of the apomictic trait depends upon some pleiotropic effect and incomplete penetrance of dominant allele controlling the apospory development (Martínez et al., 2001). Recent data indicate that apomictic seed development in plants is linked to a dynamic pattern of transcriptional activity in ovule, probably regulated through epigenetic mechanisms (Kumar et al., 2017) as well as to environmental conditions (Acuña et al., 2009; Zilli et al., 2015). Therefore, it is common to occur segregation distortion against apospory (Martínez et al., 2001) and variable levels of apomixis expressivity can be expected among generations (Acuña et al., 2011; Zilli et al., 2015).

In order to use newly developed hybrids in crosses, the reproduction mode and apospory expressivity needs to be reliably assessed to define their usefulness for crop improvement of the species and the progenies to be studied and characterized (Acuña et al., 2007, 2019; Miles, 2007). For screening the reproduction mode, molecular markers give a fast classification of plants, which is very useful for breeding purposes.

However, markers do not provide information concerning the expression level of apospory, necessary for apomictic hybrid selection (Zilli et al., 2018). On the other hand, the cytoembryological method is accurate to

identify reproduction mode and apospory expressivity in the hybrids. The objective of this study was to determine the reproduction mode of selected segregating hybrids of bahiagrass derived from intraspecific crosses through cytoembryological analysis and to evaluate the level of apospory expressivity among hybrids, in order to use them as parents for future breeding programs.

MATERIALS AND MEHODS

Plant material

The experiment was carried out at the Department of Forage Plants and Agrometeorology of the Agronomics School, Federal University of Rio Grande do Sul (UFRGS), located in the city of Porto Alegre, Rio Grande do Sul State, Brazil (latitude $30^{\circ} 1'16.13''$ S and longitude $51^{\circ}13'23.99''$ W). Crosses with *Paspalum notatum* genotypes were performed in the greenhouse to produce hybrids. The female parents were three sexual tetraploids genotypes, C4-4X (Quarin et al., 2001), Q4188 and Q4205 (Quarin et al., 2003). As male parents, the apomictic tetraploid accessions called 30N, 36N, 70N, 48N, 83N, 95N and V4 were selected after evaluation for forage production by Fachinnetto et al. (2012).

Hybridization

Crosses were performed in the greenhouse following the methodology described by Burton (1948). In the mother plants, before the anthesis, rooted culms bearing inflorescences were collected and placed in a jar with water. These culms were placed in an artificial fog chamber, with a high level of air humidity to avoid the anthers from dehiscence. In the morning of the next day, the flowers were emasculated, using sharp pointed tweezers to remove the anthers. Non-flowering spikelets were eliminated from the inflorescence. Fresh pollen from the apomictic parent was collected in paper envelopes and later dusted on emasculated inflorescences of the sexual plants. After pollination, the inflorescences were bagged and labelled. Seeds were collected at least 21 days after pollination. The progeny from each cross was referred as a family and a number was given to identify each hybrid. Nine families produced hybrids, which were selected to determine the reproduction mode.

Embryo sac observations

The reproduction mode of progenies was analyzed in inflorescences collected at anthesis (embryo sacs fully developed). The flowers of various inflorescences were dissected and fixed in FAA solution [40 ml 95% ethanol: 14 ml distilled water: 3 ml 40% formalin: 3 ml glacial acetic acid] for 24 h. Subsequently, the flowers were transferred to ethanol 70% and refrigerated. The pistils were dissected and the ovaries were dehydrated with alcohol and clarified with methyl salicylate according to the method established by Young et al. (1979), modified by Acuña et al. (2007). Slides with the ovaries were prepared and observed using a differential interference contrast microscope and some of them were photographed with a Zeiss camera. A minimum of 40 ovules per plant were examined. The reproduction mode was determined based on the observation of embryo sac morphology. The ovules were separated in sexual (presence of antipodal cells) or apomictic (without antipodal cells, multiple or single embryo sacs with the egg apparatus and the central cell) (Acuña et al., 2009; Kumar et al., 2017). The reproduction mode of the male parents was also checked using embryo sac observations.

Table 1. Identification of F₁ families of 28 selected *Paspalum notatum* genotypes, classification for reproduction mode and apomixis expressivity based on embryo sacs types observed.

