Rev. FCA UNCUYO. 2018. 50(2): 1-13. ISSN impreso 0370-4661. ISSN (en línea) 1853-8665.

Analysis of genetic variability *in vitro* regenerated buffelgrass plants through ISSR molecular markers

Análisis de variabilidad genética en plantas regeneradas *in vitro* de buffelgrass mediante marcadores moleculares ISSR

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Originales: *Recepción:* 06/10/2016 - *Aceptación:* 24/08/2017

Abstract

Genetic variability can be generated through *in vitro* culture via somaclonal variation. This tool can be potentially useful in a breeding program involving apomictic buffelgrass genotypes. The aim of this work was to evaluate inter simple sequence repeats (ISSR) as molecular markers to detect genetic variation in *in vitro* buffelgrass regenerated plants. Six plants regenerated from *in vitro* anther culture, via somatic embryogenesis were used, as well as the anther donor genotype (RN 51) as control. Of a total of 26 ISSR primers tested, 22 amplified, detecting 12% polymorphism with a divergence between 5 and 24% from RN 51. Amplification products were observed with the primers containing di-, tri- or tetra-nucleotide sequences, with or without additional nucleotides at the 3′ end. The most informative primers were those containing the repetitive sequences $GACA_n$, AG_n or GA_n. Moreover, the regenerants transplanted at field conditions differed in morphological characteristics among them and with respect to RN 51. This study confirms that ISSR are useful to identify genetic variability in *in vitro* regenerated buffelgrass plants.

Keywords
Apomixis • somatic embryogenesis • somaclonal variation • anther culture • ISSR • genetic diversity

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Resumen

El cultivo *in vitro* permite generar variabilidad genética mediante variación somaclonal. Este fenómeno puede ser potencialmente útil en un programa de mejora en genotipos apomícticos de buffelgrass. El objetivo del presente estudio fue evaluar inter secuencias simples repetidas (ISSR) como marcadores moleculares en la detección de variación genética en plantas regeneradas *in vitro* de buffelgrass. Se utilizaron seis plantas regeneradas mediante el cultivo *in vitro* de anteras, vía embriogénesis somática, y el genotipo dador de anteras (NR 51) como control. De un total de 26 cebadores ISSR probados, 22 de ellos amplificaron, detectando un 12% de polimorfismo con una divergencia del 5 al 24% con respecto al material NR 51. Se observaron productos de amplificación con los cebadores que contienen secuencias di-, tri- o tetra-nucleótidos, con o sin nucleótidos adicionales en el extremo 3′. Los cebadores más informativos fueron aquellos que contenían secuencias repetitivas $GACA_n$, AG_n o GA_n. Conjuntamente, las plantas trasplantadas a campo manifestaron características morfológicas diferentes entre ellas y con respecto al NR 51. El presente estudio confirma que el empleo de ISSR permite identificar variabilidad genética en plantas regeneradas *in vitro* de buffelgrass.

Palabras claves

Apomixis • embriogénesis somática • variación somaclonal • cultivo de anteras • ISSR • diversidad genética

Introduction

In vitro cell or tissue culture offers several advantages to plant species. The use of this biotechnological tool has resulted in regenerated plants showing spontaneous genetic variability with respect to the donor plant. This phenomenon is known as somaclonal variation (20) and may be undesirable in some *in vitro* propagated species (9). In apomictic cultivars of buffelgrass [*Pennisetum ciliare* (L.) Link syn. *Cenchrus ciliaris* L.], however, those changes can be potentially useful as a source of genetic variability that can be beneficial in breeding programs (23) of this important forage grass worldwide in arid and semiarid regions (4, 18).

Genetic variation reported in several *in vitro* regenerated species include phenotypic changes (33), alterations in chromosome number or specific regions (19), point mutations (11, 24), and activation of transposable genetic elements (21), among others. Accordingly, all these factors should be considered when identify and analyze the occurrence of a somaclone (20). Morphological characters may be one of the most practical, cost-effective and easy methods to evaluate somaclonal variation (33). Nevertheless, these studies in *in vitro* regenerated plants usually require a comprehensive approach, including several nuclear DNA analysis techniques (2, 7).

In buffelgrass, measurement of nuclear DNA content by flow cytometry (FCM) has been suggested as an alternative and plays an important role in the analysis of micropropagated plant suffering changes in ploidy levels (8). Also randomly amplified polymorphic DNA (RAPD) are mentioned to identify somaclones from induced mutations of embryogenic callus treated with ethylmethane sulfonate (EMS) and *in vitro* selection (23).

