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Research Paper

Selection based on meiotic behavior in *Urochloa decumbens* hybrids from non-shattered seed

Selección con base en el comportamiento meiótico de híbridos procedentes de semillas no dehiscentes de Urochloa decumbens

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Abstract

This study aimed to evaluate the end-products of meiosis in sexual and apomictic hybrids of *Urochloa decumbens*, so as to identify genotypes with good production of viable pollen for use in breeding programs to increase yields of pure viable seed and reduce degree of seed shattering. From 457 intraspecific hybrids of *U. decumbens* arising from crosses between 3 artificially tetraploidized sexual plants and the apomictic cultivar Basilisk, 27 hybrids from non-shattered seed were selected. Slides were prepared by smearing anthers and staining to determine the presence of abnormalities. The abnormalities found were micronuclei, microcytes and polyads. The data were compared by the Scott-Knott test at P<0.05. Data obtained enabled separation of hybrids into 4 groups depending on the presence of micronuclei and formation of polyads and into 6 groups based on the presence of microcytes in the tetrads. Among the analyzed apomictic hybrids, R179 has the attributes for viable seed production to proceed to cultivar development. Among the sexual hybrids, R161, R181, R193 and S47 are recommended as female parents for use in crossing programs.

Keywords: Abnormalities, breeding, cytogenetics, forages, intraspecific crosses.

Resumen

El estudio tuvo como objetivo evaluar los productos finales de la meiosis en híbridos sexuales y apomícticos de *Urochloa decumbens*, para identificar genotipos con buena producción de polen viable que puedan ser usados en un programa de mejoramiento genético y aumentar así los rendimientos de semilla pura viable y reducir su grado de dehiscencia. De un total de 457 híbridos intraespecíficos de *U. decumbens* que resultaron de cruzamientos entre tres plantas sexuales tetraploidizadas artificialmente y el cultivar apomíctico 'Basilisk', se seleccionaron 27 híbridos procedentes de semillas no desprendidas. Para el efecto se prepararon portaobjetos con anteras que fueron teñidas para determinar la presencia de anomalías. Las anomalías encontradas fueron micronúcleos, microcitos y políadas. Los datos se compararon mediante la prueba de Scott-Knott (P<0.05). Los resultados permitieron separar los híbridos en cuatro grupos dependiendo de la presencia de micronúcleos y la formación de políadas, y en seis grupos basados en la presencia de microcitos en las tétradas. El híbrido R179, entre los híbridos apomícticos analizados, presentó los atributos necesarios para el desarrollo de cultivares con potencial de producción de semillas viables. Entre los híbridos sexuales, se recomiendan R161, R181, R193 y S47 como progenitores femeninos en programas de cruzamiento.

Palabras clave: Anomalías, citogenética, cruces intraespecificos, fitomejoramiento, forrajes tropicales.

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Introduction

Brazil has 221.8 million head of cattle and produced 9.71 million tonnes of meat, worth R$ 523.25 billion in 2017 (ABIEC 2018), making the country one of the main producers of beef in the world. This is a result of the adoption of new technologies relating to genetics and the management and feeding of beef herds (Gomes et al. 2017).

Approximately 95% of the animals are raised on pasture (Araújo et al. 2017), so high quality forages and improvement of existing pastures are a prerequisite to efficient livestock production (Ribeiro-Junior et al. 2017). In addition, Brazil occupies a prominent position in the world as a producer and exporter of tropical forage seed (Pereira et al. 2011). In 2015, 50 thousand tonnes of certified seed was produced, with 75% destined for the domestic market and 25% for export (Rodrigues 2017).

Brachiaria (now: *Urochloa*) breeding in Brazil began when CIAT and EMBRAPA achieved compatibility between species with different ploidy levels in the late 1980s (Triviño et al. 2017). While a number of cultivars have been released, most have some limitations, so further genetic improvement is warranted. Low seed yields and seed quality are significant issues for cultivars which have been released most recently, especially the hybrids.

