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Obtaining sexual genotypes for breeding in buffel grass

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ABSTRACT

Buffel grass (*Cenchrus ciliaris* L. syn. *Pennisetum ciliare* (L.) Link) is a species that is highly tolerant to drought and is used primarily as forage in drier regions throughout the subtropics and tropics. It reproduces mainly by apomixis and the acquisition of obligate sexual genotypes or facultative apomicts with high levels of sexuality is required for performing crosses and plant improvement. The aim of this study was to obtain sexual genotypes from controlled crosses using obligate apomictic cultivars and a sexual line. Twelve putative hybrid F1 plants were selected morphologically and two of them were identified as sexual genotypes by PCR using specific primers for reproductive mechanism. Cytoembryological analysis showed 65.5 and 71.3% meiotic embryo sacs in these plants and their hybrid nature was corroborated by AFLP. Both highly sexual genotypes could be used as female parents in crosses for obtaining improved cultivars of buffel grass.

1. Introduction

Cattle production in northwestern Argentina (NOA) is essentially pastoral, and subtropical and tropical perennial grasses are the main cultivated forage resources. This region extends from Cordoba, central Argentina, to the west and northwest of the country, and is characterized by low, seasonal rainfall (average 350 mm), high temperatures and high solar radiation sunlight in summer, as well as soils with varying textures and degrees of salinity.

In Argentina, buffel grass (*Cenchrus ciliaris* L.), among subtropical pastures, has demonstrated an excellent performance, and is adapted to soil and harsh climatic conditions prevailing in the NOA. This is a mainly obligate apomictic species (Snyder et al., 1955), and the use of obligate sexual or apomictic genotypes with high levels of sexuality is the only alternative for conventional crosses (Burson et al., 2012; Hussey et al., 1993). However, Bashaw (1962) identified a source of sexuality, an off-type plant, product of a mutation that showed poor forage aptitude (Griffa et al., 2005), significant susceptibility to salt stress (Griffa, 2010; Lanza Castelli et al., 2010) and little genetic contribution for apomictic F1s of agronomic interest (Griffa et al., 2005).

Using this source of sexuality, we have obtained several apomictic F1s, one of them, named Lucero INTA-PEMAN, with superior forage characteristics compared to its parents, while the remaining hybrids obtained showed marked phenotypic similarity to the female parent (Griffa, 2002; Griffa et al., 2005). These results suggest that the possibility of obtaining promising (F1) genotype frequency by hybridization will depend largely on favorable combinations of polygenes for quantitative characteristic additive effects, and/or the occurrence of heterosis in component characters of biomass production (Griffa et al., 2006). New and improved sexual genotypes from hybridization of the introduced sexual source, with cultivars widespread in the country and genetically divergent from female parental lines (Griffa, 2010), would lead to the availability of new female parents (Valle and do Savidan, 1998) in buffel grass for breeding purposes.

Progenies obtained from controlled crosses of sexual lines and apomictic cultivars can be sexual or apomictic hybrids. The reproductive method may be determined by using specific molecular markers (PCR) that were found linked to the apomixis genomic region (ASRG) in buffel grass (Gustine et al., 1997; Jessup, 2005; Jessup et al., 2002; Lubbers et al., 1994; Ozias-Akins et al., 1993), and by embryo sac analysis (Sartor et al., 2005, 2009). However, the sexuality source is self-compatible (Bashaw, 1962; Hanselka et al., 2004), and therefore it is important to determine whether the sexual progenies obtained are hybrids. For that purpose, available molecular techniques, such as RAPD and AFLP, are widely used (de Benedetti et al., 2000; Griffa et al., 2006; Oropeza and García, 1997). The aim of this study was to obtain and characterize new and improved sexual genotypes to be used as female parents in conventional crosses in buffel grass.

2. Material and methods

2.1. Plant material

Plant material consisted of 30 plants of the introduced sexual source, arranged in 3 rows of 10 plants each, and field grown. The apomictic

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cultivars used were Biloela, Nunbank, Molopo, Americana, Nueces, Messina, Boorara, Toowomba, and *Cenchrus* sp. The sexual line was represented by plants grown from seeds provided by the Germplasm Bank of Texas A&M University College Station, TX (USA), and male parents (apomictic cultivars) were field grown in the Experimental Area IFRGV-INTA (Córdoba, Argentina).

2.1.1. Controlled crosses

Thirty female plants were cloned and field grown. Because buffel grass exhibits protogyny (Fisher et al., 1954; Snyder et al., 1955) there was no need for emasculation to produce controlled hybrids (Shafer et al., 2000). Individual panicles completely emerged from the flag leaf sheath were daily placed into acetate tubes prior to stigma exsertion. Hybridizations were made during the summer months by means of the crossing technique proposed by Sherwood et al. (1994) with modifications (Griffa, 2010). Pollen of the apomictic cultivars was collected each morning between 8 and 10 am and the inflorescences were pollinated, tagged, marked and bagged until seed maturation. Seed was harvested, dried naturally and stored cold until the following spring. Seeds collected from each pollinated panicle were planted in separate pots and the seedlings were transplanted to greenhouse and subsequently planted in the field, spaced 1 m × 1 m between them in the Experimental Area of the Institute of Plant Physiology and Genetic Resources (IFRGV) (INTA). Putative hybrid F1 plants were placed in rows, maintaining the identification of those that derived from a single pollinated panicle.