In others species, one of the approaches used in those studies is ISSR (inter simple sequence repeat) molecular markers (10, 12, 26, 30). This technique is based on polymerase chain reaction (PCR) and uses a single primer complementary to the microsatellites (28). One variant to this method consists of adding nucleotides in the 3' end of the primer, known as anchored primer (34). Each amplified band is a locus and corresponds to the DNA sequence located between two similar or inverted microsatellites. This technique has the advantage that primer design does not require previous knowledge of the DNA sequence to be amplified (5, 16). There is scarce information about the use of this type of molecular markers in buffelgrass, except for studies that determined genetic diversity in wild populations (1, 17). Here we aimed to evaluate inter simple sequence repeats (ISSR) as molecular markers to detect genetic variation in *in vitro* buffelgrass regenerated plants.

Materials and methods

Plant material

The study included six plants of the R_1 generation (plants obtained by *in vitro* anther culture, via somatic embryogenesis) which showed genetic instability in nuclear DNA content by flow cytometry (8) and anther donor plant registration number 51 accession (RN 51). All the plants were transplanted at field in the Instituto de Fisiología y Recursos Genéticos Vegetales of the Instituto Nacional de Tecnología Agropecuaria (INTA) (Córdoba, Argentina) for further evaluation.

Inter simple sequence repeats (ISSR)

Genomic DNA of each regenerated and donor plant was isolated from frozen leaves (-20°C) using the *Nucleon PhytoPure* (GE Healthcare) commercial kit and following the manufacturer's instructions. Pure DNA was resuspended in 50 µl of distilled water. DNA concentration in each sample was quantified by absorbance reading ($A_{260/280}$ nm) in a *NanoDrop* ND-1000 V3.5 spectrophotometer (NanoDrop Technologies, USA).

A total of 26 ISSR universal primers were tested; they were obtained from sequences published on line the "UBC Primer Set #9" (31) and from previous works in other plant species (14, 26, 32) (table 1, page 4).

The amplification reaction was performed in a final volume of 20 µl, containing 2 µl DNA (25 ng/µl), 1X buffer solution (Tris-HCl 6 mM pH 8.8, KCl 50 mM), 0.8 µl $MgCl_2$ (25 mM), 4 µl of each dNTP (1 mM), 4 µl of the primer (5 µM) and 0.2 U of GoTaq® DNA polymerase (Promega, USA). Amplification consisted of following steps: 94°C for 3 min, 35 cycles at 93°C for 30 s, 55°C for 1 min, 72°C for 1 min 30 s, followed by a final extension of 72°C for 5 min. To improve the band patterns of those primers that failed to amplify, different annealing temperatures were tested (14) and in some reactions, 0.5 µl (10 mg/ml) of bovine serum albumin (BSA) was added. A minimum of three reactions per primer were performed to increase resolution of the band pattern. Amplifications were conducted in an Eppendorf Mastercycler 5333 thermocycler.

Amplification products were run for two hours at 100 V in 2% agarose gels in 0.5 X TBE buffer solution. A sample volume of 12 µl was loaded per lane [10 µl amplification product + 2 µl 5X loading buffer] as well as a GeneRuler 100-pb molecular weight marker, *Plus DNA Ladder* (Thermo Scientific Inc) as reference marker to calculate the size of the bands obtained with the ISSR.

Table 1. Analysis of genetic variability of *in vitro* regenerated plants buffelgrass by ISSR.

Tabla 1. Análisis de la variabilidad genética en plantas regeneradas *in vitro* de buffelgrass mediante ISSR.

