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Microsporogenesis associated with seed yield in *Urochloa* sexual polyploid hybrids

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Abstract: *High seed yield is a fundamental characteristic of apomictic and sexual hybrids of the genus Urochloa P. Beauv. (syn. Brachiaria (Trin.) Griseb.). It has been suggested that there is an association between polyploidy, the presence of meiotic irregularities, and low seed formation in this genus. The present study aimed to correlate these characteristics in Urochloa polyploid sexual interspecific hybrids in relation to the production of pure seeds. Segregational and non-segregational abnormalities were shown to led to cause the formation of tetrads with micronuclei, microcytes, polyads, and anomalous end products. The correlation between the percentage of meiotic abnormalities and the total production of pure seeds was negative and significant. To our knowledge, this is the first research with sexual polyploid hybrids from Urochloa and is relevant to identify hybrids with less frequent meiotic abnormalities and higher seed yield, with promising female parents for the breeding program.*

Keywords: *Brachiaria, segregational irregularities, micronucleus, polyad, seed set.*

INTRODUCTION

An extensive grazing system is the basis of Brazilian livestock production, where approximately 95% of the animals are raised in pastures, both for the production of beef and dairy cattle (Araújo et al. 2017). Brazil stands out for the biggest productive herd in the world, with approximately 222 million head (ANUALPEC 2018). The genus Urochloa P. Beauv. (with several species, synonymous to syn. Brachiaria (Trin.) Griseb.) (Monteiro et al. 2016b, Paula et al. 2017) plays an exceptional role in this scenario, as it comprises approximately 85% of the cultivated pasture areas (Monteiro et al. 2016a, Corrêa et al. 2019).

The genus Urochloa is composed of approximately 110 species that display different levels of ploidy and feature aposporous-type sexuality and apomixis (Worthington et al. 2019). The most widely used species in pastures are Urochloa brizantha (syn. Brachiaria brizantha), U. decumbens (syn. B. decumbens), U. ruziziensis (syn. B. ruziziensis), and U. humidicola (syn. B. humidicola) (Paula et al. 2016, Figueiredo et al. 2019, Rocha et al. 2019). Due to the widespread use of cultivars of these species in the tropics, coupled to with Panicum maximum, Brazil has become the world´s largest producer and exporter of tropical forage seeds (Souza et al. 2017), supplying most of the Latin American markets.

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According to Rodrigues (2017), approximately 75% of certified seeds produced are used in the domestic market, and 25% are exported.

For over 30 years, the improvement of Urochloa depended exclusively on the selection of genotypes based on natural variability. Apomixis is the dominant mode of reproduction in this species, with high commercial potential (Worthington et al. 2019). However, this mode of reproduction, common in polyploid individuals, prevents self-fertilization and hybridization among existing elite genotypes or with sexual genotypes at different ploidy levels (Morais et al. 2018, Baldissera et al. 2020, Sales et al. 2021).

Cytogenetic studies have revealed that U. brizantha and U. decumbens are predominantly tetraploid (2n = 4x = 36) and apomictic (Ricci et al. 2010, Morais et al. 2018, Matias et al. 2020). Despite the predominance of tetraploidy, diploid plants have been reported () in the two species (Ricci et al. 2010). Furthermore, U. ruziziensis is diploid (2n = 2x = 18) and sexual (Pagliarini et al. 2008, Simeão et al. 2017, Morais et al. 2018).

However, chromosomes of sexual diploid accessions have been duplicated to overcome the ploidy barrier between sexual diploids and apomictic tetraploids in crosses. After hybridization, an equal proportion of sexual and apomictic hybrids were recovered, indicating a tetrasomic inheritance of apomixis in the agamic group of species, as discussed by Grimanelli et al. (1998) for Trypsacum.

As in any other forage breeding program, Urochloa species are used to obtain persistent hybrids that gather desirable characteristics from two or more high-performance parents. High seed yield is an important feature for apomictic and sexual hybrids due to its low cost in establishing pastures with the apomictic hybrid. On the other hand, it also assures large progenies from the sexual parent in crosses. Since Urochloa species are pseudogamous gametophytic facultative apomictics, pollen has to fertilize the polar nucleus for the formation of the seed endosperm in the apomictic plant. In sexual plants, seeds are formed from the fertilization of the oosphere and polar nuclei by the pollen grain.

