

Cytoembryological evaluation, meiotic behavior and pollen viability of *Paspalum notatum* tetraploidized plants

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Abstract: *This study evaluated the mode of reproduction, the meiotic behavior and the pollen viability of three tetraploid plants (2n=4x=40) originated from somatic chromosome duplication of Paspalum notatum plants. The plant WKS 3 changed the mode of reproduction after duplication and became apomictic. The plants WKS 63 and WKS 92 confirmed sexual mode of reproduction identical to that of the original genotype. The analyzed plants presented meiotic abnormalities related to tetraploidy, and the chromosome pairing were variable, but it did not hinder the meiotic products, which were characterized by regular tetrads and satisfactory pollen fertility, ranging from 88.7 to 95.7%. Results show that all plants are meiotically stable and that they can be used in intraspecific crosses in the breeding program of Paspalum notatum.*

Key words: *Chromosome duplication, cytogenetic analysis, genetic breeding, intraspecific crosses.*

INTRODUCTION

The accelerated degradation of natural pastures of the state of Rio Grande do Sul has led to the loss of genetic diversity of forage species and of quality forage supply to cattle in the state (Macedo 2009). Among the several forage species that form native pastures, *Paspalum notatum* Flugge stands out for having excellent forage value and for being present in all natural pastures of the state (Nabinger and Dall'Agnol 2008). The breeding of this species is an alternative to the use of old and/or exotic varieties, with the search of selected materials; also, they are eligible for registration at the Ministry of Agriculture, Livestock and Supply (MAPA) and subsequent seed commercialization.

In the *Paspalum* genus, there is close correlation between ploidy level and mode of reproduction; diploidy is correlated with sexual reproduction, and allogamy and tetraploidy are correlated with apomixis (Quarin 1992). Apomictic genotypes preserve the genetic diversity, which is made available, and enables crosses with sexual genotypes. The new gene combinations allow selecting individuals that solve problems related to these species, since they are more adapted to different environments, consequently mitigating risk where biotic agents, especially pests and diseases threaten the development and the production. The possibility of artificial chromosome duplication of sexual diploid plants from natural populations of variety Pensacola of *P. notatum* and their use in intraspecific crosses schemes in breeding programs (Burton and Forbes 1961) makes it possible to generate superior genotypes of apomictic

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reproduction. Therefore, it is possible to protect the developed cultivar.

Weiler et al. (2015) artificially duplicated chromosomes of three individuals of *P. notatum* (Pensacola bahiagrass) by immersing flower buds and seeds for different exposure time in various concentrations of colchicine, which is an antimitotic agent responsible for chromosome duplication. The three duplicated plants, WKS 3, WKS 63, and WKS 92, confirmed the tetraploid level of ploidy and the complete euploidy by the root tip analysis and meiotic cells diakinesis. The objective of this study was to evaluate these duplicated plants regarding the mode of reproduction, the meiotic behavior, and the pollen viability in order to use them as parents in intraspecific hybridization schemes in the breeding program.

MATERIAL AND METHODS

This work was carried out in the Cytogenetics Laboratory of the Department of Forage Plants and Agrometeorology of the Department of Agronomy of the Federal University of Rio Grande do Sul. The three plants tetraploidized by Weiler et al. (2015) were evaluated, nominated WKS 3, WKS 63, and WKS 92, regarding the cytoembryological, cytogenetic and pollen viability analysis.

Cytoembryological analysis

Analyses of the mode of reproduction of the plants were carried out with inflorescences in anthesis. Flowers were dissected and fixed in FAA (95% ethanol: 40 mL; distilled water: 14 mL; 40% formalin: 3 mL; and glacial acetic acid: 3 mL) for 24 hours. After that, they were stored in 70% alcohol under refrigeration until ovaries extraction, which went through clearing process by means of a series of alcohol dehydration with methyl salicylate, following the protocol of Young et al. (1979), modified by Acuña et al. (2007), and were stored in methyl salicylate solution (100%) until the analysis in interference contrast optical microscope. At least 30 ovaries per plant were analyzed to determine the mode of reproduction.

Cytogenetic analysis

For the analysis of meiotic behavior, inflorescences of plants were collected at several development stages, and were fixed in an absolute ethanol solution: glacial acetic acid (3: 1) for 24 hours, transferred to 70% ethanol, and stored under refrigeration (Araújo et al. 2005, Dahmer et al. 2008). For the preparation of the slides, inflorescences were dissected, stained with 1% propionic carmine, and analyzed in optical microscope. It was sought to observe cells at different stages of meiotic division, as well as the arrangement of chromosomes. All meiotic abnormalities were considered. For the verification of chromosome pairing, analyses were carried out in at least 20 cells per plant at diakinesis and metaphase I stages (Dahmer et al. 2008, Simioni and Valle 2011).

