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Meiotic stability and sexual reproduction of diploid *Paspalum notatum* Flügge accession

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ABSTRACT: *Paspalum notatum* has a good forage value and agronomic traits. The biggest problem is related to the reproduction mode, which limits crossings. This study aimed to confirm the ploidy level, analyze meiotic behavior, and determine the reproductive mode of accessions of the species. One diploid accession of *P. notatum*, the cultivar Pensacola, was used as a sexual and diploid control, and one native accession was used as a tetraploid and apomictic. Young inflorescences were used to analyze the ploidy level and meiotic behavior, and mature inflorescences were used to evaluate the reproductive mode. For accessions 92N and Pensacola, diploid and tetraploid accessions were confirmed. The meiotic behavior of the accessions was regular. The reproductive modes of accessions 92N and Pensacola were sexual, whereas the tetraploid accession was apomictic. *P. notatum* 92N is a promising species for inclusion in breeding programs because of its reproductive mode and meiotic stability.

Key words: apomixis; forage grass; genetic improvement; meiotic index; sexual reproduction

Estabilidade meiótica e reprodução sexual de um acesso diploide de *Paspalum notatum* Flügge

RESUMO: *Paspalum notatum* tem bom valor forrageiro e excelentes características agronômicas. Seu maior problema está relacionado ao modo de reprodução, o qual limita cruzamentos. O objetivo deste estudo foi confirmar o nível de ploidia, analisar o comportamento meiótico e determinar o modo de reprodução de acessos da espécie. Um acesso diploide de *P. notatum*, a cultivar Pensacola utilizada como controle sexual e diploide e um acesso native usado como controle tetraploide e apomítico foram analisados. Inflorescências jovens foram usadas para analisar o nível de ploidia e o comportamento meiótico e inflorescências maduras foram usadas para avaliar o modo de reprodução. Para o acesso 92N e a Pensacola, o nível diploide foi confirmado, bem como para o acesso tetraploide. O comportamento meiótico dos acessos foi regular. O modo de reprodução do acesso 92N e Pensacola foi sexual e o acesso tetraploide foi apomítico. *P. notatum* 92N é um material promissor para inclusão em programas de melhoramento genético devido ao seu modo de reprodução e estabilidade meiótica.

Palavras-chave: apomixia; gramínea forrageira; melhoramento genético; índice meiótico; reprodução sexual

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Introduction

The Pampa biome is recognized for its great species diversity and includes approximately 400 grasses and 150 forage legumes and plays a fundamental role in the field formations of Southern Brazil. This biome represents a pastoral natural ecosystem and its sustainable maintenance occurs through livestock activity (Andrade et al., 2019).

The native forage species of Rio Grande do Sul are the main resources used for livestock production (Carvalho et al., 2018; Andrade et al., 2019). Therefore, the meat and milk supply chain highlights the importance of cultivating native forage because they cause fewer ecological risks than exotic forage species (Overbeck et al., 2018). In addition, native species have greater productivity and adaptation potential when cultivated in their original environments (Carvalho et al., 2018). According to Andrade et al. (2019), the native grasslands of Southern Brazil contain several rhizomatous and stoloniferous grasses with good forage value, such as those of the genus *Paspalum*.

Paspalum is one of the largest genera in the Poaceae family and includes approximately 212 species in Brazil (Maciel et al., 2020). This genus is found in all Brazilian physiographic formations and its performance is recognized when destined for forage production and in native pastures. *Paspalum* is one of the main components of the field area and is widely distributed in the tropical and subtropical regions of America (Overbeck et al., 2018; Andrade et al., 2019). As reported by several authors, the species were more resistant to cold than other grasses (Motta et al., 2017).

Paspalum notatum Flügge is one of the most prevalent species in Southern Brazil and is popularly known as grama-forquilha or grama-do-rio-grande. Its center of origin is Southern Brazil, northern Argentina, and Paraguay, and it stands out due to its robustness and dissemination throughout Brazil (Rojas-Sandoval, 2018). *P. notatum* is considered a polymorphic species (Fachinetto et al., 2017) and a native forage species of wide interest because of its distribution and favorable growth habits (Fachinetto et al., 2012; Machado et al., 2017; Steiner et al., 2017).

According to Steiner et al. (2017), in addition to good forage production, *Paspalum* species also stand out for their wide genetic variability, favoring their inclusion in breeding programs. However, they reproduce by apomictic and/or sexual modes, which are closely related to the ploidy level. In general, diploidy is correlated with sexual reproduction, whereas polyploidy is associated with apomixis (Ortiz et al., 2020).