There is extensive literature confirming the strong association between polyploidy and the occurrence of irregularities during meiosis in Urochloa spp. Tetraploid plants show high rates of segregational and non-segregational meiotic abnormalities (Mendes-Bonato et al. 2007), which culminate in the formation of tetrads with abnormal microspores, suggesting the formation of sterile pollen grains (Pagliarini et al. 2006, Baldissera et al. 2020, Sales et al. 2021). Several studies have been conducted on the relationship between the percentage of abnormal tetrads and pollen fertility (Souza et al. 2015, Rocha et al. 2019, Baldissera et al. 2020), but none in relation to actual seed yield. In theory, when pollen mother cells exhibit irregular behavior during cell division and produce microspores with unbalanced genetic material, inviable pollen grains are formed (Mendes-Bonato et al. 2007, Ricci et al. 2010, Souza-Kaneshima et al. 2010, Pagliarini et al. 2012), which may result in infertile seeds.

This study investigated whether meiotic irregularities in sexual polyploid hybrids of Urochloa may produce inviable seeds. The production of viable pure seeds by the sexual polyploid hybrids of Urochloa is relevant for breeding programs at Embrapa Beef Cattle.

MATERIAL AND METHODS

Eight Urochloa sexual hybrids from the Embrapa Beef Cattle breeding program in Campo Grande, MS, Brazil, were evaluated. The hybrids were chosen from different breeding populations and, thus, had different genealogies (Table 1). Crossings, determination of reproduction mode, evaluation of hybrids in the field, and data on seed yields (Table 2) were performed at the Embrapa Beef Cattle.

Plants were monitored daily from the beginning of the seed maturation phase to determine harvest time. Harvesting was performed manually when the seeds reached the point of maturation and the onset of shattering, that is, when approximately 10% of the seeds shattered when touched. Seeds were processed and analyzed in the seed laboratory.

Seeds were weighed, homogenized in a sample divider, according to Rules for Seed Analysis (BRASIL 2009), and subjected to physical separation of empty seeds to obtain the percentage of pure seeds per plant. A South Dakota Seedburo column pneumatic blower with 0.5 HP was used, first with a 4.0 cm opening for 30 s, followed by 5.5 cm for 30 s. The pure seeds obtained were weighed on a digital scale to two decimal places, and the percentage of pure seeds

Table 1. Identity of sexual hybrids: generation and female and male parents

Hybrid	Generation	Female parent	Male parent	Ploidy level	Mode of reproduction
B13	1st	D24/2	Basilisk	2n = 4x = 36	Sexual
B1	1st	D24/2	Basilisk	2n = 4x = 36	Apomictic
R33	1st	D24/27	Basilisk	2n = 4x = 36	Sexual
460-9	3rd	BS07	Unknown*	2n = 4x = 36	Not determined
1121-9	3rd	BS14/03	Unknown*	2n = 4x = 36	Not determined
98-9	3rd	BS03/06	Unknown*	2n = 4x = 36	Not determined
676-10	2nd	BS09	Mulato II	2n = 4x = 36	Sexual
1386-10	2nd	336-T1	BRS Ybaté	2n = 4x = 36	Sexual

* Hybrid from a crossing block resulting in a half-sib population.

Table 2. Evaluated seed yield compared to total meiotic abnormalities and types of abnormalities detected for each sexual hybrid

Hybrid	Seed yield	Seed set 1	Seed set 2	Meiotic Abnormalities	Types of abnormalities and final products of meiosis (%)								
					Mcn1	Mcn2	Mcn3	Mcn4	Microcytes	Polyads	Dyads	Triads	PFA
1121-9	22.48	10.89	2.01	41.33	0	20.49	24.59	12.3	0	5.74	13.94	2.46	20.49
1386-10	34.73	9.80	2.78	39.71	29.9	35.29	18.14	16.67	0	0	0	0	0
460-9	15.60	1.60	0.51	91.77	2.75	3.92	4.31	63.53	7.84	5.1	3.653	6.67	1.96
676-10	67.61	19.84	8.52	33.1	31.91	51.06	10.64	6.38	0	0	0	0	0
98-9	39.38	27.28	8.06	68.21	6.35	18.25	6.35	65.08	3.97	0	0	0	0
B1	3.75	3.47	0.92	83.31	2.8	14.02	15.42	59.35	3.27	0	0	0	0
B13	15.73	30.02	11.85	59.56	10.87	34.06	31.88	23.19	0	0	0	0	0
R33	2.25	7.61	1.82	80.63	2.11	5.91	11.39	73.84	0	2.53	0	0	0

