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Banana somaclonal variation assessed by Amplified Fragment Length Polymorphism profiles at early cycles of *in vitro* culture

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SUMMARY

Banana micropropagation for obtaining free-virus plants frequently provokes somaclonal variation that could increase useful genetic variability in this asexually propagated crop. Both exploring the cycle of *in vitro* culture in which somaclonal variation occurs and the amount of generated polymorphism, are necessary. In this work, preliminary results of somaclonal variation during early cycles of banana *in vitro* culture are reported. Four randomly selected regenerated plants from the fifth cycle and two samples from the mother plant were analyzed. A total of 36 AFLP primer combinations were assayed, and 24 of them produced amplicons varying among 50-500 bp. The mother plant presented a total of 125 different amplicons while the regenerated plants jointly showed 131 different amplicons with a mean of 119.75 ± 3.97 per individual. High level of DNA polymorphism (24.43 %) was found among micropropagated plants and, additionally, the occurrence of somaclonal variation at earlier cycles was suggested by multivariate analysis of Principal Coordinates. In this study, somaclonal variation at early cycles of banana micropropagation was validated and the adequacy of AFLP technique to assess it at the molecular level was verified. The phenotypic effects of the detected somaclonal variations remain to be evaluated.

Key words: micropropagation, *Musa* spp., genetic variability, molecular breeding, Principal Coordinate Analysis

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SUMMARY

La micropropagación de banana para obtener plantas libres de virus frecuentemente provoca variación somaclonal que puede incrementar la variabilidad genética en cultivos de reproducción asexual. Es necesario explorar el ciclo del cultivo *in vitro* en que se produce esta variación así como cuantificar el porcentaje de polimorfismo. Este trabajo presenta resultados de variación

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somaclonal en ciclos tempranos de micropropagación de banana. Cuatro plantas tomadas al azar del quinto ciclo de regeneración y dos muestras de la planta madre se caracterizaron con 36 combinaciones de cebadores de AFLP. Veinticuatro combinaciones produjeron amplicones en un rango entre 50-500 pb. La planta madre presentó en total 125 amplicones mientras que en conjunto las plantas regeneradas mostraron 131 amplicones, con una media de 119,75 ± 3,97 por individuo. Se detectó un alto porcentaje de polimorfismo (24,43 %) en las plantas micropropagadas y, adicionalmente, análisis multivariados de coordenadas principales sugirieron la ocurrencia de variación somaclonal en ciclos de regeneración anteriores. En este estudio se validó la ocurrencia de variación somacloanl en ciclos tempranos de la micropropagación en banana y se verificó que la técnica de AFLP es adecuada para evaluarla a nivel molecular. Queda pendiente evaluar los efectos fenotípicos de estas variantes somaclonales.

Palabras clave: micropropagación, *Musa* spp., variabilidad genética, mejoramiento vegetal molecular, análisis de coordenadas principales

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INTRODUCTION

Bananas and plantains (*Musa* spp. L) are monocotyledonous, perennial herbs, cultivated in more than 130 countries in the tropics and subtropics (Resmi and Ashalatha 2007). Bananas and plantains are a good source of carbohydrates, minerals and vitamins (Kodym and Zapata-Arias, 1998). The most cultivated banana is propagated asexually because of the sterility of most edible varieties due to triploidy, hence the genetic base is narrow and diversity depends on somatic mutation to confront new resistance to fungal, bacterial and viral pathogens and numerous pests (Menon, 2016; Razani et al., 2019; Carrera et al., 2021; Rebouças et al., 2021). Since 2010 banana consumption has remained flat on a global basis at around 15 kg per capita per year. The micropropagation technology is considered to produce plants of potential superior quality. Isolation of useful variants for quality traits in high yielding genotypes, improved disease resistance and stress tolerance performance, and the possibility of rapid multiplication of a valuable genotype are some advantages of *in vitro* plant tissue culture (Suman, 2017).