Apomixis is considered a problem in the genetic breeding, registration, and protection of cultivars (Huber et al., 2016). Therefore, it is important to identify diploid materials that can be used by sexual parents. The only available diploid material of the species is the Pensacola cultivar, which belongs to the *Paspalum notatum* var. *saurae* Parodi. When one parent undergoes sexual reproduction, it is possible to increase genetic variability, allowing the recovery of elite progenies to fix alleles in the first generation (Ortiz et al., 2020).

Four wild diploid accessions of *P. notatum* have been identified (Fachinetto et al., 2018). These materials showed good forage production, with approximately four to seven times greater production than the commercial cultivar, and good persistence to climatic conditions (Fachinetto et al., 2012). In addition, these accessions exhibited wide genetic and morphological variability (Fachinetto et al., 2017). Despite the good agronomic performance of these wild diploid accessions, their reproductive modes and meiotic stability have not yet been evaluated. Thus, this study aimed to confirm the ploidy level to determine the reproductive mode of wild diploid accessions and their meiotic behavior for later inclusion in *Paspalum* breeding programs.

Materials and Methods

Seeds of four diploid accessions of *P. notatum* from the United States Department of Agriculture Germplasm Bank, previously evaluated by Fachinetto et al. (2018), were used for the analysis. These materials were identified as PI508832 (66N), PI337573 (67N), PI404863 (87N), and PI508831 (92N) (U.S. National Plant Germplasm System available at https://www.ars-grin.gov/npgs/).

The 66N and 92N accessions had a collection record on January 30, 1979, in Santa Fé (Argentina). The 67N accession was collected in Santa Fé (Argentina) in 1968, and the 87N accession was collected in 1975 in Paysandu (Uruguay).

Seeds of cv. Pensacola (sexual control) was donated by Comércio e Representações Agrícolas RELVA LTDA (Ijuõ, Brazil). The control of apomictic mode was a tetraploid accession collected at the campus of the Universidade Regional do Noroeste do Estado do Rio Grande do Sul – UNIJUÍ, Ijuí, RS, Brazil, from which was deposited a voucher in the Rogério Bueno Herbarium (HUIRB) under registration HUIRB 8038. National System for the Management of Genetic Heritage and Associated Traditional Knowledge Register Number (SISGEN) A9A11FB.

Approximately 50 seeds from each accession of *P. notatum* from the USDA and cv. Pensacola were germinated in a greenhouse. After germination, plants were cultivated and monitored until flowering.

Cytogenetic analysis

Confirmation of ploidy levels and meiotic behavior analyses were performed on the cv. Pensacola, tetraploid, and USDA accessions using pollen mother cells. Young inflorescences were collected, fixed in Carnoy 3:1 (ethanol: acetic acid) for 24 h at room temperature, and stored in ethanol 70% under refrigeration (Moreira et al., 2017). Slides were prepared using the squashing technique in acetocarmine 2% (Fachinetto et al., 2018). For each accession, at least five individuals were used and about ten cells in the diakination phase were examined under an optical microscope (Olympus BX40).

The ploidy level was determined according to the basic number of chromosomes, x = 10 (Dahmer et al., 2008). In addition, all meiotic phases were observed to analyze meiotic behavior such as chromosome associations in diakinese/metaphase I. Chromosome segregations in anaphase/telophase I and II were evaluated by counting the number of cells with normal and abnormal chromosome configurations for each meiotic phase. The meiotic index was calculated using tetrad analyses as follows: IM = normal tetrad number/total tetrad number observed × 100 (Krycki et al., 2016). Normal tetrads contained four equal numbers of cells.

Cytoembryological analysis

Inflorescences in anthesis were collected, fixed in FAA (formaldehyde alcohol acetic acid) for 24 h at room temperature, and stored in ethanol 70% under refrigeration. The ovaries were dissected and clarified (alcohol dehydration series with methyl salicylate) (Weiler et al., 2017). The ovaries were then stored in methyl salicylate solution (100%) for subsequent microscopy analysis. Embryo sacs were evaluated to identify apomixis or sexuality using the cv. Pensacola and the tetraploid accession were used as sexual and apomictic controls, respectively.

Results and Discussion

Of the four diploid accessions, only the 92N accession showed germination. Accessions 66N, 67N, and 87N were subjected to tetrazolium and showed low fertility percentages of 8%, 0%, and 7%, respectively (data not shown). The non-germination of the three accessions might be due to seed collection and storage time, as they were over 40 years old. Several factors may be involved, such as inadequate storage and environmental changes during storage.