Seed yield: pure seed production (g individual^{-1}); seed set 1: (grams of filled seeds per individual – %); seed set 2: (number of filled seeds per individual – %); Mcn1: micronucleus in one microspore (%); Mcn2: micronucleus in two microspores; Mcn3: micronucleus in three microspores; Mcn4: micronucleus in four microspores; PFA: anomalous final product.

was calculated according to the total weight produced. The production of pure seeds per plant corresponded to the total weight of the pure seeds produced per plant. All seeds produced per plant were counted to calculate the number of pure seeds. The weight of pure seeds (seed set 1) and the number of filled seeds (seed set 2) were the two parameters used to correlate meiotic abnormalities.

The hybrids for microsporogenesis studies were maintained in pots with sand, humus, and soil, at 1:1:1, in a screened house at the State University of Maringá, Maringá, PR, Brazil, where the assays were performed.

Inflorescences for the evaluation of microsporogenesis were collected while still being involved by the flag leaf. They were fixed in ethanol, chloroform, and propionic acid at 6:3:2 for 24 h. The material was washed, transferred to 70% alcohol, and refrigerated until the slides were prepared. Pollen mother cells (PMCs) were prepared by squashing in 0.5% propionic carmine. Cells were analyzed from the metaphasis I phase up to the tetrad of microspores. The cells were counted according to the meiosis phase, and the percentage of normal and abnormal cells was obtained. The images of meiocytes with the most representative abnormalities were captured using an Olympus CX 31 optical microscope and an SC 30 camera using the AnalySIS getIT program.

The percentage of meiotic abnormalities was correlated with the average production of pure seeds in each hybrid. Data on cytological and seed yield variables were analyzed using principal component analysis (PCA) to detect the structural organization of the genotypes. Graphic representations were made using FactoMineR, Factoextra, and Vegan (R Development Core Team). Pearson correlations between all characteristics were graphically represented using Past software ($p<0.05$).

RESULTS AND DISCUSSION

Analysis of microsporogenesis of the eight polyploid sexual hybrids revealed the occurrence of segregational and non-segregational meiotic abnormalities. All irregularities that occurred during meiosis culminated in a high frequency of abnormal end products. The total percentage of abnormal end products ranged between 33.1% in hybrid 676-10 and 91.77% in hybrid 460-9. Table 2 shows the percentages of abnormal end products of all hybrids.

Segregational abnormalities, that is, early or delayed migration of chromosomes to cell poles, occurred in the two meiosis phases and led to the formation of tetrads with micronuclei in one, two, three, or four microspores (Figure 1a). The occurrence of meiotic abnormalities in pollen mother cells is a consequence of the ploidy level of the species and the behavior and distribution of chromosomes in the phases preceding the formation of the meiotic product.

While micronuclei were reported in all hybrids analyzed, tetrads with microcytes (Figure 1b) were found at low frequency in hybrids 98-9, 460-9, and B1, and polyads in hybrids 1121-9, 460-9, and R33 (Figure 1c). Microcytes in tetrads are formed by an additional cytokinesis, while the polyads are established by even more additional cytokineses (Mendes-Bonato et al. 2009, Souza-Kaneshima et al. 2010). Microcytes assemble one or a few chromosomes, forming micronuclei. A microcyte is a minor microspore with very little nuclear content. In this case, each additional cytokinesis event leads to the formation of a microcyte. Hybrids 98-9, 460-9, and B1 presented only tetrads with one microcyte. The additional cytokineses that form the polyads seize the cytoplasmic and nuclear contents in similar amounts in the different microspores. Micronuclei and microcytes are considered forms of chromosome elimination that lead to chromosomal imbalance in the microspores of the tetrads, while in the polyads, the partitioning of the genome may lead to abnormal formation of all formed microspores (Sales et al. 2021).