N°	Primers	Nuc. Seq.	Temp.	PB	MB	TB	PMF (95)	PIC	AMP	SB
1	17899	$(CA)_6$ AG	55°C + BSA	2	19	21	0.10	0.21	95	350≤3000
2	AE_2	$(CA)_8$ G	55°C	1	25	26	0.04	0.21	99	100≤1400
3	D12	$(GA)_6$ CG	55°C + BSA	0	20	20	0.00	nd	100	200≤2900
4	D14	$(CAC)_3$ GC	52°C and 55°C	-	-	-	-	-	-	-
5	HB13	$(GAG)_3$ GC	52°C + BSA	0	25	25	0.00	nd	100	200≤3000
6	HB14	$(CTC)_3$ GC	55°C + BSA	0	3	3	0.00	nd	100	400≤700
7	ISSR1A	$(AG)_8$ Y*	55°C	11	18	29	0.38	0.23	84	100≤1800
8	ISSR1B	$(AG)_8$ GG	52°C + BSA	1	9	10	0.10	0.21	99	180≤550
9	ISSR4	$(GA)_8$ T	55°C + BSA	6	9	15	0.40	0.21	85	220≤1500
10	ISSR8A	$(AG)_8$ Y*T	55°C + BSA	5	23	28	0.18	0.24	92	200≤3000
11	ISSR9A	$(GATC)_3$ GC	55°C + BSA	0	21	21	0.00	nd	100	250≤3000
12	ISSR9B	$(AG)_8$ Y*C	55°C + BSA	1	24	25	0.04	0.21	97	250≤1800
13	RAF16	$(GACA)_4$	52°C + BSA	1	25	26	0.04	0.32	99	250≤3000
14	RAF4	$(GA)_9$ T	55°C	4	11	15	0.27	0.21	91	400≤2300
15	RAF9	$(CAA)_5$	55°C + BSA	0	11	11	0.00	nd	100	280≤3000
16	UBC816	$(CA)_8$ T	55°C + BSA	3	25	28	0.11	0.21	96	250≤2500
17	UBC825	$(AC)_8$ T	55°C + BSA	0	21	21	0.00	nd	100	180≤2000
18	UBC827	$(AC)_8$ G	55°C	5	21	26	0.19	0.21	92	250≤3000
19	UBC834	$(AG)_8$ CT	55°C + BSA	2	19	21	0.10	0.21	95	200≤1800
20	UBC840A	$(GA)_8$ CT	55°C	11	21	32	0.34	0.24	89	180≤3000
21	UBC840B	$(GA)_8$ TT	52°C + BSA	4	22	26	0.15	0.21	92	200≤2000
22	UBC850	$(GT)_8$ Y*C	55°C	0	17	17	0.00	nd	100	300≤1500
23	UBC861	$(ACC)_6$	52°C and 55°C	-	-	-	-	-	-	-
24	UBC863	$(AGT)_6$	52°C and 55°C	-	-	-	-	-	-	-
25	UBC864	$(ATG)_6$	55°C + BSA	1	13	14	0.07	0.21	99	180≤2000
26	UBC865	$(CCG)_6$	52°C and 55°C	-	-	-	-	-	-	-

Primers, nucleotide sequences (Nuc. Seq.), annealing temperatures (Temp.), number of polymorphic bands (PB), number of monomorphic bands (MB), number of total bands (TB), proportion of polymorphic loci (PMF(95)), polymorphic information content (PIC), amplification percentage (AMP) and size of bands (SB) generated by the ISSR primers. (*) Y= T/C. (-) Failed to amplify. nd: no data available.

Cebadores, secuencias de nucleótidos (Nuc. Seq.) temperaturas de hibridación (Temp.), número de bandas polimórficas (PB), número de bandas monomórficas (MB), número de bandas totales (TB), proporción de bandas polimórficas (PMF(95)), contenido de información polimórfica (PIC), porcentaje de amplificación (AMP) y tamaño de bandas (SB) generadas por los cebadores ISSR. Y= T/C. (-) No amplificó. nd: datos no disponibles.

PCR products were staining with ethidium bromide (10 mg/ml, GIBCO BRL) and fragments were observed under UV light (312 nm). Finally, the gel images were captured using BIORAD, Molecular Imager® Gel DocTM XR System (USA).

Phenotypic evaluation

To identify the ocurrence and observe phenotypic changes, RN 51 used as control and *in vitro* regenerated plants were transferred to an experimental plot in a completely randomized design. Thus, plant materials were transplanted at 1 m distance between them and morphological characters of leaf, stem and panicle were measured (25).

Phenotypic tests were performed during two crop seasons (2013/14 and 2014/15).

Statistical Analyses

The molecular profiles obtained using ISSR were coded by performing a matrix of binary data per primer and genotypes, where "1" and "0" indicated presence and absence of bands, respectively, and each band was regarded as a locus. Genetic relationships between individuals were evaluated using the Jaccard's similarity index [*J=a/(a+b+c)*] (15, 26, 27), where a, b and c are absolute frequencies for events (1.1), (1.0) and (0.1), respectively. To obtain a matrix of genetic distances between individuals a transformation of the Jaccard's index (sqrt (1-S)) was performe d. From the obtained data the proportion of polymorphic loci (PMF (95)) and polymorphism information content (PIC) were calculated. The PMF was calculated as the number of polymorphic loci / total number of loci, while PIC was estimated using the following equation:

$$PIC = 1 - (\sum_{i=1}^{n} p_i^2) - \sum_{j=i+1}^{n} \sum_{j=i+1}^{n} 2 p_i^2 p_j^2$$

where:
pi = the frequency of the
i = allele
n = the number of alleles (6)

The distance matrix was subjected to a principal coordinate analysis (PCoA) and to unweighted pair group method with arithmetic mean (UPGMA) hierarchical clustering. All the analyses conducted with the binary data matrix were performed using Info-Gen statistical software (3).