Pollen viability analysis

Pollen grains viability was estimated in the anthers collected from inflorescences at mature stage, fixed in 3: 1 solution (absolute ethanol: glacial acetic acid), at room temperature for 24 hours, and stored in 70% alcohol until analysis. In the preparation of the slides, pollen grains were extracted from flowers, stained with 1% propionic carmine, and observed in optical microscope. Pollen grains were considered fertile when full and well stained, while those unstained or weakly stained, were considered sterile (not viable unviable) (Singh 1993). One thousand mature pollen grains were counted in four flowers per plant, following the protocol already established and widely used (Dahmer et al. 2008, Guerra et al. 2013).

RESULTS AND DISCUSSION

Mode of reproduction analysis

The duplicated plants WKS 63 and WKS 92 confirmed having sexual mode of reproduction (Table 1), with Polygonum type embryo sac: a single meiotic embryo sac, two polar nuclei, and a cluster of antipodal cells toward the chalazal (Figure 1a). WKS 3 presented modifications in its mode of reproduction after chromosome duplication, and became apomictic. This plant had ovaries with multiple aposporic embryo sacs, which are characterized by the egg cell, one or two synergids, a binucleated central cell, and absence of antipodes (Figure 1b). Quarin et al. (2001) recorded this phenomenon by analyzing the mode of reproduction of three *P. notatum* plants artificially duplicated; two of them had

Table 1. Number and percentage (%) of meiotic (S), apomictic (A), unidentifiable (U), shriveled (Sh), embryo sacs total analyzed ovaries (T), and mode of reproduction (MR) of the three *P. notatum* tetraploidized plants

Plant	S (%)	A (%)	U	Sh	T	MR
WKS 63	30 (75)	0	8	2	40	sexual
WKS 92	24 (80)	0	5	1	30	sexual
WKS 3	0	26 (87)	4	0	30	apomictic

facultative apomictic reproduction. Based on these results, the authors state that the apomixis gene is present at the diploid level; however, it is not expressed in the plant. The ploidy-dependence may occur at a locus that controls the apomixis by means of a secondary factor that requires higher dosage of alleles to affect the expression of major locus. It is likely that the expression of apomixis in this duplicated plant is a gene dosage effect. Simioni and Valle (2011) observed the confirmation of the sexual mode of reproduction in three plants obtained by somatic chromosome duplication of sexually reproducing diploid genotype of *Brachiaria decumbens*.

Analyses of the mode of reproduction of parents and progenies are fundamental in breeding programs aimed at enabling intraspecific hybridizations. For the choice of the parents, this evaluation allows identifying the plants that will be used as female parents (plants of sexual reproduction) and as male parents (apomictic plants).

Analysis of meiotic behavior

Chromosome associations were observed at the diakinesis and metaphase I stages, which are the best stages for visualization of chromosome configurations (Dahmer et al. 2008, Simioni and Valle 2011). The three plants confirmed tetraploidy ($2n=4x=40$), as described by Weiler et al. (2015). Chromosome pairing was typical of tetraploidy, with univalent, bivalent, trivalent and quadrivalent chromosome associations (Table 2). WKS 63 (Figure 2) presented mostly quadrivalent associations. According to Ramsey and Schemske (2002), genotypes