Segregational irregularities, with the consequent formation of micronuclei, and the formation of microcytes and polyads are common in the meiosis of accessions and polyploid hybrids of Urochloa (Pagliarini et al. 2008, Paula et al. 2016, Rocha et al. 2019). The frequency of these abnormalities was variable in the different hybrids (Table 2). Cytogenetic analyses of intra- and inter-specific hybrids of Urochloa from breeding programs have revealed a high frequency of segregational meiotic abnormalities in some hybrids (Adamowski et al. 2008, Sales et al. 2021), whereas a more stable meiosis has been detected in others (Souza et al. 2015, Morais et al. 2018).

Non-segregational abnormalities were observed in hybrids B1, R33, and 460-9. Chromosome adherence was common among all three hybrids. Intense chromatin agglomeration has been described as the formation of sterile gametes in interspecific hybrids of Urochloa (Mendes-Bonato et al. 2007). In addition to this abnormality, hybrid B1 showed severe irregularities in spindle formation, while cellular fusions and cytomyxis were reported in hybrid 460-9. According to Boldrini et al. (2006), polyploid and apomictic conditions may predispose chromosome transfer between myocytes in Urochloa.

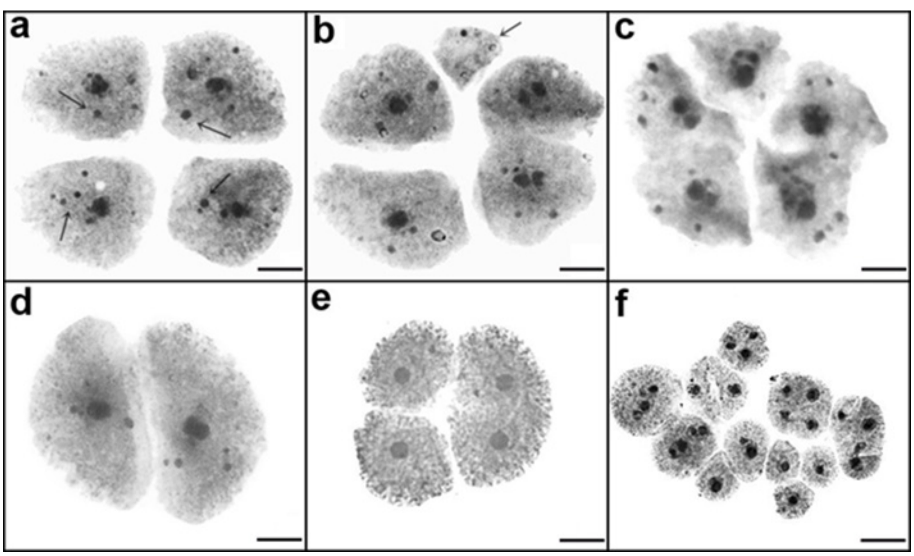

Figure 1. Abnormal end products in sexual hybrids of *Urochloa*. A– tetrad with micronuclei in the four microspores (arrows); b – tetrad with a microcyte (arrow); c – polyad; d – dyad; e – triad; f – anomalous end products. Bar: figures a, b, c, d, e: 10 µm; f: 100 µm.

All these abnormalities caused the formation of completely anomalous meiosis end products (Mendes-Bonato et al. 2009, Souza-Kaneshima et al. 2010, Pagliarini et al. 2012). However, among the hybrids analyzed, only 460-9 presented 1.66% of the anomalous final products (Table 2; Figure 1f). Dyads (Figure 1d) and triads (Figure 1e) were also detected in hybrid 1121-9, formed by failures in the first and/or second cytokinesis.