To confirm phenotypic variability in the *in vitro* regenerated materials, morphological variables of R_1 plants were compared with those of the anther-donor plant (RN 51) using *t* test. To determine the degree of grouping of R_1 and RN 51 material, a clustering analysis was performed using the average linking clustering UPGMA by applying the Euclidean distance as proximity measure. The analyses were performed using InfoStat statistical software (13).

Results

Inter simple sequence repeats (ISSR)

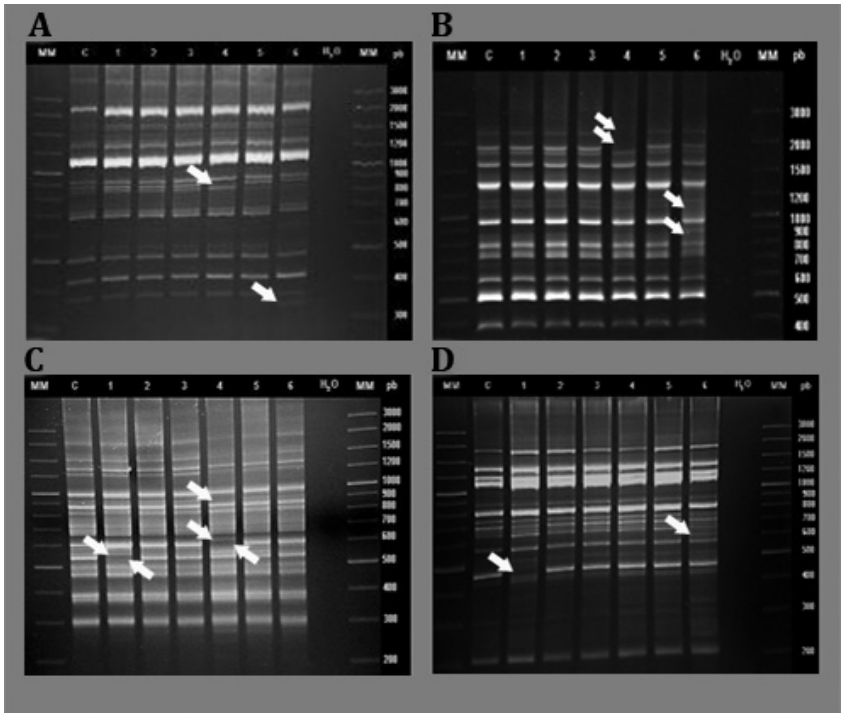
The ISSR generated amplification products in the DNA samples extracted from buffelgrass plants. Of a total of 26 primers tested, 22 amplified clear and reproducible bands. Table 1 (page 4) shows a descriptive analysis of amplification data obtained with ISSR primers. A total of 460 bands were generated, of which 58 were polymorphic (12%). Fifteen (68%) polymorphic primers were detected, with a proportion from 4 to 40% of polymorphic bands. The primers RAF16 (0.32), ISSR8A (0.24), UBC840A (0.24) and ISSR1A (0.23) produced the highest polymorphism information content. Of them, ISSR1A and UBC840A had the highest proportion of polymorphic bands (0.38 and 0.34, respectively).

Most of the polymorphic ISSR (RAF4, UBC834, ISSR8A, 17899, UBC827, UBC840A, ISSR1A, UBC816, ISSR4) generated the two types of expected data (absence and presence of alleles).

However, in comparison to donor plant, some primers only showed absence of alleles (AE_2, UBC864, ISSR1B and RAF16), whereas ISSR9B showed presence of new alleles.

The amplification profiles generated with ISSRs 17899, RAF4, UBC827 and UBC834 are displayed as an example in figure 1.

To understand the degree of similarity of molecular profiles between the *in vitro* regenerated individuals (R_1) and RN 51, the 22 ISSR that amplified were used for diversity studies and distance analyses.



MM: Molecular weight marker (100 pb Plus DNA Ladder, Thermo Scientific). C: DNA of anther donor plant (RN 51) used as control. Lanes 1 to 6: DNA of 6 *in vitro* regenerated buffelgrass plants, corresponding to the materials $R_{1\text{-}t4}$, $R_{1\text{-}t5}$, $R_{1\text{-}t6}$, $R_{1\text{-}t31}$, $R_{1\text{-}t32}$ y $R_{1\text{-}t33}$, respectively. H_2O: Negative control (a reaction containing all PCR components except the DNA template of the studied plant). The arrows indicate absence or presence of bands.