If a cultivar is to be adopted widely and have a significant impact on animal production, adequate supplies of good quality seed are essential (Valle et al. 2008). One factor affecting seed quality is the occurrence of natural shattering, i.e. seeds detaching from the raceme on reaching maturity. Seed must be retrieved from the ground, resulting in some being lost plus contamination by pathogens and impurities. Development of genotypes resistant to seed shattering would lead to increases in both seed yields and quality.

Another factor affecting seed quality and quantity is polyploidy because, when there is more than a chromosomal set in a cell, the organization of the same can be difficult at the time of pairing and segregation causing meiotic abnormalities. In the genus *Brachiaria* (now: *Urochloa*), most ecotypes studied are polyploids (Valle and Savidan 1996; Utsunomiya et al. 2005) and the vast majority reproduce by apomixis (Valle and Savidan 1996; Fuzinatto et al. 2007, 2008; Mendes-Bonato et al. 2007). Adamowski et al. (2008) revealed many important meiotic and post-meiotic abnormalities that compromise, sometimes seriously, the end-product of meiosis, causing pollen sterility. Apomixis in *Urochloa* is a pseudogamous apospory, where, despite the fact that the egg cell circumvents fertilization, the central cell requires it for endosperm formation. Therefore quality of pollen is very important to ensure the formation of viable seeds. Furthermore, hybridization is successful only if crossings are performed between parents with the same ploidy level, using a sexual genotype as a mother-plant and the apomictic or another sexual as pollen donor(s) (Barrios et al. 2013; Alves et al. 2014).

Considering the importance of selecting genotypes that combine high levels of seed retention and quantity/quality of viable seeds, the present study had as its objective evaluation of the final products of meiosis and pollen viability in hybrids of *U. decumbens*, in order to identify stable genotypes and good pollen producers for use in the *Urochloa* breeding program at Embrapa Beef Cattle Research Center (Embrapa Gado de Corte).

Materials and Methods

A base population of 457 intraspecific hybrids of *U. decumbens* was produced from crosses between 3 artificially tetraploidized sexual plants and the apomictic cultivar Basilisk. This population is maintained in an experimental field at Embrapa Beef Cattle Center, in Campo Grande, Mato Grosso do Sul (20°25'03" S, 54°42'20" W) in an allic Red Latosol type soil. From that population 27 hybrids from non-shattered seed were selected (Table 1) and were cytogenetically analyzed in the Laboratory of Cytogenetics from the Universidade Estadual de Maringá, Paraná.

The inflorescences of the hybrids were collected and fixed in a mixture of ethanol:chloroform:propionic acid (6:3:2) for 24 hours and stored in 70% alcohol at 4 °C. Three anthers from the same hermaphrodite flower, chosen randomly on the raceme, were used per slide, for the analysis of the final products of meiosis and pollen viability. Each slide was considered a replication, with 5 replications per hybrid. One hundred cells were counted per slide.

Tetrads of microspores and pollen viability were evaluated after squashing, then staining with 1% propionic carmine and analyzing under light microscopy. The pollen grains were classified into 2 groups: 1) viable pollen grains, with the exine intact and the protoplasm well stained; and 2) unviable pollen, with weak staining or shriveled and not stained.

The data on anomalous tetrads of microspores and non-viable pollen grains obtained in percentage (%) were transformed into arcsine function using the square root ($\sqrt{x}$) of the proportion of abnormal tetrads. Data were then submitted to analysis of variance using the SAS 9.2 program (SAS Institute 2009). The mean percentages were compared using the Scott-Knott test at the 5% probability level using GENES (Cruz 2001).

Table 1. Hybrids of *Urochloa decumbens* analyzed.