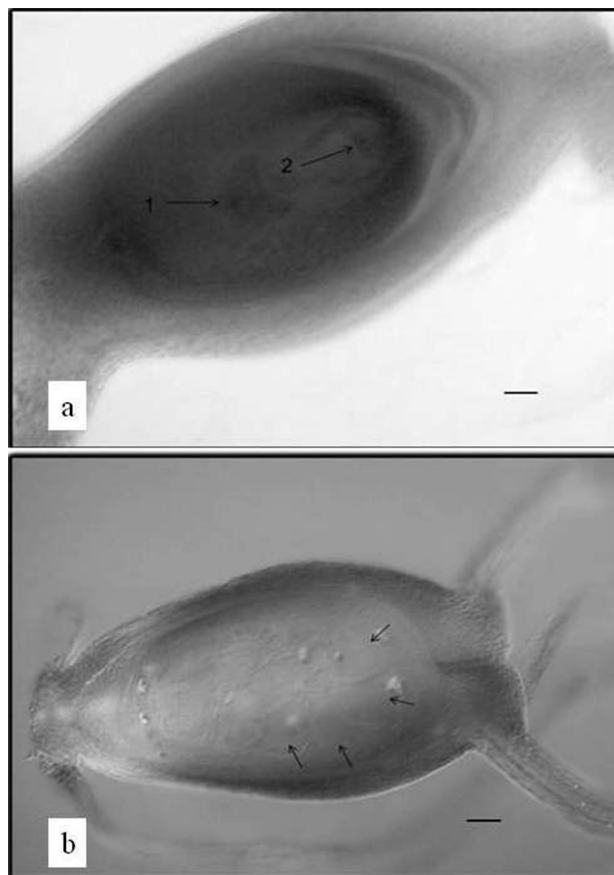


Figure 1. a) Cytoembryological aspect of a sexual ovary (duplicated plant WKS 63): antipodes (1) and polar nuclei (2); b) Cytoembryological aspect of apomictic ovary (duplicated plant WKS 3), multiple sacs (arrows). Scale: 10 μ m.

Table 2. Meiotic chromosome configurations at diakinesis phase (prophase I) of three *P. notatum* tetraploidized plants

Plant	Chromosomic number	N. of analyzed cells	Mean n. of observed associations (per cell) (reach*)			
			I	II	III	IV
WKS 63	40	58	0	0.59	0	9.66
			0	(0-6)	0	(8-10)
WKS 92	40	31	2.23	15.26	0.16	1.71
			(0-34)	(3-20)	(0-2)	(0-4)
WKS 3	40	21	0	9.86	0.29	4.86
			0	(2-17)	(0-2)	(0-9)

* Minimum and maximum limits of associations observed in the total cells analyzed.

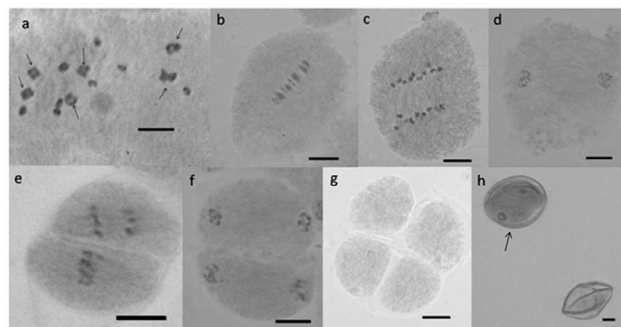


Figure 2. Meiotic aspects of the tetraploidized plant WKS 63. a) Diakinesis. It is observed the presence of quadrivalent associations (arrows). b) Normal metaphase I. c) Normal anaphase I. d) Normal telophase I. e) Anaphase II with asynchrony. f) Normal telophase II. g) Microsporocyte (Tetrad). h) Viable pollen grain (stained; arrow), and unviable pollen grain (non-stained). Scale: 10 μ m.

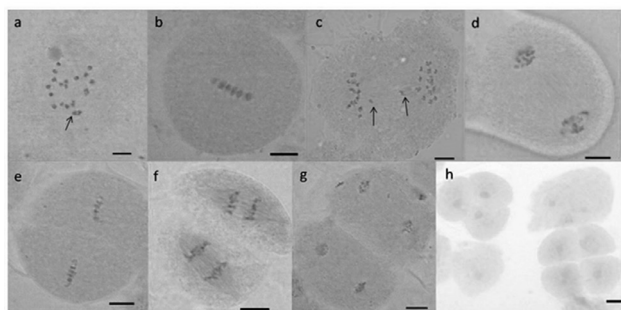


Figure 3. Meiotic aspects of the tetraploidized plant WKS 92. a) Diakinesis. It was observed the presence of one quadrivalent association (arrow). b) Normal metaphase. c) Anaphase I with the presence of laggard chromosomes (arrows). d) Normal telophase I. e) Normal metaphase II. f) Normal anaphase II. g) Normal telophase II. h) Microsporocyte (triad and tetrad). Scale: 10 μ m.

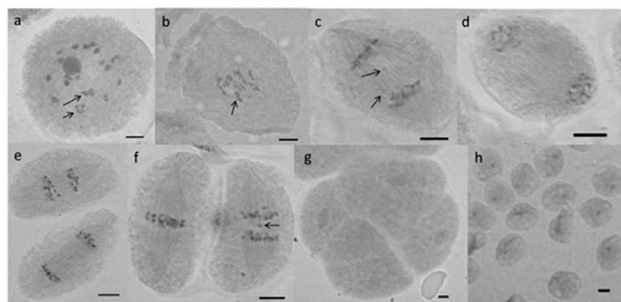


Figure 4. Meiotic aspects of the tetraploidized plant WKS 3. a) Diakinesis. It was observed the presence of two quadrivalent associations (arrows). b) Metaphase I. Presence of chromosomes in early ascension (arrow). c) Anaphase I with the presence of laggard chromosomes (arrows). d) Normal telophase I. e) Normal anaphase II. f) Anaphase II with asynchrony. It was observed the presence of laggard chromosome (arrow). g) Microsporocyte (polyads with micronuclei). h) Viable pollen grains (stained). Scale: 10 μ m.

with polysomic inheritance have tendency for multivalent formation.