Reports in the literature on the correlation between meiotic abnormalities and seed yield are scarce and outdated. According to Kempanna and Seetharam (1972), there is no evidence that alteration during meiosis causes seed sterility in triticale (× Triticosecale Wittmack) whereas, Smith and Murphy (1986) reported that irregularities in the meiotic process were partially responsible for the decline in seed yield in Medicago sativa L.. In the case of the genus Urochloa, there are several recent reports associating polyploidy and irregularities during cell division with pollen inviability. However, there is no well-defined correlation between meiotic alterations and the production of fertile seeds. Lutts et al. (1991) studied the relationship between uni-, bi-, and multivalent in metaphase I with pollen viability and seed yield and concluded that chromosome associations cannot be used to evaluate the fertility level of F_1 hybrids from the crossing of U. ruziziensis × U. decumbens and U. ruziziensis × U. brizantha. Meanwhile, most studies report that a low frequency of meiotic abnormalities is associated with viable pollen production and consequent fertility in Urochloa, while the high frequency of irregularities is associated with inviable pollen featuring low production of fertile seeds (Adamowski et al. 2008, Souza et al. 2015, Paula et al. 2016, Rocha et al. 2019, Baldissera et al. 2020). Souza et al. (2015) reported that some hybrids of U. decumbens showed a peculiar behavior because 100% of the tetrads were affected, although pollen sterility was lower than expected. This could be explained by the fact that not all tetrad microspores were abnormal. Therefore, normal microspores would supply the need for viable pollen for the formation of the embryo and endosperm in sexual hybrids or the endosperm in apomitic ones.

There was no significant correlation between seed yield and the two types of seed set in this study: seed set 1 and seed set 2. However, the correlation between the percentage of meiotic abnormalities and total production of pure seeds (seed yield) was negative and significant (correlation = -0.72; $p < 0.04$) (Figure 2), indicating that meiotic performance affected seed yield. The correlation between meiotic abnormality and seed set, taking into account either seed weight (seed set 1 in grams) or the number of filled seeds (seed set 2) was negative, -0.41 and -0.40, respectively. This may indicate some influence of meiosis problems on the pure seed yield, although not statistically significant (Figure 2). The correlation between characters does not denote a causal effect of one on the other, such as the presence of meiotic abnormalities that determine the lowest seed yield. Instead, it detects a certain association between them. The percentage of irregular microspore tetrads in Medicago sativa L. was negatively correlated with seed fertility only when irregularity levels were high (Smith and Murphy 1986). According to Usandizaga et al. (2020), meiotic irregularities are closely correlated with pollen fertility, although they do not appear to be a limiting factor for the fertility of crosses in Acroceras macrum. In the same study, pollen viability of tetraploid cytotypes with open pollination was positively correlated with the seed set ($r = 43$, $p = 0.0034$).

This research showed that the formation of only one or two micronuclei positively correlated with seed yield, but these variables were not associated with seed set (Figure 2). Theoretically, microspores with micronuclei would be genetically imbalanced, while those without micronuclei would be normal.

The occurrence of one or two micronuclei in a tetrad would result in a ratio of 3:1 and 2:2 for viable and inviable pollen grains, respectively. According to Baldissera et al. (2020), tetrads that presented micronucleus in three or four microspores were more detrimental to the production of viable pollen than those with micronuclei in only one or two microspores. However, the lack of association between the variables Mcn1 and Mcn2 with the seed set does not reinforce this hypothesis.

Non-significant correlations were obtained between the other types of meiotic abnormalities found in the final products in relation to seed yield and the two types of seed sets (Figure 3), despite the fact that these abnormalities consisted of irregular cell division, which would cause the formation of abnormal gametes, either with more chromosomes or with chromosomal elimination.

It is important to highlight that not all meiotic abnormalities were seen simultaneously and in the same proportion in all hybrids evaluated. Variables that better differentiate hybrids from each other are indicated in the PCA biplot (Figure 2), which considered all variables and all hybrids. Hybrids 676-10, 1386-10, and B13, which were positioned in the