In Argentina, Formosa province is an important banana producer in a suboptimal geographical area. Hence, somaclonal variation could increase genetic variability by obtaining plants with better adaptability to the rigorous climate for this fruit in this subtropical region. Previous to this practical application, it is necessary to evaluate the period of somaclonal occurrence during micropropagation and its frequency in Argentinian conditions, given that biotic and abiotic factors affecting the growth and development of explant donor plants can modify the *in vitro* culture response. In order to asses somaclonal variation, the objective of this exploratory research was to characterize through Amplified Fragment Length Polymorphism (AFLP) four regenerated banana plants from the fifth cycle of *in vitro* culture together with two samples of the mother plant as a reference.

MATERIALS AND METHODS

In the present study, the banana clone BCCI, provided by INTA Chaco-Formosa collection, was micropropagated. Four samples randomly taken from a batch of 32 explants at the fifth cycle of *in vitro* regeneration were analysed, together with 2 samples of the mother plant, with 36 combinations of AFLP primers to reveal somaclonal variation. First, the explant (1 cm^3) was excised from the decapitated shoot apex of BCCI and cultured following the standard protocol for *in vitro* initiation (Bairu et al., 2006) with 2 mg l^{-1} BAP, 0.023 mg l^{-1} of IBA, and 5.6 g l^{-1} agar. Then, after one week in dark chamber and two weeks under light photoperiod, the growing shoot was cut in four pieces, each of them being transferred to the multiplication medium (Figure 1) composed by 5 mg l^{-1} of BA and 0.18 mg l^{-1} of IAA. Cultures were incubated at 22 ± 2°C under a 12 hours photoperiod provided by continuous cool-white fluorescent lights. After 21 days, and during four additional cycles, multiplied shoots were isolated following the previous method mentioned and transferred to new tubes with fresh multiplication medium. The average number of shoots produced was 4, 9, 9, 23 and 32 at the first, second, third, fourth and fifth cycle, respectively.

Four plants of these 32 total regenerated at the fifth cycle were randomly chosen for molecular characterization together with two samples of the mother plant. The AFLP protocol described by Senerchia et al. (2015) was followed with minor modifications. First, the extracted genomic DNA was labeled in 1% (wt/vol) agarose gel and run with TAE buffer at 80 Volts for 40 minutes. Visualization was achieved by using ethidium bromide stain to measure the quality. The quantity was evaluated with *NanoDrop®*. The genomic DNA was diluted to 200-500 ngand digested with 8 units of EcoRI and 2 units of MseI in a final volume of 15 µL incubated at 37°C for 2 hours and 20 minutes at 65°C. Ligation of the digested fragments to the specific universal EcoRI and MseI adapters, both at final concentration of 0.14 *p*M, was achieved by incubation at 37°C for 2 hours with 1 unit of T4 ligase in a final volume of 35 µL. Pre-amplification samples were prepared with 1 µl of the digested-ligated DNA, 5 mM of each primer+1 (EcoRI+1: 5'-GACTGCGTACCAATTCA-3' and MseI+1: 5'-GATGAGTCCTGAGTAAC-3'), 10 mM dNTPs, and 0.5 unit of Taq polymerase in a final volume of 20 µl. The PCR conditions were 28 cycles of 45 seconds at 94°C, 45 seconds at 56°C and 1 min at 72°C each one. Once preamplified, the solution was 1:20 diluted in sterile water. The

