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Differences in stomatal and pollen grain dimensions and pollen viability between *Paspalum rawitscheri* populations

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ABSTRACT

The objective of this study was to evaluate stomatal and pollen grain size and to estimate pollen viability of individuals from different populations of *Paspalum rawitscheri* (Parodi) Chase ex G.H. Rua & Valls. To analyze stomatal size, slides were made of the adaxial leaf epidermis using the epidermal impression method. The height and width of 100 stomata per population were analyzed. Pollen was obtained from inflorescences to evaluate pollen grain size and pollen viability. Pollen grains were stained with 2% acetic orcein, 2% acetic carmine, or Alexander's reactive stain. Per population, 1600 grains of pollen were observed for viability, and 50 grains of pollen were measured. There were significant differences between populations in stomatal height and pollen grain height and width. The populations also differed in pollen viability, with the Santa Maria population showing the lowest viability. The differences in stomatal and pollen grain size suggest genetic variability in the evaluated populations. Moreover, low pollen viability in one population indicates that its decline may be related to low fertility.

Keywords: Grass; Ploidy; Fertility; Threatened species

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1 INTRODUCTION

Paspalum (Poaceae; Panicoideae) is an important genus of grasses with high forage value. It has about 350 species, distributed in different regions of the Americas (DENHAM, 2005; RUA et al., 2010). Species in this genus are widely studied in plant breeding programs for plant hybridization and polyploidization techniques (WEILER et al., 2015; MOTTA et al., 2016). They show high polyploidy, ranging from diploid to hexaploid (SOSTER, 2009; FACHINETTO, 2010); this high variation in ploidy level is one of the main characteristics of the genus.

Cytogenetic analyses are used to characterize germplasm by assessing ploidy level, meiotic and mitotic behavior, and pollen grain fertility (NOLASCO, 2011; POZZOBON et al., 2011; BRUNO, 2015). For polyploidy analysis, the chromosome counting method and the evaluation of morphological characters, such as stomatal dimensions and pollen grain diameter, can be used (TEDESCO et al., 1999; MARINHO, 2013). Some studies have confirmed that polyploidized plants tend to have larger stomata, and may also show changes in stomatal density (HODGSON et al., 2010; RAIZER, 2017).

In addition, the evaluation of pollen grain viability provides important information that can be used in different areas of study, such as genetics, palynology, and taxonomy (FRESCURA et al., 2012). Analysis of pollen viability gives relevant information that is used in plant breeding programs, e.g., using viable pollen grains to demonstrate fertilization success (CABRAL et al., 2013). As the genus *Paspalum* has high rates of polyploidy, and is used in breeding and hybridization programs, it is interesting to evaluate pollen viability in species of this genus.

Paspalum rawitscheri (Parodi) Chase ex G. H. Rua & Valls is distributed in the southern region of Brazil. Studies on this species are scarce, with little or no information on its mode of reproduction, phenology, and ploidy levels. In addition, the species is included in the endangered species list for the State of Rio Grande do Sul (SEMA, 2014) and as endangered (EN) in the Brazilian Red List (MMA, 2014), making the study of its biology extremely important. Thus, the objectives of this study were to

evaluate stomatal and pollen grain dimensions and estimate the pollen viability of individuals from different populations of *P. rawitscheri* from Rio Grande do Sul.

2 MATERIALS AND METHODS

2.1 Plant Collection

The plant material used for the analyses was collected from three different *P. rawitscheri* populations, each from one of the following municipalities of Rio Grande do Sul State (RS), Brazil: Santa Maria (29°37'37.5"S 53°52'27.1"W; elevation 222 m), Campestre da Serra (28°40'42.7"S 51°03'50.3"W; elevation 770 m), and São Martinho da Serra (29°24'54.8"S 54°61'09.6" W; elevation 305 m). Six individuals from the populations were collected between November 2016 and February 2017, and deposited in the SMDB herbarium of the Federal University of Santa Maria (UFSM). For the analysis, plants collected in the field were replanted and cultivated in the greenhouse of the Department of Biology at UFSM. The analyses were carried out at the Plant Cytogenetics and Genotoxicity Laboratory (LABCITOGEN).

2.2 Measurement of Stomatal Dimensions

Fresh leaves collected from six *P. rawitscheri* individuals cultivated in the greenhouse were used for the evaluation of stomatal parameters. The leaves were collected in the afternoon, and in the same day the slides were prepared. The preparation of the slides followed the adaxial leaf epidermal printing methodology, with universal instant bonding (Super Bonder®; SEGATTO et al., 2004).