Cytogenetic analysis

The ploidy levels of the 92N accession, the cv. Pensacola, and the tetraploid accession were confirmed using pollen mother cell analyses. Homogeneity was observed in the number of chromosomes within the accessions. All the cells of 92N accession and cv. Pensacola are diploid (2n = 2x = 20 chromosomes) (Table 1). According to Fachinetto et al. (2018), the 92N accession can be considered a wild diploid because of its place of origin, which is the same as that of cv. Pensacola which belongs to the *P. notatum* var. *saurae*. The expected chromosome number was confirmed to be tetraploid (2n = 4x = 40; Table 1), which was observed in all the cells analyzed.

Chromosome number analysis has demonstrated that the tetraploid form of *P. notatum* is the most common in Brazil (Dahmer et al., 2008). Diploid accessions originated from the Provinces of Corrientes, Entre Rios, and Santa Fé in Argentina, which is the collection site for the 92N accession (Fachinetto et al., 2018). Generally, diploids are sexual and stable during meiosis; however, polyploids reproduce by obligatory or facultative apomixis (Ortiz et al., 2020).

In our analysis, the accessions showed regular meiotic behavior during meioses I and II, in which all the cells were regular (Table 1). The meiotic index showed little variation between the tetraploid accessions and cv. Pensacola and 74.21% and 74.45%, respectively. *P. notatum* 92N, the meiotic index was 62.08%. Normal tetrads were found (with four equal cells) (Figure 1A-C) and dyads and triads (Figure 1D-F). These are products of the absence or failure of the first and/or second cytokines.

Paspalum spp. exhibit a high meiotic index, as shown by Krycki et al. (2016) who evaluated three plants of *P. notatum* and observed a meiotic index ranging from 88.7% to 95.7%. Plants are considered meiotically stable if they have a meiotic index of 80% or higher (Krycki et al., 2016). Although 92N accession had a meiotic index lower than the reference value, it showed regularity during microspore formation,

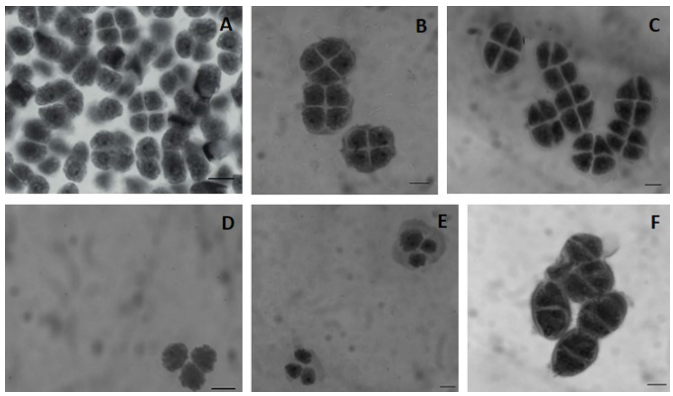

Figure 1. Tetrads in *Paspalum notatum*. A) Tetrad 92N accession. B) Tetrad cv. Pensacola. C) Tetrad tetraploid accession. D) Triad 92N accession. E) Triads cv. Pensacola. F) Dyads tetraploid accession. Scale 50µm.

Table 1. Meiotic behavior and chromosome number in *Paspalum notatum*.

Accession	Regularity	Number of cells in each meiotic phase				Meiotic index (%)	Chromosome number
		Diakineses/ Metaphase I	Anaphase I/ Telophase I	Metaphase II -	Anaphase II/ Telophase II		
P. notatum 92N	Normal	153	2	2	-	62.08	2n=2x=20
	Abnormal	-	-	-	-		
cv. Pensacola	Normal	251	243	-	-	74.45	2n=2x=20
	Abnormal	-	-	-	-		
Tetraploid	Normal	396	56	-	8	74.21	2n=4x= 40
	Abnormal	-	-	-	-		

as evidenced by the analysis of meiotic behavior, which is considered a stable accession for this criterion.

Meiotic regularity is a determining factor, and according to Ragalzi et al. (2021), when there is chromosomal irregularity, seed production can decline, harming the selection of better plants. The identification of genetic alterations among populations within a species is a basic aspect of plant breeding programs. However, meiotic irregularities make it impossible to produce new cultivars through recombination in breeding programs.