Hybrid	Reproduction mode	Female parent	Male parent
R158	Apomictic	D24/27	cv. Basilisk
R168	Apomictic	D24/27	cv. Basilisk
R169	Apomictic	D24/27	cv. Basilisk
R176	Apomictic	D24/27	cv. Basilisk
R177	Apomictic	D24/27	cv. Basilisk
R179	Apomictic	D24/27	cv. Basilisk
R184	Apomictic	D24/27	cv. Basilisk
R187	Apomictic	D24/27	cv. Basilisk
R189	Apomictic	D24/27	cv. Basilisk
S48	Apomictic	D24/27	cv. Basilisk
T87	Apomictic	D24/27	cv. Basilisk
X113	Apomictic	D24/45	cv. Basilisk
Y22	Apomictic	D24/45	cv. Basilisk
Y23	Apomictic	D24/45	cv. Basilisk
Z8	Apomictic	D24/45	cv. Basilisk
R 161	Sexual	D24/27	cv. Basilisk
R163	Sexual	D24/27	cv. Basilisk
R165	Sexual	D24/27	cv. Basilisk
R167	Sexual	D24/27	cv. Basilisk
R171	Sexual	D24/27	cv. Basilisk
R181	Sexual	D24/27	cv. Basilisk
R193	Sexual	D24/27	cv. Basilisk
S47	Sexual	D24/27	cv. Basilisk
Y21	Sexual	D24/45	cv. Basilisk
Z9	Sexual	D24/45	cv. Basilisk
X119	-	D24/45	cv. Basilisk
X122	Sexual- sterile	D24/45	cv. Basilisk

The most representative abnormalities in the tetrads and pollen grains were photographed under an OLYMPUS CX 31 capture microscope with attached SC 30 camera, using the AnalySIS getIT software, with 400× magnification.

Results

Many abnormalities were observed in the final products of meiosis of hybrids of *U. decumbens* analyzed, the main ones being 1, 2, 3 and 4 micronuclei in the microspores (Figures 1a–1d), microcytes (Figures 1e–1f) and polyads (Figures 1g–1i).

Cytogenetic analysis revealed the presence of micronuclei and microcytes in the same tetrad (Figures 1e–1f) and polyads with micronuclei (Figures 1g–1i).

The analyses of the tetrads of microspores from these hybrids are presented in Table 2.

The meiotic abnormalities in tetrads were expressed as percentages of abnormal cells and the significant differences between the irregularities of the hybrids were tested by the Scott-Knott test. In the analysis of variance for meiotic abnormalities, the mean square for the hybrid effect was significant by the F-test with 5% probability of error; therefore, there are differences between hybrids in the frequencies of chromosomal irregularities in the tetrads of microspores (Table 2). For the variables related to abnormalities in tetrads, the estimated coefficient of variation (CV) was high for the presence of micronucleus in 1 microspore (38.3%), microcytes (41.9%) and polyads (86.7%). These high values for CV can be explained by differences in the numbers of cells found with these abnormalities in each slide (replication).

Based on the Scott-Knott test it was possible to separate the hybrids into 4 groups (A, B, C and D) concerning the presence of micronuclei in 1, 2 and 3 microspores and 3 groups concerning the presence in 4 microspores. The groups differ on the basis of minimum significant difference while the hybrids within the groups are similar.

Table 2. Analysis of variance of the meiotic abnormalities observed in the 27 hybrids of *Urochloa decumbens.*

Source	DF	Mean Square of meiotic abnormalities					
		1	2	3	4	5	6
Hybrid	26	0.109*	0.123*	0.072*	0.156*	0.208*	0.73*
Error	108	0.008*	0.007*	0.007*	0.018*	0.007*	0.006*
Total	134						
CV%		38.3	22.4	16.5	21.6	41.9	86.7

1 = micronuclei in 1 microspore; 2 = micronuclei in 2 microspores; 3 = micronuclei in 3 microspores; 4 = micronuclei in 4 microspores; 5 = microcyte; 6 = polyad.
*Significant by the F-test (P<0.05).