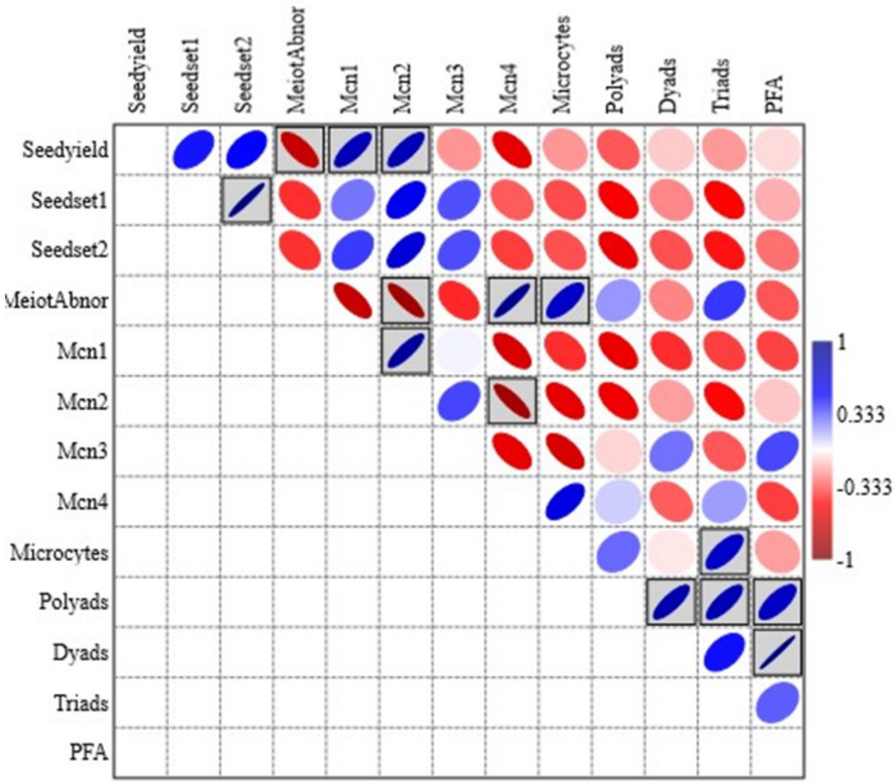

Figure 2. Pearson´s correlations between meiotic abnormalities and seed production. Correlations represented in closed squares are significant (p<0.05). Seed yield: pure seed production (g individual^{-1}); Seed set 1: (grams of filled seeds per individual %); Seed set 2: (number of filled seeds per individual %); Mcn1: Micronucleus in a microspore; Mcn2: Micronucleus in two microspores; Mcn3: Micronucleus in three microspores; Mcn4: Micronucleus in four microspores; PFA: Anomalous final product.

same quadrant, presented a more stable meiotic behavior than the other hybrids. These hybrids presented only tetrads with micronuclei in their microspores with a higher frequency of micronuclei in two of them. Hybrids 676-10 and B13 proved to be very good seed producers, with high seed sets (Table 2). However, hybrid 1386-10 revealed an intermediate behavior in relation to seed yield (Table 2). Despite its polyads, dyads, triads, and anomalous final products, hybrid 1121-9 also strangely presented an intermediate seed yield and was positioned within a different quadrant (Table 2). Another strange behavior was detected in hybrid 98-9, whereby there was a high frequency of micronuclei in four microspores and the presence of tetrads with microcytes, along with a high seed yield, indicating that these abnormalities might not completely impair viable pollen grains. According to Ricci et al. (2010) and Souza et al. (2015), a high frequency of abnormal tetrads may not be correlated with high pollen sterility. This may indicate that the abnormalities were not sufficiently severe to induce infertility.

Hybrids R33 and B1 presented an unstable meiotic behavior, with a high frequency of micronuclei in the four microspores, tetrads with microcytes in hybrid B1, and pentads in hybrid R33, coupled with low seed yield. The high frequency of micronuclei in three or four microspores, tetrads with microcytes, and polyads may cause a low percentage of viable pollen, as reported by Baldissera et al. (2020), in their analysis of sexual and apomictic hybrids of Urochloa, which would help explain the low seed yield. Hybrid 460-9 had the highest proportion (91.77%) of meiotic abnormalities in the tetrads of microspores and a low seed set, regardless of the type.

