

Reproductive analyses of intraspecific *Paspalum notatum* Flüggé hybrids

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Abstract: *Paspalum notatum* is an important forage native to Southern Brazil and one of the most promising fodder species in terms of productivity, quality, resistance and growth speed. The objective of this study was to evaluate the reproductive mode and fertility of hybrid progenies resulting from artificial crosses among artificially duplicated sexual plants, selected intraspecific hybrids of the UFRGS breeding program and apomictic ecotypes of the species. The resulting F_1 progenies were represented by 24 plants with high pollen viability (varying from 82.15 to 99.67%) and 1.2:1 segregation for the sexual: apomictic reproduction mode, indicating predominantly sexual hybrids, due to distorted segregation. In the apomictic plants, most chromosomes paired as bivalents in diakinesis. Since the low presence of abnormalities observed in the meiotic chromosome pairing and the high pollen viability of the hybrids, all plants were confirmed as promising parents for future crosses.


Keywords: Reproduction mode, meiotic behavior, pollen viability.

INTRODUCTION

Paspalum notatum, a perennial summer forage grass, is the main herbaceous product of most native pastures of Rio Grande do Sul State. The species has two forms of dispersion, via above-ground rhizomes or seeds, making it resistant to animal trampling and competitively advantageous even at locations with degraded soil and pasture (Nabinger and Dall'Agnol 2008). The native germplasm is tetraploid and displays apomictic reproduction (Forbes and Burton 1961). By the apomictic reproduction mode, the seeds are clones of the mother plant (Ortiz et al. 2013), which can be either a drawback or a valuable tool for crop improvement (Kumar et al. 2017). Historically, the breeding of apomictic plants was limited by selection, evaluation and natural perception of the occurrence of the most vigorous and highest-yielding apomictic ecotypes (Miles 2007). Many of the currently planted cultivars originate from this form of breeding (Hojsgaard et al. 2016). However, it depends on research and natural selection of superior ecotypes, which greatly limits their potential. This alternative is less viable, because unchanged natural germplasm does not provide ideal cultivars even in forage plants (Miles 2007); the pastures without variability are advantageous for animal management but represent a serious risk when planted over wide expanses of land (Simioni and Valle 2009). Since sexual diploids are frequently found in the genus *Paspalum* (Martínez et al. 2007), it is possible to improve apomictic species by making crosses with relatives of sexual reproduction,

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exploiting the existing variability in polyploid types (Quarin et al. 2001). The commercial cultivar “Pensacola” of *P. notatum* (Forbes and Burton 1961) and four accessions originate from Argentina and Uruguay (Fachinetto et al. 2018) are diploids and can be used as a source of sexuality in breeding.

For the crossbreeding, the sexual genotypes must share the same ploidy level as wild autotetraploids, requiring previous chromosomal duplication of the maternal parents (Quarin et al. 2001, Miles 2007). Completely sexual tetraploid plants were obtained directly from sexual diploids by colchicine treatment (Quarin et al. 2001, Weiler et al. 2015). With the induced polyploids and the native apomictic ecotypes from Rio Grande do Sul State, Weiler et al. (2018) carried out intraspecific crosses. Progenies of 198 hybrid plants were established for agronomic assessment and the 28 most productive plants in terms of herbage accumulation and production of leaves were selected. Cytoembryological analyses and molecular markers were used to assess the reproduction mode of the hybrids, indicating segregation for apomictic and sexual genotypes (Weiler et al. 2017), also reported by Kumar et al. (2010b) and Kumar et al. (2017) in *Cenchrus ciliaris*, which reinforces the possibility of producing apomictic cultivars with desirable traits and using sexual genotypes as mother plants in future crosses. Barbosa et al. (2019) established them in lines and evaluated in two physiographic regions of Rio Grande do Sul (Central Depression and Campain), showing the productive potential of some of these genotypes. The selected hybrids, the native ecotypes and the duplicated plants were then used as parents in new crossing cycles, generating hybrid progenies whose reproductive evaluations are presented here.

Evaluation of fertility and analyses of the reproductive mode of the generated hybrids are necessary in breeding program, defining their usefulness and enabling intraspecific hybridizations (Krycki et al. 2016).