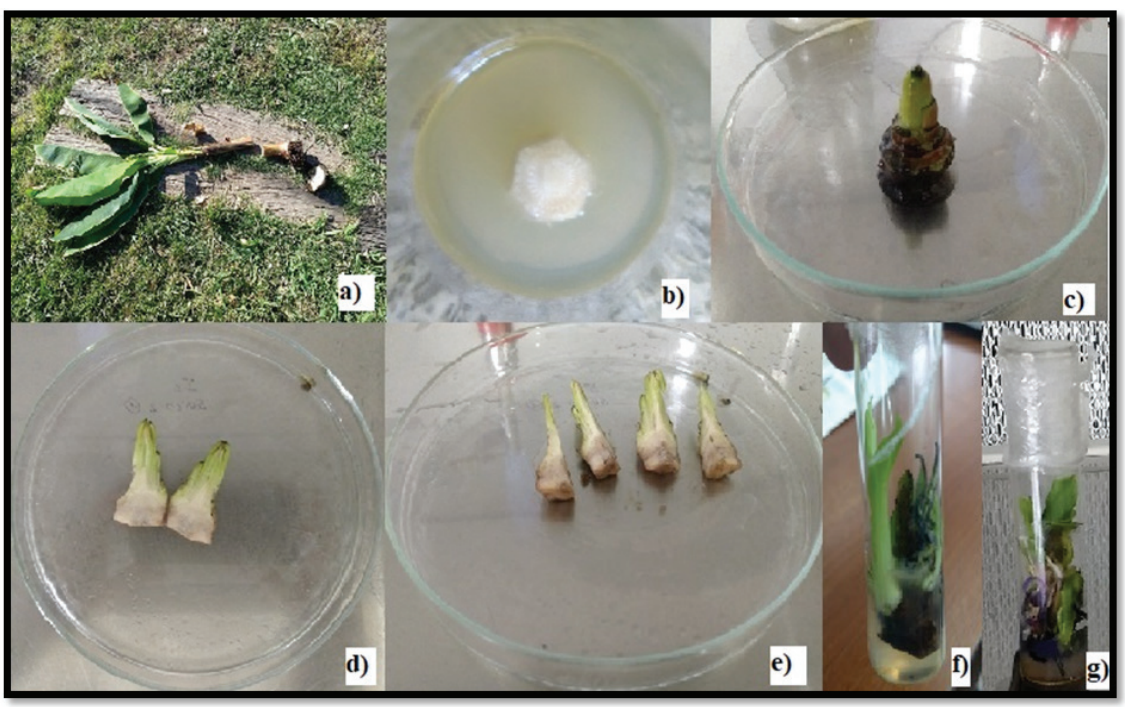

Figure 1. a) Source of explant from the mother plant BBC1 clone after one day of dehydration. b) BCCI explant in the initiation medium. c) Shoot grown from the unitiated explant before being cut for transferring to the multiplication medium. d) Cut of the shoot for disabling the apical dominance. e) First four explants for culturing in the multiplication medium. f) Shoot multiplication after 21 days in the first cycle of *in vitro* culture, g) Examples of shoot multiplication in following cycles of micropropagation

selective amplification samples were prepared using 3 µl of this dilution and the same mix used for pre-amplification. In the present study, 36 primers combinations of the primers+3 presented in Table 1 were assayed. A touchdown PCR for selective amplifications was programmed, starting with a cycle of 30 seconds at 94°C, 30 seconds at 65°C and 1 minute at 72°C. During the next 10 cycles, the annealing temperature was reduced to -0.7°C per cycle, until reaching 56°C. Then, 23 cycles of 30 seconds at 94°C, 1 min at 56°C and 1 minute at 72°C each were repeated.

As fluorophore-labelled primers NED™, VIC™, and FAM™ were used, the amplified bands, or amplicons, were resolved through fragment analysis capillary electrophoresis by ABI3730XL sequencer Macrogen™. Amplicons were analyzed using the GeneMarker® program. Monomorphic and polymorphic amplicons of molecular weights ranging from 500 and 50 base pairs were recorded in the samples analyzed. Polymorphic amplicons were identified according to their presence or absence in each sample, assigning values of 1 or 0, respectively. The two samples taken from the mother plant were the reference for estimating the repeatability of the molecular characterization and then the reliability of the putative polymorphism in the regenerated plants. The number of total and polymorphic amplicons was recorded and the percentage of polymorphism was calculated. Finally, a binary matrix of 1/0 was constructed and analyzed by Principal Coordinates Analysis with InfoGen software (Balzarini and Di Rienzo, 2003).