Regarding the presence of micronuclei in just 1 microspore, hybrids of Group D (Table 3) presented the lowest frequencies of this abnormality. However, based on the parameters established by Love (1951), this group should be considered unstable, since more than 10% of abnormal tetrads were detected, with the presence of micronuclei in all 4 microspores (Table 3). For the presence of micronuclei in 2 microspores of the tetrad, Groups C and D (Table 3) were those with fewer than 10% of abnormal tetrads, while also presenting high frequency of micronuclei in the tetrad. The same is true for micronuclei in 3 microspores where hybrids R187 and R189, despite having fewer micronuclei in 3 microspores, showed 33 and 68% of micronuclei in the tetrads, with high frequency of microcytes and polyads. The only hybrid that presented fewer than 10% of tetrads with micronuclei in the 4 microspores was R181, although this hybrid did not differ statistically from other hybrids of Group C.

Hybrids have been classified into 6 groups from A to F on the basis of the presence of microcytes in tetrads (Table 3).

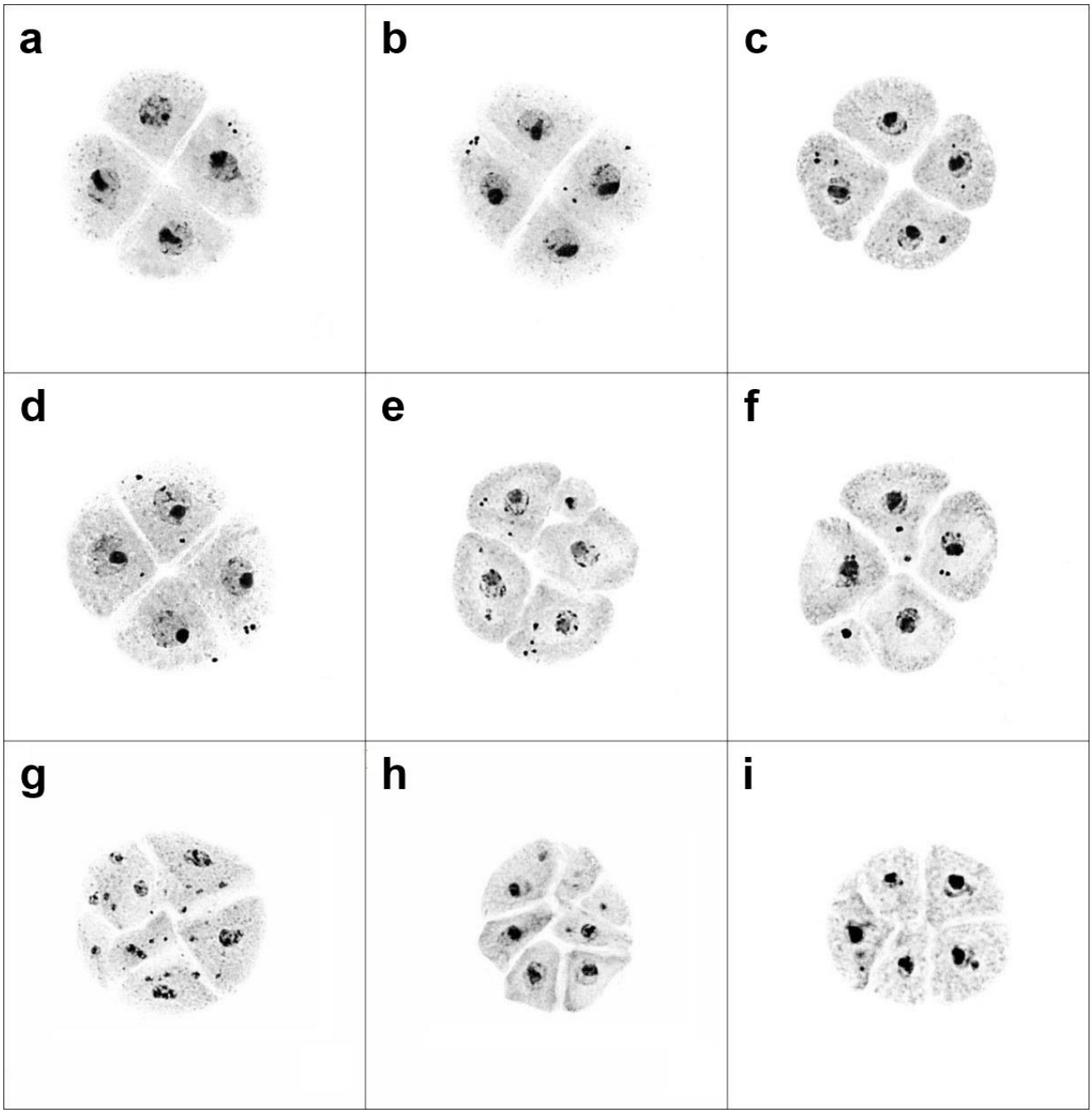