The objective of this study was to determine the reproduction mode and pollen viability of segregating hybrid progenies of *P. notatum* and to analyze the meiotic chromosome pairing of the plants with apomictic reproduction mode to confirm its reproductive stability, in order to be used as future parents in breeding programs. Hybrid plants with reproductive stability and high forage yield in field trials will be candidates for registration as new cultivars.

MATERIAL AND METHODS

The hybrids analyzed in this study belong to the families ‘KC’ (‘WKS63’ x ‘Bagual’), ‘KD’ (‘WKS92’ x ‘Bagual’), ‘KE’ (‘D25’ x ‘Bagual’), ‘KF’ (‘D16’ x ‘D3’) and ‘KN’ (‘B2’ x ‘C2’). The genotypes ‘WKS63’ and ‘WKS92’ are polyploids artificially generated by Weiler et al. (2015); ‘Bagual’ is a native ecotype of the species; and those tagged ‘B2’, ‘C2’, ‘D3’ and ‘D25’ are intraspecific hybrids selected for their high yield potential in field evaluations (Weiler et al. 2018, Barbosa et al. 2019). The first group of parents mentioned are sexual and the second apomictic. Of the 139 resulting seeds, 24 adult plants (named KC1, KD1, KD2, KD5, KD6, KD7, KD9, KE1, KF1, KF2, KF3, KF4, KF6, KF7, KF8, KF9, KF10, KF15, KF16, KF17, KN2, KN3, KN4 and KN5) were evaluated (Table 1).

Evaluation of the reproduction mode

The progenies reproduction mode was analyzed in inflorescences collected at anthesis and were fixed in FAA [40 ml 95% ethanol: 14 ml distilled water: 3 ml 40% formalin: 3 ml glacial acetic acid] and refrigerated in 70% alcohol until ovary dissection, alcohol dehydration and methyl salicylate clearing (Young et al. 1979, Acuña et al. 2007). About 40 ovaries per plant were examined under

Table 1. Mode of reproduction of F₁ hybrid plants

Genotype	S ^a	A ^a	SA ^a	UI ^b	T ^b	Mode of reproduction
KC1	35	0	0	5	40	sex*
KD1	16	3	0	21	40	fac apo**
KD2	14	3	0	23	40	fac apo
KD5	60	3	0	17	80	fac apo
KD6	28	0	0	32	60	sex
KD7	60	2	0	18	80	fac apo
KD9	23	0	0	17	40	sex
KE1	30	0	0	44	74	sex
KF1	18	0	0	22	40	sex
KF2	35	0	0	45	80	sex
KF3	7	6	0	67	80	fac apo
KF4	21	0	0	18	39	sex
KF6	13	1	0	66	80	fac apo
KF7	30	0	0	10	40	sex
KF8	0	31	10	8	49	fac apo
KF9	32	0	0	41	73	sex
KF10	14	3	0	23	40	fac apo
KF15	28	0	0	22	50	sex
KF16	12	5	0	58	75	fac apo
KF17	37	0	0	45	82	sex
KN2	26	0	0	54	80	sex
KN3	26	2	0	12	40	fac apo
KN4	19	0	0	61	80	sex
KN5	23	6	0	51	80	fac apo

*sex=sexual; **fac apo = facultative apomictic; ^atypes of embryo sacs: meiotic (S); apomictic (A); sexual and apomictic in the same ovary (SA), unidentified (UI); ^btotal number of ovaries analyzed (T).

differential interference contrast microscopy. To determine the reproduction mode, the ovules were separated in sexual or apomictic, according to the embryo sac morphology.

Meiotic chromosome pairing and pollen viability analysis

The meiosis of pollen mother cells (PMCs) of apomictic plants derived from intraspecific crosses was analyzed.

Young inflorescences were collected and fixed in a 3:1 solution (absolute ethyl alcohol: glacial acetic acid) for 24 h at room temperature and then transferred to 70% (v/v) aqueous alcohol and stored in a freezer until slide preparation. The microsporocytes (PMCs) were prepared from the inflorescences by squashing and staining with 1% propionic carmine and examined under an optical microscope (40x). Chromosome pairing was monitored by counts at meiosis phase I, in diakinesis, in at least 20 cells per plant (Dahmer et al. 2008, Simioni and Valle 2011).