The presence of dyads and triads suggested the presence of unreduced gametes. In the 92N accession, these formations were observed, suggesting the presence of 2n gametes. 2n gametes may be of great importance in plant breeding and can be used in two ways: for sexual polyploidization and/or for transferring desirable genes from diploid species to polyploid cultivars (García et al., 2020).

Cytoembryological analysis

Cytoembryological analysis was performed using the 92N accession cv. Pensacola and the tetraploid accession were used as controls for sexual and apomictic reproductive modes, respectively. Analyses demonstrated that the 92N accession and cv. Pensacola had embryo sacs compatible with sexual reproduction, whereas the tetraploid accessions had embryo sacs compatible with apomixis (Figure 2). These results agree with those of Ortiz et al. (2017) who suggested that diploidy is associated with sexual reproduction and allogamy, whereas polyploidy is associated with apomixis. Recent studies have shown that apomixis in *Paspalum notatum* is controlled by several genes, the expression of which can be affected by quantitative inheritance and genetic distance (Marcon et al., 2019).

Sexual plants possess antypodes and polar nuclei in a single embryonic sac. The tetraploid accessions showed multiple embryonic sacs, which are characteristic of apomixis. Plants with an apomictic reproduction mode have multiple aposporic embryo sacs characterized by egg cells with no antipodes or synergies (Weiler et al., 2017). Experimental crosses and progeny tests have revealed that tetraploid races are apomictic (Kaushal et al., 2019). However, in a wide collection of *P. notatum*, cytoembryological analysis has revealed that most tetraploid genotypes of different origins exhibit residual sexuality (Ortiz et al., 2020).

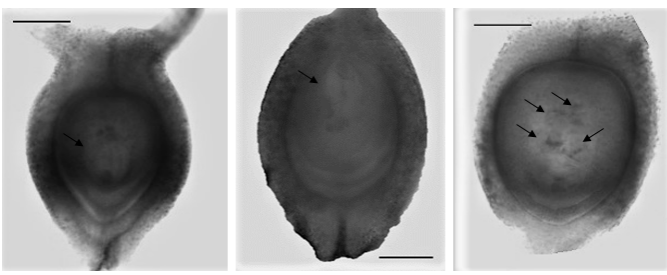

Figure 2. Embryo sacs of *Paspalum notatum* accessions. A) P. notatum 92N. B) cv. Pensacola. Arrow indicates the single embryo sac. C) Tetraploid accession. Arrows indicate the multiple embryo sacs. Scale: 50µm.

According to Fachinetto et al. (2017), the main methodology for genetic breeding involves the selection of natural variability and germplasm collections. *P. notatum* is widely used for breeding and is one of the few cultivars of the genus Pensacola that incorporates the native ecotypes of South America. Apomictic species require plants in a totally sexual reproductive mode so that they can have genetic variability (Ortiz et al., 2020). Thus, the determination of the reproductive mode and confirmation of the ploidy level and meiotic behavior can contribute to breeding programs for *P. notatum*. The 92N accession had superior agronomic traits and can be used as an alternative to cv. Pensacola, allowing for increased genetic variability and new plants with improved characteristics compared to apomictic and sexual plants. Apomictic species are considered ideal for breeding because hybridization with sexual plants offers the possibility of new genetic combinations and allele fixation in the F1 progeny. According to Fachinetto et al. (2012). total dry matter production was approximately seven times higher than that of cv. Pensacola and five times dry matter yield of leaves, In addition, the 92N accession is considered a wild diploid (Fachinetto et al., 2018). The morphological characterization of this accession is described in the study development by Fachinetto et al. (2017).

Conclusions

Our results demonstrated that 92N accession is a promising candidate for the *Paspalum* breeding programs because it possesses a sexual reproduction mode and regular meiotic behavior, in addition to the other favorable agronomic traits described in the literature. This accession allowed us to obtain new maternal genitors with greater aggregation of favorable alleles for promising new recombinations. Additionally, to facilitate the generation of variability, it was possible to select fixed F1 clones with heterosis.

Compliance with Ethical Standards

Author contributions: Conceptualization: TRS, JMF; Data curation: TRS, JMF; Formal analysis: TRS, MD, JAGS, EAP, JMF; Investigation: TRS; Methodology: TRS, JMF; Project administration: MD, JMF; Resources: MD, JMF; Supervision, JMF: MD, JAGS, EAP; Validation: MD, JAGS, EAP, JMF; Visualization: TRS; Writing – original draft: TRS; Writing – review & editing: MD, JAGS, EAP, JMF.

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