The plants WKS 92 (Figure 3) and WKS 3 (Figure 4) presented less meiotic abnormalities when compared with WKS 63, with most of the chromosomes in bivalent and sporadic quadrivalent associations, showing tendency for regularization of the chromosome pairing and genetic control of the pairing in these newly formed tetraploids. According to Dahmer et al. (2008), in the case of apomictic ecotypes, there is the need of meiotic regularity, since they are pseudogamic, so that to it is possible to ensure sufficient pollen fertility to form the endosperm. This is the case of WKS 3 in the present experiment.

Several studies on tetraploid species and accessions have registered regular meiosis and wide variability of the emergence of several uni, bi, tri and tetravalent chromosome associations. The predominance of bivalent associations were observed in 36 (Dahmer et al. 2008) and five (Moraes-Fernandes et al. 1973) *P. notatum* accessions, 24 accessions of different *Paspalum* species (Pagliarini et al. 2001), 53 *Paspalum nicorae* accessions (Reis et al. 2008), three polyploidized plants of *Brachiaria decumbens* (Simioni and Valle 2011), six accessions of different *Brachiaria* species (Araújo et al. 2005), one *Brachiaria ruziziensis* accession (Risso-Pascotto et al. 2005) and a *Paspalum durifolium* accession (Quarin 1994). In contrast, other authors reported accessions in which most of the associations were uni- or multivalent: *B. brizantha*, *B. decumbens* and *B. ruziziensis* (Valle and Savidan 1996), *B. decumbens* cv. Basilisk (Junqueira-Filho et al. 2003), and *Panicum maximum* (Caetano et al. 2006, Pessim et al. 2010).

Table 3 shows a total of 4082 microsporocytes analyzed for meiotic behavior in the three duplicated plants in this experiment. In 442 cells, there were no irregularities from prophase I to the meiotic products, representing a mean percentage of meiotic abnormalities of 10.83% in the three plants. Tetraploid plants generally have meiotic abnormalities related to irregular chromosomes segregation, which generates genetically unbalanced microspores, and thus hinders the fertility of pollen grains (Pagliarini and Pozzobon 2004). Multiple associations in diakinesis and abnormalities related to irregular chromosome segregation (early ascension) were the most frequent abnormalities in both divisions in the three plants. It was also noted laggard chromosomes and asynchrony in the three plants. Few bridges and micronucleus were observed. The meiotic products were mostly normal, with few dyads, triads and polyads, which resulted in excellent pollen viability of these plants.

Table 3. Meiotic abnormalities recorded in the three *P. notatum* tetraploidy plants

Individual	Stage	N. of analyzed cells	N. of abnormal cells (%)	Main abnormalities (N. of cell and %)	
WKS 63	Diakinesis	58	58(100)	Multiple associations: 58 (100)	
	Metaphase I	502	69 (13.75)	Early ascension: 38 (7.57) Laggard: 31 (6.18)	
	Anaphase I	89	19 (21.35)	Laggard: 13 (14.61) Bridges: 3 (3.37) Asynchrony: 3 (3.37)	
	Telophase I	170	1 (0.59)	Laggard: 1 (0.59)	
	Metaphase II	162	18 (11.11)	Early ascension: 4 (2.47) Laggard: 2 (1.23) Asynchrony: 12 (7.41)	
	Anaphase II	34	14 (41.18)	Laggard: 1 (2.94) Bridges: 1 (2.94) Asynchrony: 12 (35.29)	
	Telophase II	156	6 (3.85)	Asynchrony: 6 (3.85)	
	Meiotic Products	279	10 (3.58)	Polyads: 1 (0.36) Triads: 9 (3.22)	
	Total number of cells		1450	195 (13.45)	
	WKS 92	Diakinesis	31	30 (96.78)	Multiple associations: 30 (96.78)
Metaphase I		585	38 (6.5)	Early ascension: 34 (5.81) Laggard: 4 (0.69)	
Anaphase I		136	11 (8.09)	Laggard: 10 (7.35) Bridges: 1 (0.74)	
Telophase I		70	4 (5.71)	Laggard: 2 (2.86) Micronucleus: 1 (1.43) Asynchrony: 1 (1.43)	
Metaphase II		115	9 (7.83)	Early ascension: 2 (1.74) Asynchrony: 7 (6.09)	
Anaphase II		33	7 (21.21)	Laggard: 3 (9.09) Asynchrony: 4 (12.12)	
Telophase II		48	1 (2.08)	Asynchrony: 1 (2.08)	
Meiotic Products		812	9 (1.11)	Dyads: 2 (0.25) Triads: 7 (0.86)	
Total number of cells		1830	109 (5.96)		
WKS 3		Diakinesis	21	21 (100)	Multiple associations: 21 (100)
	Metaphase I	226	118 (52.21)	Laggard: 1 (0.44) Micronucleus: 1 (0.44)	
	Anaphase I	72	7 (9.72)	Laggard: 5 (6.94) Bridges: 2 (2.78)	
	Telophase I	91	2 (2.2)	Bridges: 2 (2.2)	
	Metaphase II	28	1 (3.57)	Asynchrony: 1 (3.57)	
	Anaphase II	88			
	Telophase II	56	4 (7.14)	Asynchrony: 4 (7.14)	
	Meiotic Products	251	15 (5.98)	Polyads: 11 (4.38) Triads: 4 (1.59)	
	Total number of cells		833	168 (20.17)	