Studies of multivalent chromosomal associations formed in diakinesis have revealed that forages of the genus Urochloa have been considered segmental allopolyploids (Boldrini et al. 2006, Adamowski et al. 2008). According to Sotomayor-Ríos and Shank (2000), fertility in polyploids decreases as meiotic irregularities increase. Allopolyploids would become fertile due to the polyploidization process that allow a regular meiotic procedure, as observed in diploids (Dar

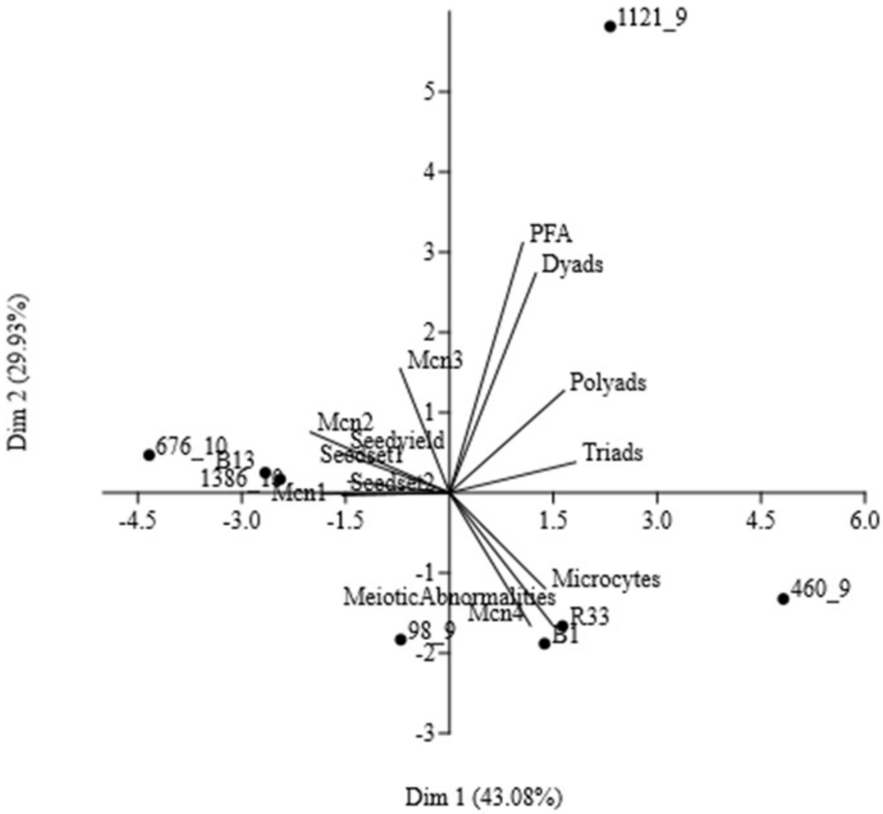

Figure 3. Graphic representation of Principal Component Analysis (PCA) for meiotic abnormalities and seed production evaluated in eight hybrids of Urochloa spp. Seed yield: pure seed production (g individual^{-1}): Seed set 1: (grams of filled seeds per individual %); Seed set 2: (number of filled seeds per individual %); Mcn1: Micronucleus in a microspore; Mcn2: Micronucleus in two microspores; Mcn3: Micronucleus in three microspores; Mcn4: Micronucleus in four microspores; PFA: Anomalous final product.

et al. 2017). However, this does not appear to relate to neo-polyploids, since they present several meiotic abnormalities that could render them infertile (Ramsey and Schemske 2002, Comai 2005).

Irregularities that occur during meiosis and form abnormal end products induce the formation of genetically unbalanced microspores and, consequently, the production of inviable pollen grains. The low viability of pollen, among other biotic and abiotic stresses, may cause low seed yields. According to Adamowski et al. (2008), hybrids with a high proportion of irregularities during meiosis may have their seed yield completely compromised due to unbalanced gametes. However, it is still necessary to determine the effect of these abnormalities on the viability of pollen grains of each hybrid and whether they lead to low seed yield when other factors are controlled. Although further research is still required, the results have shown an association between meiotic irregularities and the production of fertile seeds. The evaluation of meiotic abnormalities and the production of pure seeds in the sexual polyploid hybrids of Urochloa is an unprecedented study that is relevant to identify hybrids with less meiotic abnormalities and greater seed yield and is highly promising for a female parent in breeding programs at the Embrapa Beef Cattle.

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