The pollen viability of both sexual and apomictic progenies plants was assessed. To this end, mature inflorescences were collected, fixed and prepared based on the same protocol as for meiotic analyses. Mature pollen was analyzed, of which the stained grains were considered viable and wrinkled or colorless grains unviable (Singh 1993). About 1000 mature pollen grains per plant were counted in four flowers per plant (250 pollen grains per plant), following the protocol established and used (Dahmer et al. 2008, Guerra et al. 2013, Krycki et al. 2016). The pollen viability estimate was calculated by the percentage of viable grains: number of grains considered viable divided by the total number of grains multiplied by 100 (Tedesco et al. 2019).

RESULTS AND DISCUSSION

Evaluations of the reproduction mode

The reproduction mode of all 24 plants was determined (Table 1). In the sexual plants (Figure 1a) all the ovules observed have only one embryo sac per ovule, resulting from the functional reduction of the megaspore, containing one egg cell and two polar nuclei near the micropyle and an antipodal cell cluster approaching the chalaza as previously reported (Quarin et al. 2001, Kumar et al. 2010a). Plants with two or three multiple embryo sacs, a binucleate central cell (the polar nucleus) and without antipodes were classified as apomictic (Figure 1b).

Among the analyzed hybrids, 13 sexual and 11 apomictic genotypes were found. The sexual to apomictic ratio of the genotypes was 1.2:1, close to the expected 1:1 for tetrasomic monogenic inheritance with dominant apospory. In other evaluations of the reproductive mode of segregating *P. notatum* hybrid progenies, were found sex:apo ratios of 2.8:1 (Martínez et al. 2001); 6.5:1 (Stein et al. 2004); 4.6:1 and 4.3:1 in the first and second generations, respectively (Acuña et al. 2011) and 1:2.1 (Weiler et al. 2017). Some results showed a distortion, trending to a deviation towards sexual reproduction. According to Martínez et al. (2001), the apo-locus in *P. notatum* is linked to a lethal allele responsible for distorted segregation rates. Burton (1982) showed that the environment has little or no effect on the apomictic expression in *P. notatum*. Zilli et al. (2015) reported segregation rates between sexual and apomictic plants ranging from 1:1 to 7:1 between *P. notatum* families of intraspecific crosses. These authors concluded that the segregation for apomixis depends on the genetic structure of sexual and apomictic parents.

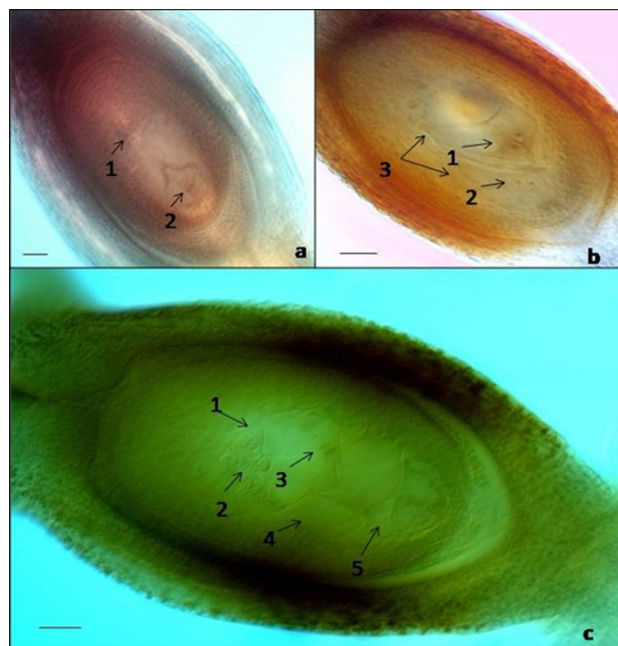


Figure 1. Morphology of embryo sacs. a) Sexual ovary of the KE1 plant with antipodes (1) and polar nuclei (2). b) Apomictic ovary of plant KF16 with polar nuclei (1 and 2) and two multiple sacs (3). c) Mixed ovary of plant KF8: sexual sac (1) with antipodes (2) and polar nuclei (3) and two apomictic sacs (4 and 5). Scale: 10 µm.

The plant KF8 had 31 exclusively apomictic ovaries and 10 embryo sacs with a sexual as well as an apomictic sac (Figure 1c). Plants with both reproduction modes represented (co-exist in same ovule or in different ovules of the same plant) are considered as facultative apomictis (Kaushal et al. 2019, Yadav et al. 2019).