MM: Marcador de peso molecular (100 pb Plus DNA Ladder, Thermo Scientific). C: ADN de planta dadora de anteras (NR 51) utilizada como control. Calles 1 al 6: ADN de 6 plantas regeneradas *in vitro* de buffelgrass, correspondiente a los materiales $R_{1\text{-}t4}$, $R_{1\text{-}t5}$, $R_{1\text{-}t6}$, $R_{1\text{-}t31}$, $R_{1\text{-}t32}$ y $R_{1\text{-}t33}$, respectivamente. H_2O: Control negativo (reacción de PCR conteniendo todos los componentes menos el ADN molde de la planta en estudio). Las flechas indican ausencia o presencia de bandas.

Figure 1. Detection of genetic variability using ISSR *in vitro* regenerated buffelgrass plants. Agarose gel electrophoresis (2 %) of the PCR product obtained with the following ISSR: (A) 17899, (B) RAF4, (C) UBC827, and (D) UBC834.

Figura 1. Detección de variabilidad genética utilizando ISSR en plantas regeneradas *in vitro* de buffelgrass. Electroforesis en gel de agarosa al 2 % del producto de PCR obtenido con los siguientes ISSR: (A) 17899, (B) RAF4, (C) UBC827 y (D) UBC834.

The results obtained from the sqrt (1-S) transformation of Jaccard's similarity index indicates that the distance coefficients vary between 0.05 and 0.24 (5 and 24%, respectively), between *in vitro* regenerated plants and control (table 2).

The analysis including only the coefficients of the *in vitro* regenerated plants showed values ranging between 0.11 and 0.29 (11 and 29%, respectively), indicating not only divergence from RN 51 but also differences between *in vitro* regenerated plants.

The results of the principal coordinate analysis (PCoA) show that the most important principal components (PCs) (PC 1 and PC 2) (figure 2, page 8), which account for almost 62% of the total variability, shows two groups; one of them is composed of a single genotype ($R_{1\text{-}t31}$), which showed the greatest differences among the *in vitro* regenerated materials in PC 1. In turn, PC 2, differentiates two groups, one of them including the material used as anther donor or control (C) and the other including two regenerants that were very different from the rest ($R_{1\text{-}t32}$ and $R_{1\text{-}t33}$).

Figure 3 (page 8) showed the results of the dendrogram generated by UPGMA clustering method. The former cophenetic analysis yielded a correlation coefficient of 0.97. These results further highlight the differences compared with PCoA, because if considering a distance of 0.13 as an arbitrary cut-off value (50% of the maximum distance), $R_{1\text{-}t32}$ and $R_{1\text{-}t33}$ individuals not formed a group and the $R_{1\text{-}t4}$ individual was separated from the group that included RN 51 (C).

Table 2. Distance matrix of six *in vitro* regenerated buffelgrass plants and genotype RN 51 (C), obtained from the transformation sqrt (1-S) of the Jaccard's similarity index based on 22 ISSR primers.

Tabla 2. Matriz de distancia de seis plantas regeneradas *in vitro* de buffelgrass y del genotipo NR 51 (C), obtenida de la transformación sqrt (1-S) del índice de similitud de Jaccard basado en 22 cebadores ISSR.

	C	$R_{1\text{-}t31}$	$R_{1\text{-}t32}$	$R_{1\text{-}t33}$	$R_{1\text{-}t4}$	$R_{1\text{-}t5}$	$R_{1\text{-}t6}$
C	0						
$R_{1\text{-}t31}$	0.24	0					
$R_{1\text{-}t32}$	0.18	0.29	0				
$R_{1\text{-}t33}$	0.18	0.29	0.22	0			
$R_{1\text{-}t4}$	0.14	0.27	0.21	0.21	0		
$R_{1\text{-}t5}$	0.12	0.25	0.19	0.2	0.16	0	
$R_{1\text{-}t6}$	0.05	0.23	0.18	0.17	0.13	0.11	0

C: Apomictic genotype used as anther donor control plant. $R_{1\text{-}t4}$, $R_{1\text{-}t5}$, $R_{1\text{-}t6}$, $R_{1\text{-}t31}$, $R_{1\text{-}t32}$ and $R_{1\text{-}t33}$: *in vitro* regenerated buffelgrass plants.

C: Genotipo apomíctico utilizado como planta control dadora de anteras. $R_{1\text{-}t4}$, $R_{1\text{-}t5}$, $R_{1\text{-}t6}$, $R_{1\text{-}t31}$, $R_{1\text{-}t32}$ y $R_{1\text{-}t33}$: plantas regeneradas *in vitro* de buffelgrass.