Two individuals per population were analyzed (Table 1); one slide per individual was prepared, and in each of the slides 50 stomata were analyzed. The analysis was performed under the 40× objective of an optical microscope with a micrometer eyepiece, using the fields of the slides with the largest apparent number of stomata per area. Two measurements were made for each stoma: width (polar diameter) and height (equatorial diameter).

Table 1 – Individuals of *Paspalum rawitscheri* used for measurement of stomatal and pollen grain dimensions.

Stomata			Pollen grain		
Population	**Individual**	**Identification***	**Population**	**Individual**	**Identification***
Santa Maria	1	Essi, L. 749A	**Santa Maria**	1	Essi, L. 749B
	2	Essi, L. 749B		2	Essi, L. 749D
Campestre da Serra	1	Essi, L. 766D	**Campestre da Serra**	1	Essi, L. 766C
	2	Essi, L. 766K		2	Essi, L. 766M
São Martinho da Serra	1	Essi, L. 1049A			
	2	Essi, L. 1049N			

*Identical identification numbers followed by different letters represent different individuals from the same collection location.

2.3 Measurement of Pollen Grain Dimensions

For the measurement of pollen grain dimensions, inflorescences were collected from greenhouse-cultivated plants belonging to the populations from Santa Maria and Campestre da Serra (Table 1). Plants from the São Martinho da Serra population did not flower in the greenhouse during the experiments, making it impossible to analyze their pollen grains.

Inflorescences were collected and fixed in 3:1 ethanol:acetic acid for 24 h, and then stored in 70% alcohol under refrigeration. The slides were prepared using the crushing technique described by Guerra & Souza (2002), and stained with 2% acetic orcein dye.

Two slides per population (one slides from each individual) and 50 pollen grains per culm were analyzed, totaling 100 pollen grains per population. The evaluation was made under a 40× objective of an optical microscope with a micrometer eyepiece. Two measurements were taken for each pollen grain: width (polar diameter) and height (equatorial diameter).

2.4 Pollen Viability

To evaluate pollen viability, inflorescences were also collected from greenhouse-cultivated plants belonging to the Santa Maria and Campestre da Serra populations (Table 1).

Inflorescences were collected and fixed in 3:1 ethanol:acetic acid for 24 h and then stored in 70% alcohol under refrigeration. The slides were prepared using the crushing technique described in Guerra and Souza (2002). For the pollen viability analysis, three different colorimetric methods were used: 2% acetic orcein, 2% acetic carmine, and Alexander's reactive stains. Pollen viability of *P. rawitscheri* was estimated since no studies were found with information on the fertility of the species or its mode of reproduction.

The pollen grains stained dark pink (or purple) with 2% acetic orcein were considered viable and those poorly stained or unstained were considered unviable. Pollen grains stained red with 2% acetic carmine were considered viable and those minimally stained or unstained were considered unviable.

In contrast, in the test using Alexander's reactive stain, viable pollen grains were stained a purple color and unviable were stained a blue-green color. These colors occur due to two components present in Alexander's reactive dye, acidic fuchsin and malachite green, which interact with the protoplasm and cellulose of the pollen grain wall (MUNHOZ et al., 2008). For each population, 1600 pollen grains were analyzed for each stain.

2.5 Statistical Analysis

Data for stomatal and pollen grain dimensions and pollen viability were analyzed separately using the Tukey's test at 5% probability of error with the Sisvar 5.6 program (FERREIRA, 2014).

3 RESULTS AND DISCUSSION

The stomatal (Figure 1) and pollen grain dimensions were the lowest for the Santa Maria population (Table 2), while the Campestre da Serra population had the highest values for pollen grain dimensions and stomatal height, with stomatal width similar to that for the São Martinho da Serra population. These differences indicate

the possibility of differences in the ploidy level between Santa Maria and Campestre da Serra individuals.

Figure 1 – The stomata of *Paspalum rawitscheri* individuals from Santa Maria, Rio Grande do Sul (RS) (A); São Martinho da Serra, RS (B); and Campestre da Serra, RS (C). The scale represents 2.5 µm.