RESULTS AND DISCUSSION

Twenty four of the 36 AFLP primer-3 combinations were successful to generate molecular profiles. A total of 131 amplicons were obtained, 99 of them being monomorphic among the mother and the regenerated plants. An example of these results is shown in Figure 2. Hence a 24.43% of polymorphism was detected. Both BCC1, the mother plant, samples presented 125 amplicons and their AFLP profiles were identical. Instead, the four regenerated plants presented a total of 131 amplicons but their AFLP profiles were different. Some amplicons were common but others were specific of each individual. Hence, the mean number of amplicons per individual was 119.75 ± 3.97.

Regarding multivariate analysis, the first two Principal Coordinates accounted for a 71.1%. The biplot generated from both these coordinates is shown in Figure 3. A different position of the mother and the regenerated plants according to their molecular polymorphism assessed by AFLP profiles can be observed in the biplot. In fact, the regenerated plants 48 and 57 from the fifth cycle of *in vitro* culture are next to each other and closer to both mother plant samples than the regenerated plants 67 and 70. Interestingly, regenerated plants 48 and 57 were derived from the same regenerated plant 5 in the second cycle of *in vitro* culture while regenerated plants 67 and 70 were derived from the same regenerated plant 6 in the same second cycle. According to these observations, somaclonal variation has probably initiated at the earliest cycles of micropropagation and continued occurring during the subsequent cycles of *in vitro* culture. Additionally, the hypothesis about the occurrence of somaclonal variations at earlier cycles of *in vitro* culture is supported by observations from Youssef et al. (2011). In that report, authors analyzed somatic embryogenesis in 'Williams' and 'Great Dwarf' banana cultivars and reported the absence of 10 and 5 bands in the regenerated plants from a first cycle of *in vitro* culture assessed by AFLP when compared to the explant donors.

In a previous work using the standard AFLP technique (Ermini et al., 2018), 6 primer combinations

Table 1. Primers +3 used for selective amplifications

MseI (primer) 5'➔3'	+3 bp ending	EcoRI (primer) 5'➔3'	+3 bp ending
GATGAGTCCTGAGTAA	CAG	GACTGCGTACCAATTC	ACT
GATGAGTCCTGAGTAA	CTC	GACTGCGTACCAATTC	ACC
GATGAGTCCTGAGTAA	CCC	GACTGCGTACCAATTC	AGC
GATGAGTCCTGAGTAA	CGT	GACTGCGTACCAATTC	ATG
GATGAGTCCTGAGTAA	CCT	GACTGCGTACCAATTC	AAT
GATGAGTCCTGAGTAA	CGG	GACTGCGTACCAATTC	ATA
GATGAGTCCTGAGTAA	CTT	GACTGCGTACCAATTC	AAC
		GACTGCGTACCAATTC	AAA
		GACTGCGTACCAATTC	AAG
		GACTGCGTACCAATTC	ACA