Figure 1. Meiotic abnormalities observed in tetrads of microspores, due to irregular segregation of chromosomes and genome asynchrony in tetraploid hybrids of *Urochloa decumbens*: a) micronuclei in 1 microspore; b) micronuclei in 2 microspores; c) micronuclei in 3 microspores; d) micronuclei in 4 microspores; e-f) tetrads with micronuclei in the microspores and microcytes; and g-h-i) polyads with microspores of different sizes and with micronuclei (400× magnification).

Hybrids in Groups A and B are expected to have higher frequency of unbalanced gametes and thus higher pollen infertility. According to the parameters established by Love (1951), hybrids of Groups D, E and F can be considered stable cytogenetically.

Hybrids were separated into 4 groups, from A to D, on the basis of the frequency of polyads (Table 3). Except for hybrid R187 with 25% of polyads, these occurred in much lower frequencies, probably not compromising pollen fertility.

Pollen viability of *U. decumbens* hybrids was tested using propionic carmine at 1% (Figures 2a–2c). Pollen grains of different sizes and staining patterns were observed in the hybrids analyzed, but in many cases it was not possible to accurately determine whether pollen grains were viable or non-viable.

Table 3. Grouping of the 27 *Urochloa decumbens* hybrids evaluated based on similar behavior regarding mean percentages of incidence of abnormal cells observed at the end of meiosis.