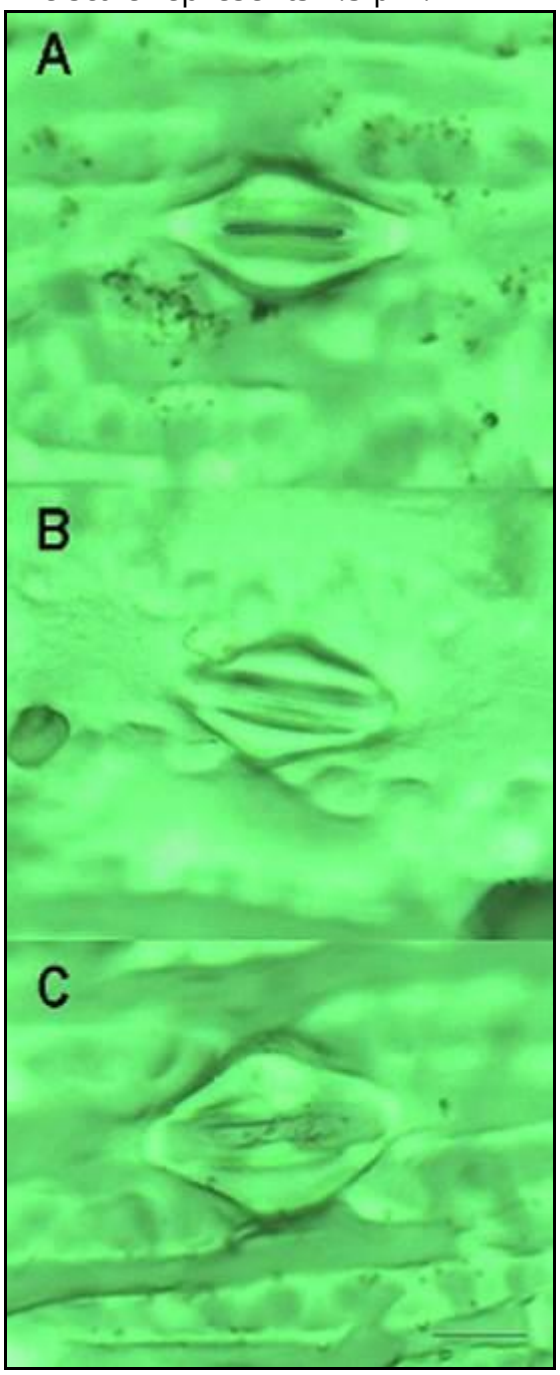

Table 2 – Average height and width of stomata and pollen grains of *Paspalum rawitscheri*.

POPULATION	Stomatal height (µm)	Stomatal width (µm)	Pollen grain height (µm)	Pollen grain width (µm)
Santa Maria	1.65[c]	3.34[b]	3.15[b]	3.93[b]
Campestre da Serra	1.90[a]	3.57[a]	3.66[a]	4.03[a]
São Martinho da Serra	1.81[b]	3.65[a]	-	-

*The same letters in the same column indicate that the averages do not differ statistically from each other (Tukey test; $p < 0.05$).

Khazaei et al. (2010) showed that stomata in *Triticum* species (*T. monococcum* L., *T. durum* Desf., and *T. aestivum* L.) were wider in polyploids than in diploids. In a study of induction and identification of polyploidy in *Hymenaea courbaril* L. var. *stilbocarpa* (Hayne) Lee et Lang, root meristems exposed to 3 µM of the herbicide trifluralin for 96 h, which caused genome duplication, had the largest average stomatal size compared to the treatments (24 h, 48 h, and 72 h exposure to 3 µM of trifluralin; BONA et al., 2016).

Studies such as the above confirm that there may be a relationship between ploidy level and morphological characters, and that the larger the chromosome number, the larger the stomatal size. On the other hand, Vichiato et al. (2006) found that tetraploid plants of *Dendrobium nobile* Lindl. had smaller polar and equatorial diameters than diploid plants of the same species.

In this study, there was between-individual variation in stomatal height and pollen grain height in the *P. rawitscheri* population from Santa Maria (Table 3). In the case of the Campestre da Serra population, all measures analyzed differed significantly between individuals (Table 3). These results show that there is variability between *P. rawitscheri* individuals in stomatal and pollen grain parameters.

Table 3 – Average height and width of stomata and pollen grains in individuals of *Paspalum rawitscheri*.

POPULATION	INDIVIDUAL	Stomatal height (µm)	Stomatal width (µm)	Pollen grain height (µm)	Pollen grain width (µm)
Santa Maria	1	1,74[a]	3,34[a]	2,94[b]	4,02[a]
	2	1,56[b]	3,34[a]	3,37[a]	4,04[a]
Campestre da Serra	1	1,62[b]	3,27[b]	3,52[b]	4,00[a]
	2	2,18[a]	3,86[a]	3,81[a]	3,86[b]
São Martinho da Serra	1	1,74[b]	3,99[a]	-	-
	2	1,88[a]	3,31[b]	-	-

*The same letters in the same column indicate averages that do not differ significantly from each other (Tukey's test; $p < 0.05$).