Phenotype evaluation

Of the six plants that were transplanted to the field (R_1), five exhibited very different phenotypes from RN 51, and the remaining one did not survive. Plants $R_{1\text{-}t5}$ and $R_{1\text{-}t6}$, whose DNA content increased (6), showed significant differences ($P < 0.05$) in four morphological variables: plant height (PH), internode length (INTL), stem width (SW) and number of branches (NBR) (table 3, page 9).

The lower INTL, without modifying the number of nodes (NN), gives these plants a stunt appearance (lower PH). Moreover, the plants have a lower number of branches (NBR). The material $R_{1\text{-}t5}$ exhibits scarce panicles and the other one ($R_{1\text{-}t6}$) has a lower panicle length (PanL) ($P < 0.05$). Both regenerants produce few seeds (data not shown). $R_{1\text{-}t6}$ also presented a decrease in flag leaf length (FLL), flag leaf width (FLW) and flag leaf sheath length (FSL) ($P < 0.05$).

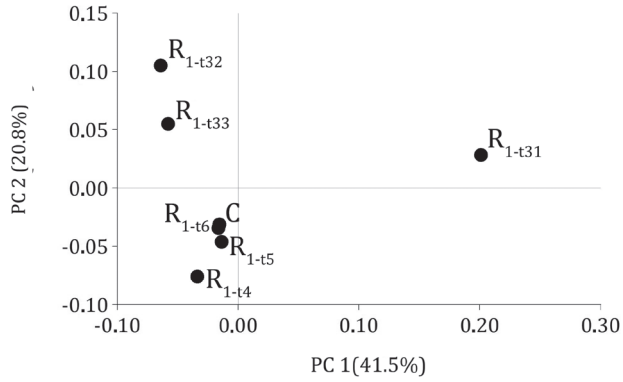


Figure 2. *Biplot* of six *in vitro* regenerated buffelgrass plants ($R_{1\text{-}t4}$, $R_{1\text{-}t5}$, $R_{1\text{-}t6}$, $R_{1\text{-}t31}$, $R_{1\text{-}t32}$ and $R_{1\text{-}t33}$) and RN 51 genotype used as control (C), obtained from sqrt (1-S) transformation of Jaccard's similarity index based on 22 ISSR primers.

Figura 2. *Biplot* de seis plantas regeneradas *in vitro* de buffelgrass ($R_{1\text{-}t4}$, $R_{1\text{-}t5}$, $R_{1\text{-}t6}$, $R_{1\text{-}t31}$, $R_{1\text{-}t32}$ y $R_{1\text{-}t33}$) y del genotipo NR 51 utilizado como control (C), obtenido de la transformación sqrt (1-S) del índice de similitud de Jaccard basado en 22 cebadores ISSR.

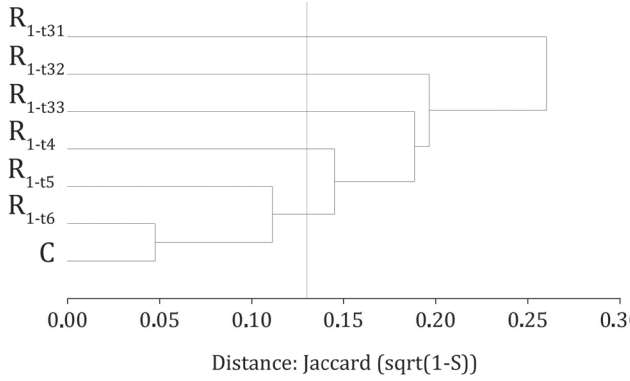


Figure 3. Dendrogram of six *in vitro* regenerated buffelgrass plants ($R_{1\text{-}t4}$, $R_{1\text{-}t5}$, $R_{1\text{-}t6}$, $R_{1\text{-}t31}$, $R_{1\text{-}t32}$ and $R_{1\text{-}t33}$) and RN 51 genotype used as control (C), obtained via UPGMA method through the analysis of ISSR markers over a distance matrix calculated from the sqrt (1-S) transformation of the Jaccard's similarity index.

Figura 3. Dendrograma de seis plantas regeneradas *in vitro* de buffelgrass ($R_{1\text{-}t4}$, $R_{1\text{-}t5}$, $R_{1\text{-}t6}$, $R_{1\text{-}t31}$, $R_{1\text{-}t32}$ y $R_{1\text{-}t33}$) y del genotipo NR 51 utilizado como control (C), obtenido por el método UPGMA mediante el análisis de marcadores ISSR, sobre una matriz de distancia calculada de la transformación sqrt (1-S) del índice de similitud de Jaccard.