Cross combination				Number of ovaries containing					
Sexual parent	female parent	Apomictic male parent	ID	Total number of considered ovules	SES	AES	AbES	Apomixis expressivity (%)	Mode of reproduction
C4-4X		83N	115	31	31	-	9	-	sex*
C44X		83N	515	25	25	-	15	-	sex
C4-4X		95N	116	34	34	-	6	-	sex
C44X		95N	216	33	33	-	7	-	sex
C44X		95N	316	30	30	-	11	-	sex
Q4188		V4	127	39	39	-	6	-	sex
Q4188		30N	321	32	32	-	11	-	sex
Q4188		30N	421	33	21	12	8	36.4	fac apo**
Q4188		30N	521	39	39	-	4	-	sex
Q4188		30N	421	28	28	-	12	-	sex
Q4188		30N	1021	33	8	25	7	75.8	fac apo
Q4188		83N	325	32	32	-	12	-	sex
Q4188		83N	225	36	2	34	8	94.4	high apo***
Q4188		83N	725	33	9	24	8	72.7	fac apo
Q4188		95N	926	29	2	27	11	93.1	high apo
Q4188		95N	126	31	12	19	10	61.3	fac apo
Q4205		36N	132	36	5	34	7	94.4	high apo
Q4205		36N	332	35	35	-	6	-	sex
Q4205		95N	336	25	25	-	15	-	sex
Q4205		95N	536	29	29	-	12	-	sex
Q4205		95N	1036	38	38	-	12	-	sex
Q4205		95N	136	30	2	28	14	93.3	high apo
Q4205		95N	436	31	-	31	11	100	high apo
Q4205		95N	736	28	6	22	15	78.6	fac apo
Q4205		95N	936	29	10	19	14	65.5	fac apo
Q4205		V4	137	38	38	-	4	-	sex
Q4205		V4	437	36	36	-	7	-	sex
Q4205		V4	337	30	-	30	12	100%	high apo

ID (identification); **SES**: sexual embryo sacs; **AES**: apomictic embryo sacs; **AbES**: aborted or immature embryo sacs; *sex=sexual; **fac apo = facultative apomictic; *** high apo = highly apomictic.

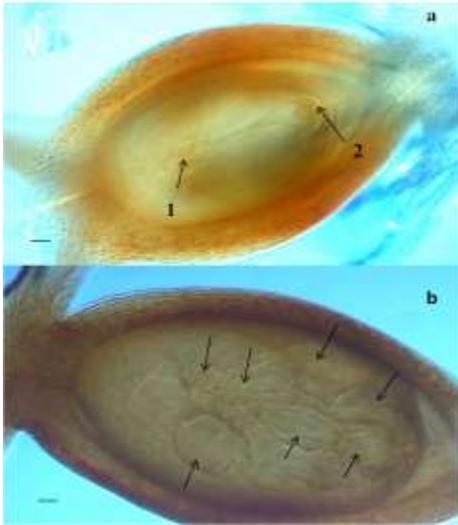


Figure 1. Morphology of embryo sacs of bahiagrass tetraploids hybrids. (a) Sexual ovary with antipodes (1) and egg cell (2). (b) Aposporous ovary with multiple sacs (arrows). Scale: 10 μm .

Evaluation of the reproduction mode and apospory expressivity

The plants were classified as sexual, highly apomictic, and facultative apomictic based on the number of ovules with aposporous embryo sacs. Aborted or immature ovules were not counted (Marcón et al., 2019); only embryo sacs identified as sexual or apomictic were used for classification. Plants with almost all ovules (>90%) bearing aposporous embryo sacs were classified as highly apomictic; plants with less than 90% (1 - 89%) of aposporous embryo sacs on the same inflorescence were classified as facultative apomictic whereas plants without aposporous embryo sacs, bearing only single reduced embryo sacs were classified into sexual. Acuña et al. (2009) and Ortiz et al. (2020) classify the aposporic hybrids as 'obligate' or 'highly apomictic' when almost all ovules (>90%) show one or several aposporous embryo sacs (AESs).

RESULTS AND DISCUSSION

From the crosses, we obtained 81 tetraploid hybrids of sexual and apomictic parents, corresponding to 21 families (C4-4X x 30N; C4-4X x 36N; C4-4X x 48N; C4-4X x 70N; C4-4X x 83N; C4-4X x 95N; C4-4X x V4; Q4188 x 30N; Q4188 x 36N; Q4188 x 48N; Q4188 x 70N; Q4188 x 83N; Q4188 x 95N; Q4188 x V4; Q4205 x 30N; Q4205 x 36N; Q4205 x 48N; Q4205 x 70N; Q4205 x 83N; Q4205 x 95N and Q4205 x V4). The hybrid progenies were transplanted to the field, at the Agronomic Experimental Station of the Federal University of Rio Grande do Sul (UFRGS), Rio Grande do Sul State, Brazil (latitude 30°05'S and longitude 51°39'W) and evaluated to estimate their agronomic value. Five cuts were taken during two years. The variable total production of dry mass of leaves (LDM) showed the highest phenotypic

correlation with the production of total dry mass (TDM). The 28 selected hybrids from nine families (Table 1) presented higher forage production than the ecotypes used as parents and also from the standards. Cumulative TDM varied from 191.0 (g. plant⁻¹) (plant 225) until 414.1 g.plant⁻¹ (plant 437), while the cultivar Pensacola produced 76.1 g.plant⁻¹. The reproduction mode and apospory expressivity were then assessed in these selected hybrids.