In a study by Vieira (2014), the diameter of the stomatal complex in P. stellatum Hum. & Bonpl. ex Flüggé was analyzed for different ploidy levels and two types of environment—wet and dry. Among the accessions analyzed, stomatal widths of cytotypes 937 (2n = 48) and 973 (2n = 52) were the largest. However, cytotype 1010 (2n = 60) had a smaller width. Stomatal height of cytotypes 255 (2n = 20), 973 (2n = 52), and 1019 (2n = 52) was greater compared to those of the other accessions, while cytotype 1010 (2n = 60) had stomata with the lowest height. There was a significant difference between environments only for the width measure. This study also shows that there may be differences in stomatal size and pollen grain diameter between individuals of the same species.

In addition to the analysis of stomatal and pollen grain size, pollen viability was also verified using three different staining methods (Figure 2). The acetic orcein stain gave the highest pollen viability results for the two populations evaluated, Santa Maria (99.75%) and Campestre da Serra (99.5%; Table 4).

Figure 2 - *Paspalum rawitscheri* pollen grains. Viable pollen grain (A) and unviable pollen grain (B) stained with 2% acetic orcein; viable pollen grain (C) and unviable pollen grain (D) stained with 2% acetic carmine; viable pollen grain (E) and unviable pollen grain (F) stained with Alexander's reactive stain. The scale represents 2.5 µm.