Micronuclei in microspores													Microcytes			Polyads		
1			2			3			4									
Hybrid	%	Group	Hybrid	%	Group	Hybrid	%	Group	Hybrid	%	Group		Hybrid	%	Group	Hybrid	%	Group
R181	28.0	A	R193	33.8	A	Y22	38.6	A	R189	68.0	A		S48	42.0	A	R187	25.0	A
R179	23.0	A	R179	30.1	A	X113	36.5	A	R163	64.5	A		R176	28.0	B	X122	7.8	B
R 161	19.0	A	X119	28.9	A	R177	32.9	A	R169	56.2	A		R187	33.0	B	S48	6.6	B
R193	19.0	A	R181	26.8	A	R158	30.4	A	R167	53.8	A		X122	23.0	B	R189	5.5	B
Y23	14.0	B	R171	26.5	A	Z9	30.0	A	Y22	50.0	A		R 161	15.0	C	R167	2.8	C
R171	11.0	B	Z9	24.8	A	X119	29.7	A	S47	48.2	A		R189	17.0	C	R163	2.3	C
X119	11.0	B	T87	24.5	A	R184	29.6	A	X122	43.5	B		R167	10.0	D	Y23	2.0	C
R165	10.0	B	R 161	24.4	A	Z8	28.6	A	Z8	43.3	B		Y21	8.6	D	R169	1.3	C
R168	8.3	B	R158	23.8	A	R171	28.0	A	R184	42.0	B		R163	3.3	E	Y21	1.3	C
R176	7.8	B	Y23	21.6	A	R165	27.7	A	R177	39.5	B		R165	1.1	E	R168	1.2	C
R158	7.7	B	R165	21.3	A	R193	27.1	A	S48	37.9	B		R168	3.1	E	R176	0.5	D
Z9	6.2	C	R184	19.8	B	T87	27.0	A	X113	37.1	B		R169	6.1	E	R 161	0.2	D
Z8	6.1	C	X113	18.7	B	S47	26.2	A	Y21	37.0	B		R171	1.3	E	R165	0.2	D
R184	5.4	C	S47	18.2	B	Y21	25.5	B	T87	34.5	B		T87	2.0	E	R171	0.2	D
T87	5.3	C	R177	17.9	B	Y23	24.6	B	R187	33.3	B		Y23	2.5	E	R181	0.2	D
Y21	4.5	C	R176	16.9	B	R179	24.1	B	Z9	33.1	B		Z8	2.0	E	Z9	0.2	D
R177	4.2	C	Y21	16.5	B	R167	22.9	B	R158	30.2	B		R158	0.6	F	Z8	0.1	D
S47	4.0	C	R168	14.8	B	R169	21.3	B	R168	29.5	B		R177	0.8	F	R158	0.0	D
X113	3.7	C	Z8	13.8	B	R176	20.9	B	R165	28.1	B		R179	0.1	F	R177	0.0	D
R167	2.6	C	Y22	10.3	B	X122	19.4	B	R171	25.7	B		R181	0.0	F	R179	0.0	D
R169	1.6	D	R169	8.0	C	R168	19.1	B	X119	24.8	B		R184	0.0	F	R184	0.0	D
R163	0.7	D	R163	7.8	C	R163	19.0	B	Y23	24.3	B		R193	0.0	F	R193	0.0	D
X122	0.4	D	R167	6.1	C	R 161	15.6	C	R176	18.6	C		S47	0.0	F	S47	0.0	D
S48	0.2	D	X122	3.6	C	R181	13.2	C	R193	12.1	C		X113	0.2	F	T87	0.0	D
Y22	0.2	D	S48	1.6	D	S48	10.2	C	R179	11.7	C		X119	0.5	F	X113	0.0	D
R187	0.0	D	R187	0.3	D	R189	5.1	D	R 161	10.9	C		Y22	0.0	F	X119	0.0	D
R189	0.0	D	R189	0.3	D	R187	1.8	D	R181	7.1	C		Z9	0.7	F	Y22	0.0	D

1 = micronuclei in 1 microspore; 2 = micronuclei in 2 microspores; 3 = micronuclei in 3 microspores; 4 = micronuclei in 4 microspores. Grouping based on significance by the Scott-Knott test (P<0.05).