Table 3. Characters of external morpholoogy of leaf, stem and panicle analyzed in 5 *in vitro* regenerated buffelgrass plants.

Tabla 3. Caracteres de morfología externa de hoja, tallo y panoja analizados en 5 plantas regeneradas *in vitro* de buffelgrass.

Material	FLL cm	FLW cm	FSL cm	PH cm	PanL cm	TFPL cm	EPL cm	INTL cm	SW mm	NN	NBR	FL/ BR
RN 51	33.3	1.2	7.2	126.8	11.9	19.4	8.3	10.8	3.9	9.8	6.8	2.4
$R_{1\text{-}t32}$	31.0	1.3	6.2	102.5*	7.75*	15*	5.6	6.02*	3.2	9.2	6.2	2.8
$R_{1\text{-}t33}$	-	-	-	105.8*	-	-	-	6.33*	2.7	12.5	7.7	-
$R_{1\text{-}t4}$	28.7	0.9*	6.4	92.8*	8.5*	17.5	8.7	7*	2.4*	9.6	5.6	3.4
$R_{1\text{-}t5}$	-	-	-	57.4*	-	-	-	4*	2.1*	10.0	2.8*	-
$R_{1\text{-}t6}$	22.4*	0.7*	3.2*	64.2*	6.3*	15.2	6.4	4.5*	2.1*	8.4	3.6*	1.0

(*) Indicates significant differences ($P < 0.05$) within each column in the morphometric characters analyzed in *in vitro* regenerated materials with respect to the anther donor plant (RN 51). Material: *in vitro* regenerated R_1 plants. Morphological variables: flag leaf length (FLL), flag leaf width (FLW), flag leaf sheath length (FSL), plant height (PH), panicle length (PanL), total floral penduncle length (TFPL), exerted peduncle length (EPL), internode length (INTL), stem width (SW), number of nodes (NN), number of branches (NBR) and number of flowers/branch (FL/BR).

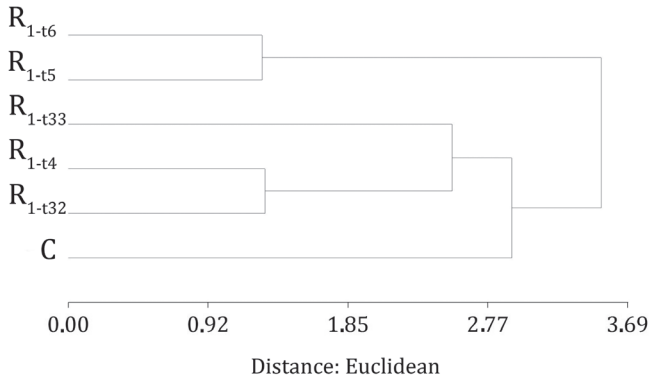
(*) Indica diferencias significativas ($P < 0,05$) dentro de cada columna en los caracteres morfológicos analizados de los materiales regenerados *in vitro* con respecto a la planta dadora de anteras (NR 51). Material: plantas R_1 regeneradas *in vitro*. Variables morfológicas: longitud de lámina de hoja bandera (FLL), ancho de lámina de hoja bandera (FLW), longitud de vaina (FSL), altura de planta (PH), longitud de inflorescencia (PanL), longitud total del pedúnculo floral (TFPL), longitud del pedúnculo excerto (EPL), longitud inter-nodal (INTL), ancho tallo (SW), número de nudos (NUD), número de ramas (RAM) y número de flores/ramas (FL/RA).

The plants exhibiting a reduction in DNA content ($R_{1\text{-}t4}$, $R_{1\text{-}t32}$ and $R_{1\text{-}t33}$) (8) also showed a statistically different increase in phenotypic traits with respect to control ($P < 0.05$) (table 3). All the plants exhibited a decrease in PH. Plant $R_{1\text{-}t4}$ had reduced FLW (thinner leaves), as well as PanL, INTL and SW. Plant $R_{1\text{-}t32}$, also had smaller panicles, and smaller length of exerted peduncle (EPL) and INTL. Plant $R_{1\text{-}t4}$ exhibited particularly lighter-colored foliage.

Because $R_{1\text{-}t33}$ and $R_{1\text{-}t5}$ plants had few panicles with immature inflorescences, PanL, TEPL, EPL and flowers/branch (FL/BR) variables and characters of leaf (FLL, FLW, FSL) were not considered in the analysis of clusters. The results of this analysis show that *in vitro* regenerated plants are phenotypically different from genotype RN 51. The dendrogram shows that the group including regenerants $R_{1\text{-}32}$, $R_{1\text{-}t4}$ and $R_{1\text{-}t33}$ are the most similar to material RN 51, and that $R_{1\text{-}t5}$ and $R_{1\text{-}t6}$ are the most different materials (figure 4, page 10).