In a study with *P. malacophyllum*, Hojsgaard et al. (2013) described the competition between sexual and apomictic development of ovules and seeds with both embryo sacs in the same ovary, suggesting that this might be due to the genetic and epigenetic effects, causing instabilities in developmental programs. Epigenetic control of apomixis is an emerging theory being investigated on sexual plants in which mutations in epigenetic pathway genes resulted into apomixis-like phenotypes (Kumar 2017). The key factors reducing sexuality are the faster growth and parthenogenetic development in the aposporous pathway.

Meiotic chromosome pairing analysis

Based on counting of chromosome associations in diakinesis in pollen mother cells, all assessed apomictic hybrids confirmed polyploid.

Meiotic chromosome pairing was predominantly bivalent, but quadrivalent chromosome associations were also observed (Table 2; Figure 2). In bivalent associations, the chromosome matching of the evaluated tetraploid hybrids tended to be regular, although Quarin et al. (2001) claimed that quadrivalent associations predominate in apomictic tetraploids. In an evaluation of the meiotic behavior of plants ‘WKS63’ and ‘WKS92’ used as female parents in this experiment, Krycki et al. (2016) observed the predominance of bivalent associations in diakinesis and metaphase I, while uni, tri and quadrivalent associations also occurred, although sporadically.

In the study of Dahmer et al. (2008) in 36 apomictic *P. notatum* accessions collected in the state of Rio Grande do Sul, meiotic configurations at diakinesis and metaphase I varied from plants with only bivalents up to plants with high frequency of quadrivalents. The authors stated that all the examined accessions were potentially male-fertile and could be used as male genitors in crosses into the breeding program.

Table 2. Meiotic configurations of the diakinesis phase (prophase I) of apomictic F₁ hybrids

Genotype	Chromosome number	Number of analyzed cells	Mean of observed associations (range ^a)			
			I ^b	II ^b	III ^b	IV ^b
KD1	40	50	0	2.92	0	0.84
			0	(0-20)	0	(0-11)
KD2	40	22	0	7.05	0	1.73
			0	(0-20)	0	(0-10)
KD5	40	33	0	3.15	0	0.45
			0	(0-20)	0	(0-9)
KD7	40	44	0	5.18	0	0.57
			0	(0-20)	0	(0-10)
KF3	40	72	0	2.85	0	0.22
			0	(10-20)	0	(0-3)
KF6	40	67	0	2.27	0	0.3
			0	(9-20)	0	(0-5)
KF8	40	49	0	1.71	0	0.16
			0	(14-20)	0	(0-3)
KF10	40	32	0	3.91	0	0.47
			0	(10-20)	0	(0-5)
KF16	40	35	0	2.13	0	0.19
			0	(14-20)	0	(0-3)
KN3	40	26	0	2.08	0	0.12
			0	(16-20)	0	(0-2)
KN5	40	44	0	2.89	0	0.34
			0	(10-20)	0	(0-5)

^a Minimum and maximum limits of associations observed in the total cells analyzed; ^b Types of chromosome associations: I (univalent), II (bivalent); III (trivalent); IV (quadrivalent).

Other studies with tetraploid forage grass species ($2n = 4x = 40$) showed that some kind of abnormality can generally be observed in meiotic chromosome pairing of polyploid genotypes, mainly multivalent associations in diakinesis: Quarin (1994), Pagliarini et al. (2001), Araújo et al. (2005), Risso-Pascotto et al. (2005), Caetano et al. (2006), Reis et al. (2008), Simioni and Valle (2011).

According to Sybenga (1992), some abnormalities are possible and inherent to polyploid plants, generating pollen with an unbalanced chromosome number, a phenomenon that can affect the male fertility of these plants. This episode can induce the formation of cells that expose chromosome lagging, bridges, precocious ascension, and several other rare abnormalities.

Pollen viability analysis

The pollen viability of all 24 analyzed genotypes was high (82.15% - 99.67%), both for sexual and facultative apomictic plants (Table 3). Twenty-one hybrid genotypes had over 90% viable pollens, i.e. 72% of the total. These results demonstrate that the meiotic changes observed in pollen mother cells (data not shown) did not affect pollen viability. According with Pagliarini and Pozzobon (2004), apomictic *P. notatum* is pseudogamic, the pollen fertilizes only the endosperm, thus requiring a certain pollen viability.