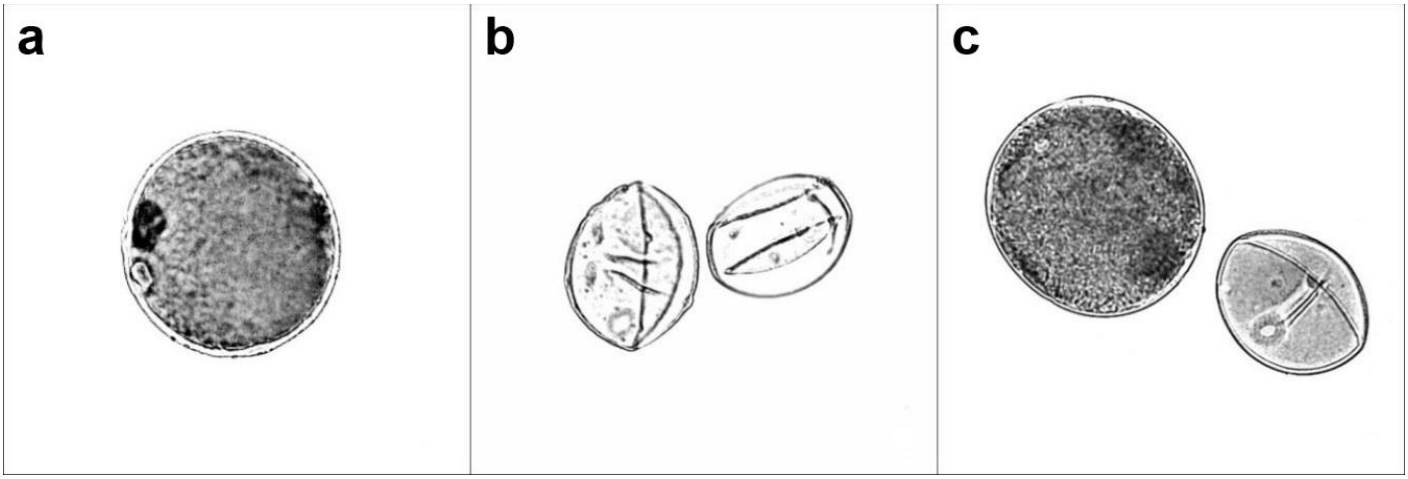

Figure 2. Pollen viability of the 27 *Urochloa decumbens* tetraploid hybrids determined by staining with 1% propionic carmine: a) viable pollen grain strongly stained; b) non-viable pollen grains unstained; c) viable and non-viable pollen grains (400× magnifications).

Discussion

Micronuclei are a consequence of segregation irregularities occurring in different phases of meiosis. As reported by Risso-Pascotto et al. (2004), micronuclei in 1 or more microspores are the most common cytological abnormality resulting from irregular chromosome segregation in higher plants. When formed, the

micronuclei can remain in tetrads of microspores even after the dissolution of the callose wall and the release of microspores impairing normal gamete formation (Valle and Pagliarini 2009). Micronuclei can also be eliminated from the tetrads as microcytes by cytokinesis. In the hybrids analyzed, the elimination of micronuclei by additional cytokinesis gave rise to microcytes in tetrads and polyads.

Micronuclei in microspores of tetrads, tetrads with microcytes and polyads have often been reported in meiotic studies of interspecific hybrids of *Urochloa* (Risso-Pascotto et al. 2004; Mendes-Bonato et al. 2007), which, depending on the frequency of occurrence, results in the formation of unbalanced gametes. We expected that intraspecific hybridizations would produce fewer anomalies in meiosis than with interspecific hybrids, since chromosome sets were supposedly homologous. The occurrence of abnormalities in these intraspecific hybrids of *U. decumbens* could be due to the recent artificial replication of the chromosomes of their female parent. Artificial chromosome duplication using colchicine can cause loss of chromosomes or chromosomal rearrangements such as deletions or inversions, as well as sterility and abnormal growth (Luckett 1989).

The analysis of meiotic behavior of artificially tetraploidized accessions of *U. decumbens*, *U. brizantha* and *U. ruziziensis* has shown a rate of meiotic abnormalities varying from 5 to 60%, and a high rate of abnormalities in interspecific hybrids using tetraploidized parents (Fuzinatto et al. 2007; Souza et al. 2015). These culminated in abnormal tetrads and in the formation of a high rate of unviable pollen grains.

Love (1951) indicated that the analysis of tetrads easily proved the degree of stability of the meiotic process, since it demonstrated the pattern of chromosome behavior during the phases of meiosis. According to this author, a plant with 90–100% of normal tetrads is considered stable, whereas plants with fewer than 90% of normal meiotic products limit breeding, because this hampers production of viable seeds.