Discussion

Different molecular profiles were detected in *in vitro* regenerated buffelgrass plants using ISSR molecular markers, suggesting some degree of variability (16, 17, 34). There is scarce information available on the use of this type of markers in somaclonal variation in buffelgrass.



In vitro regenerated buffelgrass plants: $R_{1\text{-}t4}$, $R_{1\text{-}t5}$, $R_{1\text{-}t6}$, $R_{1\text{-}t31}$, $R_{1\text{-}t32}$ and $R_{1\text{-}t33}$. The genotype RN 51 was used as control (C). Cophenetic correlation = 0.938.

Plantas regeneradas *in vitro* de buffelgrass: $R_{1\text{-}t4}$, $R_{1\text{-}t5}$, $R_{1\text{-}t6}$, $R_{1\text{-}t31}$, $R_{1\text{-}t32}$ y $R_{1\text{-}t33}$. El genotipo NR 51 fue utilizado como control (C). Correlación cofenética = 0,938.

Figure 4. Dendrogram constructed using UPGMA method through the analysis of morphological markers, with Euclidean distance being applied as proximity measure.

Figura 4. Dendrograma construido utilizando el método UPGMA mediante el análisis de marcadores morfológicos, aplicando la distancia Euclídea como medida de proximidad.

Studies conducted with ISSR in other *in vitro* propagated species reported very different polymorphic levels. For example, a 50% polymorphism level was detected in variants of *Camellia sinensis* L. (30); lower levels, of 5 and 3.9%, were recorded in micro-propagations of *Musa* spp. and *Dictyospermum ovalifolium*, respectively (10, 26). Here, total polymorphic loci were found to be 12%, which is similar to results reported for *Codonopsis lanceolata* (15.7%) or in clones of *Camellia* spp. (12.8%) (12, 15).

Likewise, the generated polymorphism showed a diferentiation degree of 5 to 24% with respect to the "control" pattern, as observed in somaclones of *Zea mays* L. (24). In addition, it is important to note that using the same type of molecular marker similar levels of polymorphism were obtained in wild populations of buffelgrass (1).

Amplification products were observed with the primers containing di-, tri- or tetra-nucleotide sequences, with or without additional nucleotides at the 3′ end. While most of the ISSR produced bands using the method implemented here, some of them failed to amplify. These primers were then tested at lower annealing temperatures, since molecular patterns were found to be highly modified by this factor (14), but some of these ISSR also failed to amplify. Lower temperatures could be used (16) or other additional factors should be considered, which were not studied in this work (5, 34).

The most informative primers were those containing repetitive sequences $GACA_n$, AG_n or GA_n, with the addition of one or three nucleotides at the 3′ end. Similar results were described in a study conducted to determine genetic diversity in wild bufflegrass populations (17).

Hence, this results confirm that the motifs $(GA)_n$ and anchors at the 3′ end can be included in the ISSR markers used for different studies of this species.

The polymorphism detected by ISSR in *in vitro* regenerated buffelgrass plants may be attributed to genetic changes in a restricted portion of the genome, because microsatellites and their continuous bases are the hybridization site of the marker primer (16, 34). This portion of the genome usually undergoes changes during the DNA replication-repair process (22, 29), and some authors suggest that the generated polymorphism may be due to deletions or insertions that occur at the primer recognition site (24). Regardless of the type of mutations occurring during *in vitro* plant regeneration, they can generate gain or loss of alleles (28), as shown in this work.

Finally, plants transplanted to the field exhibited very different morphological characteristics among them and from the genetic material used as control (RN 51), thereby confirming changes in the phenotype.

This study, along with a previous one (8), clearly reveal that a group of *in vitro* regenerated plants undergo genetic and morphological changes with respect to the anther donor material, suggesting that *in vitro* culture technique is a source of somaclonal variation (20).

These observations suggest that, besides the changes detected by flow cytometry at the nuclear DNA content levels (8), *in vitro* regenerated plants might also have undergone specific mutations in the genome (24). It is possible that these changes restricted to a few nucleotides cannot be detected using flow cytometry. For this reason, ISSR molecular markers should be included to explore genetic variations in *in vitro* regenerated buffelgrass plants.

Conclusions

The use of ISSR allowed to identify genetic variability in *in vitro* regenerated buffelgrass plants.

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Acknowledgments

We thank I. Teich for assistance with statistical analyses. This research was supported by the projects INTA-AEFP-PE No. 1126072 and CONICET (PIP 112 201101 0031).