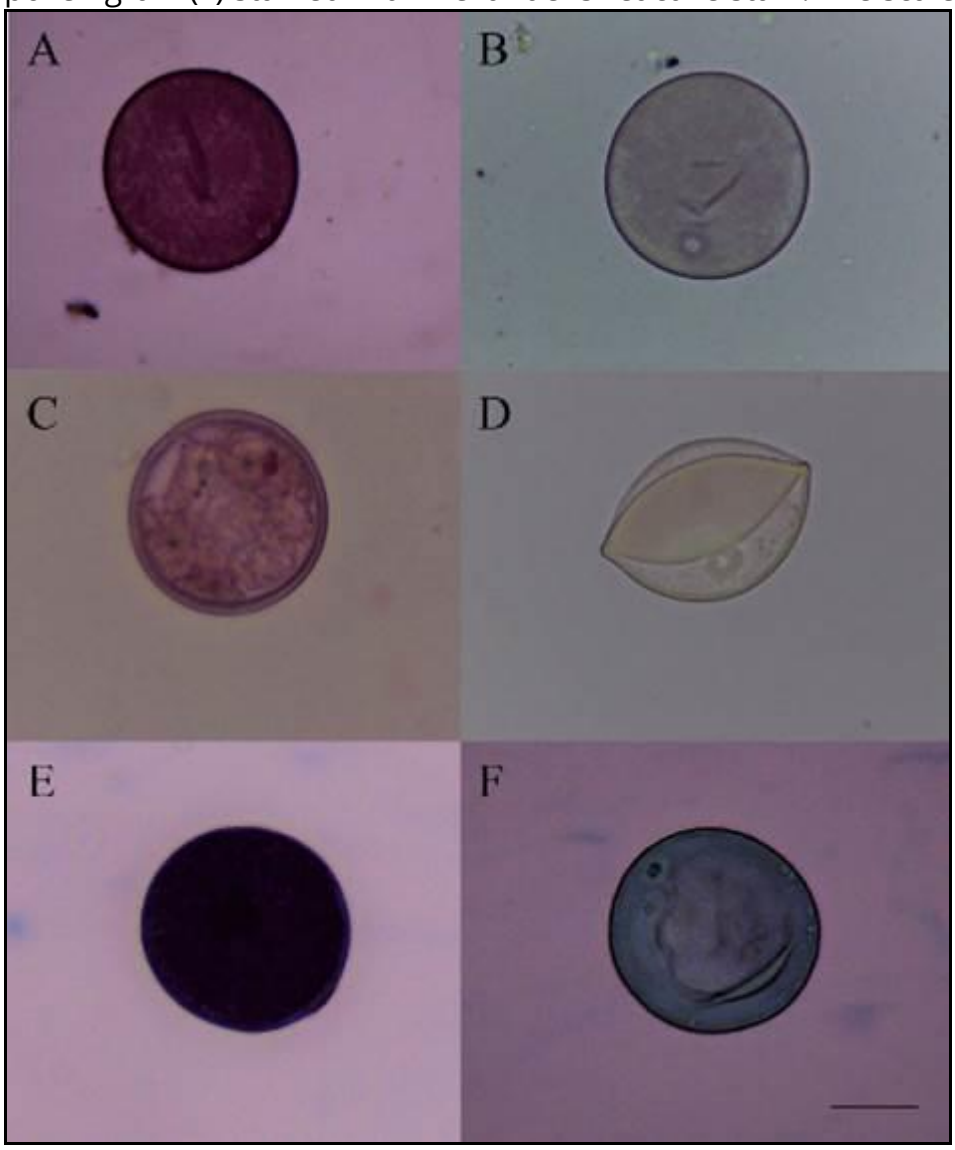

Table 4 – Assessment of pollen viability in *Paspalum rawitscheri* using three different stains.

POPULATION	**COLORIMETRIC METHODS (% viable pollen)**		
	2% Acetic Orcein	2% Acetic Carmine	Alexander's Reactive
Santa Maria	99.75[3]	47.31[3]	3,34[a]
Campestre da Serra	99.5[3]	76.06[a]	3,27[b]
Average	99.63[A]	61.69[B]	3,86[a]

*The same letters in the same column indicate averages that do not differ significantly from each other (Tukey's test; $p < 0.05$).

The overall mean of percent pollen viability for the 2% acetic orcein stain differed significantly from the overall means for the other two stains. Other studies have also shown that orcein normally demonstrates the highest viability rate in pollen grains of different species.

When comparing pollen viability of *Crotalaria juncea* L. accessions from the state of Rio Grande do Sul, Coelho et al. (2012) demonstrated significantly higher viability of pollen grains stained with 2% acetic orcein (98.96%) than those stained with Alexander's reactive stain (83.37%). Although overall averages were equally high for both stains, this study also showed that in three of the ten *C. juncea* accessions analyzed, orcein staining indicated 100% pollen viability.

Studies such as this corroborate the fact that the 2% acetic orcein stain may overestimate pollen viability, making it difficult to distinguish between viable and unviable pollen grains (FRESCURA et al., 2012; HISTER & TEDESCO, 2016).

In this study, the viability result obtained with 2% acetic carmine stain for the Santa Maria population was low (47.31%) when compared to the Campestre da Serra population (76.06 %). However, this difference was not statistically significant.

Acetic carmine was an effective colorimetric method for differentiating viable and unviable pollen in our study, confirming results obtained in other studies. Ribeiro (2016) evaluated pollen viability with 2% acetic carmine stain for different species of *Mesosetum* Steud.: *M. alatum* Filg., *M. ansatum* (Trin.) Kuhlm., *M. bifarium* (Hack.) Chase, *M. chaseae* Luces, *M. compressum* Swallen, *M. elytrochaetum* (Hack.) Swallen, and *M. rottboellioides* (Kunth) Hitchc. There was a high rate of differentiation between viable and unviable pollen, with *M. ansatum* showing the highest rate of viability (99.16%) and M. chaseae the lowest (27.69%), demonstrating that 2% acetic carmine is a reliable stain for pollen viability assessment.

When *P. rawitscheri* pollen grains were stained with 2% acetic orcein and 2% acetic carmine, the viability percentages did not differ significantly between the two populations. However, when stained with Alexander's reactive stain there was a significant difference between populations (Table 4).

Auler et al. (2006) found a similar result for pollen viability in eight populations of *Baccharis trimera* (Less.) DC. from the states of Rio Grande do Sul and Santa Catarina. Only Alexander's reactive stain could reveal differences in pollen viability between five of the evaluated populations (Santa Maria, Arroio Grande, Corsan Biological Reserve, São Vicente do Sul, and EPAGRI Caçador), showing that it was more efficient than acetic orcein and propionic carmine. In the present study also statistically significant differences in pollen viability between the two *P. rawitscheri* populations were demonstrated with Alexander's reactive stain.

In the two populations evaluated, Santa Maria had a lower average percentage of pollen viability (30.13%) compared to Campestre da Serra (79.31%; Table 4). The low viability found in the Santa Maria population suggests low fertility in *P. rawitscheri* individuals that may be related to the decline of this species.

4 CONCLUSION

There may be genetic variability between *P. rawitscheri* populations in Santa Maria and Campestre da Serra, since these populations show significant differences in stomatal and pollen grain size. Additional support for this conclusion is provided by the large geographical distance between the two populations, which may be a barrier to gene flow between them.

Because they showed significant variation in both stomata and pollen grain measurements, *P. rawitscheri* populations may have distinct ploidy levels, which is quite common for species of the genus *Paspalum*.

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