Podio et al. (2012) analyzed five natural apomictic tetraploid accessions and three artificially induced sexual tetraploid of *P. notatum*, and found that, in the apomictics, 55.6% of the cells at anaphase I are normal, and in the sexual accessions, 70.3% of the cells at anaphase I are normal. Abnormalities were mostly laggard chromosomes, chromatin bridges, and the presence of micronuclei, which appeared in 44.3% of the apomictic and in 29.66% of the sexual plants. In telophase I, both apomictic and sexual accessions presented chromosomes clustered in the poles of the cells, as well as small size micronuclei, suggesting they were composed of chromosome fragments. It can be inferred that meiotic behavior is characteristic of each genotype, as previously reported by Stein et al. (2004) for the species.

Pessim et al. (2010) observed high meiotic stability in hybrid genotypes and parents of *P. maximum*, with abnormalities ranging from 6.7 to 14.2%, such as irregular chromosome segregation, chromosome stickiness, and absence of cytokinesis. However, they did not affect pollen viability.

Analyses of pollen viability

The three duplicated plants showed high pollen viability: WKS 3, WKS 63 and WKS 92 recorded 92.3%, 88.7% and 95.7% of stained pollen grains, respectively. Studies reported pollen viability of ecotypes and of native apomictic accessions of *P. notatum* in the state of Rio Grande do Sul: Dahmer et al (2008) found pollen viability ranging from 81.0 to 91.47% in the ecotype "Bagual", and of 86.0 to 98.0% in the ecotype "André da Rocha". Moraes-Fernandes et al. (1973) reported pollen fertility ranging from 0 to 84.3%; and Reis et al. (2008) found pollen viability ranging from 88.99 to 95.06% in 53 accessions, despite the numerous meiotic irregularities found. The high pollen viability of apomictic plants is expected, since seed formation occurs only if there is fertilization of the polar nuclei of the embryo sac by one of the gametic nuclei of pollen grain, due to pseudogamy, typical in species of *Paspalum* and *Brachiaria*, which present this mode of reproduction (Pagliarini and Pozzobon 2004).

Cytological analysis are important tools in breeding programs for the selection of compatible and fertile parents which do not present meiotic abnormalities that may hinder gametes viability (Simioni and Valle 2011). This work allowed observing satisfactory meiotic regularity of the three duplicated plants, and made them viable as parents in the breeding program.

Guerra et al. (2016) also recorded cytological stability of 35 cherry trees accessions (*Eugenia involucreta* DC) collected in the state of Rio Grande do Sul; the average of meiotic cells considered normal was 82.12% and the average pollen viability was 92.44%. The authors concluded that such native access can be used directly in commercial orchards, and also as male parents in directed crosses in breeding programs such as those presented in this work.

With the study of the mode of reproduction, it was possible to direct the plants for intraspecific crosses: WKS 3, of apomictic reproduction, was used as pollen donor, and WKS 63 and WKS and 92 were used as female parents in the hybridizations. In further stages of the program, the hybrid progeny will be evaluated in agronomic trials under field conditions, in order to select genotypes that meet the demands of increased forage yield and that can contribute to the preservation of southern fields, preventing their degradation. This work represents a progress for the development of the Brazilian southern farming, with the use of well-adapted materials, diversifying forage production.

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