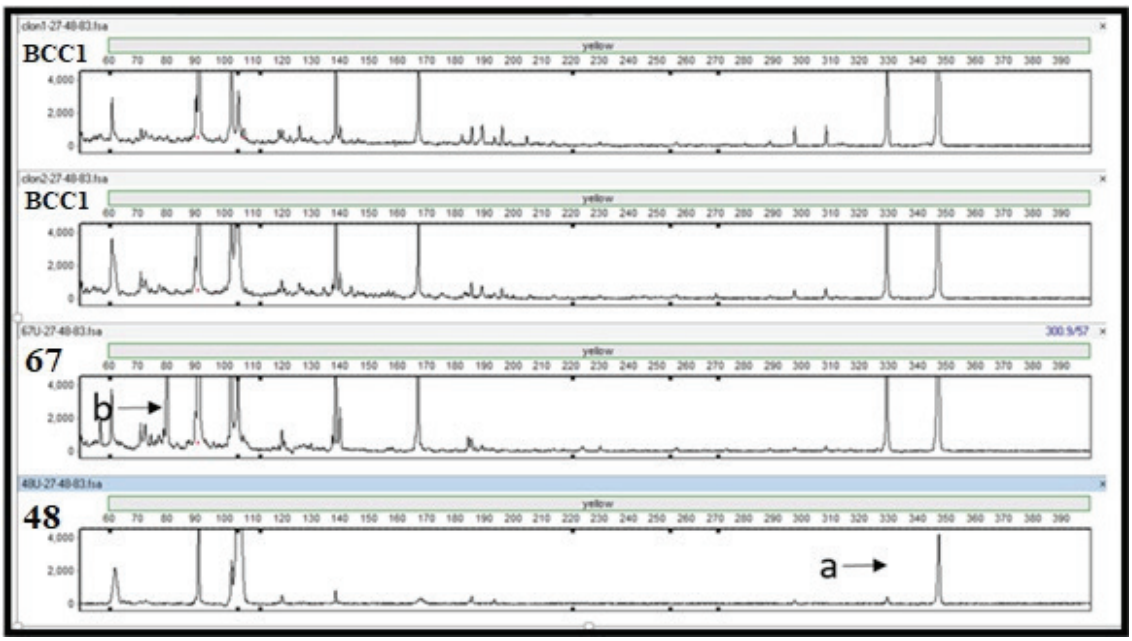

Figure 2. Example of AFLP analysis for assessing somaclonal variation at the molecular level. The two first rows show the AFLP profiles of the two samples from the mother plant. The two last rows show the AFLP profiles of regenerated plants 67 and 48 from the fifth cycle of *in vitro* culture. Arrows identified with letter "a" in the profile of plant 48 and with letter "b" in the profile of plant 67 show molecular polymorphisms compared to the mother plant and also among regenerated plants

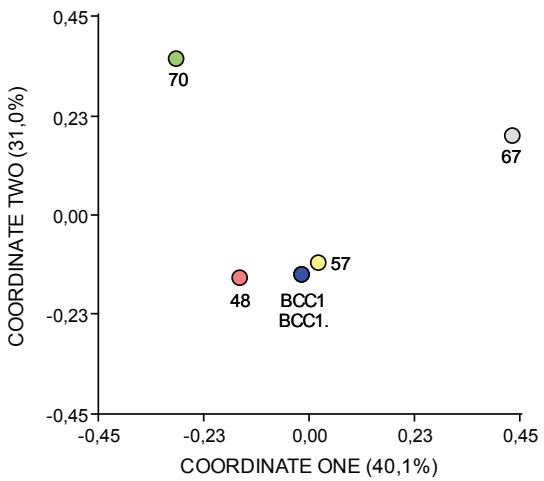

Figure 3. Biplot from Principal Component Analysis with all AFLP amplicons. Mother plant samples are identified as BCCI and BCCI., respectively, and 48, 57, 67, and 70 are regenerated plant from the fifth cycle of *in vitro* culture

were successful to asses the genetic diversity of 52 genotypes selected for their interesting agronomic performance in the subtropical and suboptimal environment of the Argentinian Formosa province. The mother plant BCCI was included among these clones and the amount of genetic diversity detected in that work (100 %) was similar to the 95.50 % of polymorphism reported by Opara et al. (2010). However, regenerated plants usually present more molecular polymorphism than unregenerated plants, as informed by Bairu et al. (2006), Deepthi et al. (2007) and the present study.

As a conclusion, this study validated the occurrence of somaclonal variation at early cycles of banana micropropagation and verified the adequacy of the AFLP technique to assess it at the molecular level. The phenotypic effects of the detected somaclonal variations remain to be evaluated.

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