Classification of reproduction mode

As expected, the hybridization between induced sexual tetraploid and apomictic plants results in F₁ plants that segregated for reproduction mode. Abnormal, atrophied and sterile ovaries was found in almost all plants (Table 1). Ovules bearing both sexual and apomictic embryo sacs were not observed. In the same family, some hybrids were sexual while others were apomictic, because a single dominant Mendelian factor controls apospory in bahiagrass, where sexuality behaves as recessive (aaaa).

The male parents used in the crosses confirmed the apomictic reproduction as expected. All the parents presented almost all ovules (>90%) bearing aposporous embryo sacs and were classified as highly apomictic. Sixteen plants were classified as sexual because only a single reduced embryo sacs were observed in their ovules. Twelve plants had embryo sac morphology compatible with apomictic mode of reproduction and were classified as apomictic (Table 1 and Figure 1). The apospory expressivity was variable in the 12 apomictic hybrids, with high and low levels observed. Six apomictic plants (named 421, 1021, 725, 126, 736 and 936) showed intermediate level of expressivity and were classified as facultative apomictic. The remaining six plants (named 225, 926, 132, 136, 436 and 337) were classified as highly apomictic because aposporous embryo sacs were observed in more than 90% of their ovules (Table 1), representing 21.43% of the total hybrids.

Acuña et al. (2009) reported the mode of reproduction and apospory expressivity of 173 hybrids from different families of bahiagrass. Of these, 19 plants were highly apomictic; 16 were classified as facultative apomictic and 138 plants were classified as sexual. Apomictic progenies represented 20% of the plants. The ratio between sexual and apomictic tetraploid hybrids of bahiagrass in the first-generation (FGTH) and in the second-generation (SGTH) was determined by Acuña et al. (2011). For the FGTH it was from the 4.3:1 (sexual:apomictic) and for the SGTH was of 4.6:1. Although similar, there was a strong distortion with an excess of sexual progeny, but the segregation patterns for apomixis remained constant through hybridization cycles. The expressivity was variable between generations and only 3% of the progenies were classified as highly apomictic, maybe due

environmental differences between years of evaluation.

Zilli et al. (2015) also recorded variable expressivity for apospory in hybrids of bahiagrass. For 32% of the hybrids classified as aposporic, were observed highly levels of expressivity, between 81 and 100%. The variable expression of apomixis among the progenies remains uncertain. It is possible that a single genomic region is responsible for triggering the trait. However, others important elements as genetic or epigenetic factors maybe affecting the expressivity (Acuña et al., 2009; Zilli et al., 2015; Ortiz et al., 2020). Genetic analyses in *Paspalum* showed that apomixis occur by various mechanisms, which share three common developmental components: apomeiosis, parthenogenesis and unbalanced endosperm formation (Kumar et al., 2017; Ortiz et al., 2020). The single locus is blocked in terms of recombination, hemizygoty and heavy cytosine methylation. Podio et al. (2014) evaluated the DNA methylation state of the apomixis-controlling region (ACR) in *Paspalum simplex* and *Paspalum notatum*. The study suggested that factors controlling repression of parthenogenesis might be inactivated in apomictic *Paspalum* by DNA methylation, while demethylation induces a significant depression of parthenogenesis. Environmental conditions/stress provide evidences to support the theory that apomixis is epigenetically regulated. So, it's possible to reach desirable variability and adaptive advantages without altering DNA sequence (Rodrigues and Koltnow, 2005).

Identification of highly apomictic, facultative apomictic and sexual hybrids has an important role in the use in the next phases in a breeding program. Sexual genotypes can be used in further crosses, while highly apomictic hybrids identified in this research can potentially produce uniform progenies and stability in the successive reproductive cycles (Acuña et al., 2019). However, variable levels of apospory expressivity can reduce the probability of fixing hybrids exhibiting heterosis for traits of interest by apomixis. Acuña et al. (2011) asserted that the low proportion of highly apomictic hybrids derived from artificial hybridization difficult the selection of superior genotypes highly apomictic. Even so, the apomictic hybrids selected in this research encouraged us to develop a breeding scheme that allows the register, protection and later the released of the apomictic hybrids as new cultivars.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors appreciate CAPES (Brazilian Federal Agency for Support and Evaluation of Graduate Education), CNPq

(Brazilian Council for Scientific and Technological Development) and the South-Brazilian Association for the Promotion of Forage Research (SULPASTO) for financial support and grants.

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