2.1.2. Preliminary evaluation and selection of putative F1 hybrid plants

Preliminary evaluation of putative F1 hybrid plants was performed by the character plant height, a component that in forage production has a direct influence on dry matter production (Daher et al., 2004), has high coefficient of genetic determination (CGD = 89%) (Griffa, 2002) and is stable in different environments. It also has a high discriminating power among cultivars and is easily measured (Griffa et al., 2011). Plant weight and height are widely used in genetic improvement of *C. ciliaris* (Griffa, 2002; Griffa et al., 2011). Those putative F1 hybrid plants that were different from either both parents or from the female parent were selected for further morphological characterization.

2.1.3. Morphological evaluation of putative F1 hybrid plants

Previously selected plants were propagated vegetatively and three plants per clone were obtained. The selected clones were evaluated in the field, using a completely randomized design with three replications. For that purpose, morpho-agronomic characters (Table 1) with capacity to discriminate genotypes in buffel grass were used, most of which had coefficient of genetic determination between 50 and 89% (Griffa, 2002). Seven vegetative and reproductive characters were measured in 18 main tillers with panicles at harvest (six tillers per individual cloned/three cloned individuals/plant selected). The dimensions of the flag leaf were evaluated in 18 other main tillers with panicles of approximately 2 cm, emerging from the flag leaf sheath. At the end of the growing season, in April, (six months after being planted) the adult plants were cut to 10 cm of soil and weighed to record total fresh weight (Table 1).

2.1.4. Statistical analysis

An ANOVA was performed with the morphological characters; the means were further compared using the multiple comparisons Di Rienzo, Guzman, and Casanoves test (DGC) (Di Rienzo et al., 2002) with a confidence level of 5%. Multivariate analysis of clusters was conducted to observe the phenotypic divergence among the materials and between the materials and their respective parent through the measured characters. In all cases, statistical analyses were made using InfoStat software (Di Rienzo et al., 2011).

Table 1
Morphological characters evaluated, mode of data collection, and ontogenetic stage at data collection in *Cenchrus ciliaris*.

Morphological character	Ontogenic stage
-*Plant height (PLH)* (measured in m from ground level to the apex of the panicle of a main tiller). -*Length of panicle (LP)* (measured in cm from the base to the apex of floral peduncle of a main tiller). -*Internodal length (INTL)* (distance in cm between 3rd and 4th node). -*Tiller diameter (TD)* (measured in mm on a main tiller immediately below the 3rd node) -*Number of nodes per tiller (NUD)* (measured on a main tiller) -*Number of vegetative branches per tiller (VB)* (number of secondary axes of a main tiller without panicles) -*Number of reproductive branches per tiller (RB)* (number of secondary axes of a main tiller with panicles)	Harvest
-*Flag leaf lamina length (FLL)* (measured in cm from ligule to the distal tip of lamina) -*Flag leaf lamina width (FLW)* (measured in cm at 2 cm from the base of lamina) -*Flag leaf sheath length (FSL)* (measured in cm from the node to the base of ligule) -*Total leaf length (TLL)* (length of lamina plus length of sheath of flag leaf in cm)	Panicle emergence
-*Fresh weight (FW)* (weight of plant in kg, measured immediately after the cut)	Post-harvest

2.2. Reproductive mechanism identification

2.2.1. PCR technique

Because materials obtained from F1 hybrids may be apomictic or sexual genotypes, the selected F1s were evaluated by PCR with the following specific markers linked to the apomixis sequence genomic region (ASGR) (Jessup, 2005): PCAB10, Q8H and UGT197 (Table 2).

Reagents and the PCR program adjusted in buffel grass (Griffa, 2010) were the following: Mastermix final volume of 20 μl, 10× buffer with 15 mM $MgCl_2$, 25 mM $MgCl_2$, 1 mM dNTP, 5 mM of each primer (forward and reverse), GoTaq DNA polymerase from Promega (5 U/μl) and 25 ng/μl of sample DNA. The thermal cycler program was as follows: 94 °C for 3 min and 10 cycles of 94 °C for 30 s, 64 °C for 30 s (−1 °C/cycle) and 72 °C for 45 s, followed by 36 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, and then storage at 4 °C. PCR products were visualized in 2% agarose gel.

2.2.2. Clearing embryo sacs

Selected putative F1 hybrid plants, found sexual by PCR, were additionally analyzed cytoembryologically. Immature inflorescences, in partial emergence of the flag leaf and without exertion of stigmas and anthesis, were fixed in FAA (18 parts Ethanol 70%:1 part Formaldehyde 37%:1 part glacial acetic acid) for 24 h. Pistils were dissected out of the florets and cleared using the method of Young et al. (1979). One hundred mature ovules per genotype were directly mounted for

Table 2
Markers linked to apomixis, forward and reverse sequences and weight of the amplified fragment in base pairs (bp).

Marker	5′————————> 3′	bp
PCAB10	F: TTCGAAATCGCATAGGTGAG R:GAGCCTTTCTTTATTTACCCAGTG	211
Q8H	F: GAGCTTGNCCAATCGGGAAA R: ATGGTGATGGATCTTTTGGAC	800–850
UGT197	F: GGATGAATAAAACGGTGTTGGGAG R: GAACAACCGCACAAGTGAGAGAA	850