In a study with *Urochloa decumbens*, Mendes-Bonato et al. (2002) estimated a mean pollen sterility of 52.73%, based on the high rates of meiotic abnormalities in accessions of the species analyzed. However, despite the high sterility rate, seed production was not affected, leading to the conclusion that these plants were apomictic. The percentage of viable grains (61.36% - 64.86%) of freshly induced polyploidy *Urochloa decumbens* plants, was considered satisfactory by Simioni and Valle (2011), who stated that these plants could be used as parents in polycross blocks in the species breeding program. Tetraploid hybrids derived from crosses between *U. ruziziensis* and *U. brizantha* had mean pollen viability indices of 52.76% (Risso-Pascotto et al. 2005).

The pollen viability of the parents of F_1 hybrids was described as high by Krycki et al. (2016). The genotypes subjected to chromosome duplication, 'WKS63' and 'WKS92' had 88.7 and 95.7 of fertile pollen, respectively. The viability of the other parents also exceeded 85%, except for genotype 'D25', with a fertility of 74.7%. On the other hand, in the study of Dahmer et al. (2008), the pollen viability of *P. notatum* accessions ranged from 81 to 91.5%; in F_1 hybrids, Podio et al. (2012) found from 66.1 to 84.7%. and in *P. leptum*, Reis et al. (2008) found fertile pollens at rates of 88.99 - 95.06%.

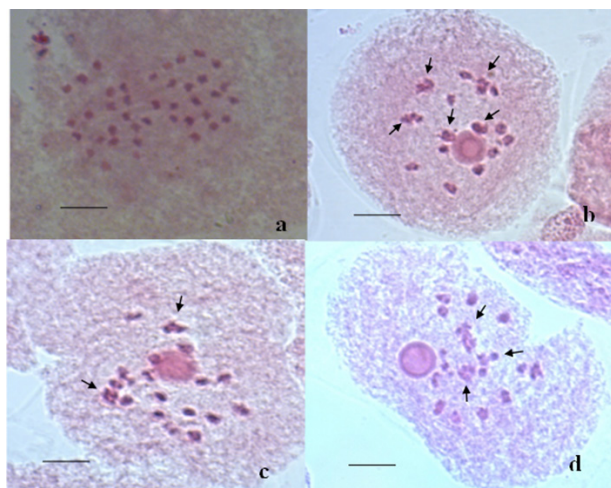


Figure 2. Chromosome associations in meiotic cells (meiosis I): a) plant KF16 (anaphase I with 40 chromosomes); b) plant KF6 (diakinesis with 10 bivalents and 5 quadrivalent associations); c) plant KF3 (diakinesis with 16 bivalents and 2 quadrivalent associations); d) plant KN5 (diakinesis with 14 bivalents and 3 quadrivalent associations). The arrows indicate the quadrivalent associations. Scale: 10 μ m.

Table 3. Pollen viability of F_1 genotypes, apomictic and sexual progenies

Genotype	Pollen viability (% stained pollen)
KC1*	91.04
KD1**	99.67
KD2**	95.29
KD5**	96.69
KD6*	96.25
KD7**	86.95
KD9*	93.79
KE1*	89.40
KF1*	86.14
KF2*	91.14
KF3**	93.81
KF4*	91.01
KF6**	89.43
KF7*	91.78
KF8**	94.28
KF9*	82.15
KF10**	86.78
KF15*	89.76
KF16**	98.63
KF17*	98.66
KN2*	94.55
KN3**	96.21
KN4*	98.64
KN5**	92.72

* Sexual; ** Facultative apomictic.

Comparing the percentages pollen viability data of experiments with species, accessions and hybrids of the genera *Urochloa* and *Paspalum*, viability of the latter was higher, although in all studies with *Urochloa*, the indices found in the evaluation were considered sufficient to validate them as parents for hybridization programs.

Analysis of the F₁ progenies in this study showed low presence of abnormalities in the meiotic chromosome pairing and high pollen viability. For the future crosses, the apomictic hybrids can be the male parents and the sexual plants can be used as female parents.

Besides that, the hybrid progenies were evaluated in field trials to verify the yield potential (data not show). Combined with the results of this study, genotypes with reproductive stability and satisfactory forage yield under field conditions will be included in new breeding cycles in our breeding program and, after testing in other evaluation phases, they may become cultivars candidates, with possibility of registration and protection.

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