Although Love´s meiotic index is widely used to determine the meiotic stability and consequently the fertility of a plant, a more detailed analysis of the final products of meiosis may result in much more accurate information, especially for polyploid plants, which have a high rate of abnormalities in tetrads of microspores. This can be explained by the fact that a tetrad with micronuclei in 1 microspore can theoretically have 3 other normal microspores. These hybrids would thus produce viable pollen in the ratio of 3:1 (viable:unviable pollen). According to Souza et al. (2015), genotypes with a high frequency of micronuclei in only 1 microspore would be more promising, since the other 3 microspores of the tetrad may contain balanced genetic material.

Using this basis for selection, the best hybrids would be those with no micronuclei or a high frequency of tetrads with micronuclei in only 1 microspore. That was not the case in the hybrids studied, where the important criteria were to select hybrids with fewer micronuclei throughout and also absence of microcytes and polyads.

The formation of microcytes in tetrads and polyads is much more serious than the presence of micronuclei in microspores of the tetrad. When additional cytokinesis forms microcytes and polyads, all microspores are abnormal due to uneven division of the genomes. Tetrads with microcytes and polyads generate unbalanced pollen grains of different sizes.

Pollen viability is an accepted measure of male fertility and can be estimated by staining methods using mature pollen grains. Although several authors, e.g. Ricci et al. (2010); Simioni and Valle (2011); Souza et al. (2015), have already tested pollen viability in *Urochloa* using this staining method and were able to discriminate between viable and non-viable pollen grains, the method is often unreliable, because in addition to meiotic irregularities, pollen viability can be affected by failures in the microgametogenesis process (Twel 1995), natural water loss that occurs during the collection and storage of inflorescences (Tecchio et al. 2006) and the storage time of the inflorescences (Stanley and Linskens 1974). According to Souza et al. (2002), pollen grain is fully viable at the opening of the flower, and as time progresses, the viability decreases, reducing its efficiency.

Hybridizations performed in the *Urochloa* breeding program of Embrapa Beef Cattle use sexual genotypes as mother plants and apomictic ones as pollen donors (Mendes-Bonato et al. 2004). According to Souza et al. (2015), sexual hybrids that have a low frequency of abnormalities in tetrads and good viable pollen production may be included in polycross blocks with other sexual hybrids for the recombination of alleles or used in crosses with other elite apomictic genotypes to generate new populations from which to select future apomictic cultivars. Superior apomictic hybrids can be evaluated agronomically to select new cultivars or can be used as pollen donors in new crosses.

Among the apomictic hybrids analyzed, R179 could be regarded as a good pollen donor, since it had a high percentage of tetrads with micronuclei in only 1 or 2 microspores (Group A), and a low percentage of tetrads with micronuclei in the 4 microspores (Group C), tetrads with microcytes (Group F) and polyads (Group D). Apomictic hybrids R187, R189 and S48, however, with high rates of tetrads with micronuclei in the 4 microspores,

microcytes and polyads must be discarded as parents for crossing. Among the sexual hybrids R161, R181, R193 and S47 may be considered for crossing blocks and the next generation evaluated to confirm potential fertility.

Absence of seed shattering was a key factor in the domestication of major grasses because humans could collect seed throughout the long summer season, making them preadapted candidates for domestication (Kislev et al. 2004). The inheritance of non-shattering behavior, which in some grasses seems to be controlled by few genes or transcription factors (Konishi et al. 2006; Li et al. 2006), is an important trait to be a focus in evaluation of the intraspecific hybrids of *U. decumbens* analyzed. Given the importance of this character for the improvement of this forage, the detailed analysis of the tetrads of microspores and pollen viability is essential in selecting hybrids that could produce larger quantities of fertile seeds that could be harvested conventionally. Hybrids resistant to shattering would improve significantly the harvesting of viable seed for either the breeding program or commercial purposes. Furthermore, selection of future cultivars with better potential production of directly harvested seed should reduce cost of seed, resulting in greater adoption rates and contributing to pasture diversification and sustainability (Fonseca and Martuscello 2011).

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