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Variations of nuclear DNA content of *Musa* (AAA) CV. Williams micropropagated in high concentration of N6-benzyladenine

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Abstract

Shepherd and Dos Santos (1) had reported that the *Musa* plants micropropagated with N^6-benzyladenine (BA) show higher percentage of mitotic abnormalities than plants propagated by classical methods. In this research, adventitious shoots of Williams (AAA), obtained by induction in 20 mg/L BA, were characterized with flow cytometry. Nuclei were isolated from banana leaves and stained using propidium iodine (50 μg/L). The distribution of the relative nuclear DNA content of these *in vitro* plants was according to the following intervals: 8% between 3 to 3.25, 27% 3.25 to 3.5, 38% 3.5 to 3.75, 23% 3.75 to 4 and 4% 4.25 to 4.5. These results show the possibilities to obtain somaclones with different nuclear DNA contents from triploid cultivar for *Musa* improvements programs.

Keywords: N^6-benzyladenine, triploid bananas, somaclonal variation, flow cytometry, DNA content.

Cambios en el contenido de ADN de *Musa* (AAA) CV. Williams micropropagadas en alta concentración de N6-benciladenina

Resumen

Shepherd y Dos Santos (1) reportaron que plantas micropropagadas en N^6-benciladenina tienen mayores porcentajes de anormalidades mitóticas que las propagadas por métodos clásicos. En esta investigación, se caracterizaron por citometría de flujo, brotes adventicios del cultivar Williams (AAA) inducidos en BA (20 mg/L). Los núcleos fueron aislados de hojas de bananas y teñidos con ioduro de propidio (50 μg/L). La distribución del contenido relativo de ADN nuclear de estos brotes fue según los siguientes intervalos: 8% desde 3 a 3,25; 27% 3,25 a 3,5, 38% 3,5 a 3,75, 23% 3,75 a 4 y 4% 4,25 a 4,5. Estos resultados muestran la posibilidad de obtener somaclones con diferentes contenidos de ADN nuclear a partir de un cultivar triploide para programas de mejoramiento de *Musa*.

Palabras clave: N^6-benciladenina, bananas triploides, variación somaclonal, citometría de flujo, contenido de ADN.

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Introduction

Conventional breeding in bananas is a very difficult task due to lack of sexual reproduction (2). Mutation induction with gamma irradiation of *in vitro* plants had been successfully used by the International Atomic Agency laboratories for many years (3). However many laboratories of developing countries do not have the possibilities to use gamma irradiation for mutation induction programs, then i*n vitro* mutagenesis by somaclonal variation in tissue culture could be an alternative for *Musa* improvement.

Shoots induction from sucker tips with N^6-benzyladenine (BA) is the most common method for *in vitro* propagation in the genus *Musa.* Somaclonal variation is becoming commonly observed in this type of propagation (4). Shepherd and Dos Santos (1), Sandoval *et al.* (5) and Shepherd and Da Silva (6) report aneuploid cells, minichromosomes, and increments in chromosome number from 33 to 36. Additionally Giménez, *et al.* (7) used flow cytometry to study a somaclonal variant of Williams (AAA) obtained by adventitious shoot induction in 15 mg/L BA. In this report, the authors characterized a tetraploid like somaclone obtained from the triploid cultivar Williams (AAA) by adventitious shoots induction in high BA (15 mg/L) concentrations.

Since the application of flow cytometry for nuclear DNA estimations of *Musa* (8), this technique is becoming an excellent alternative for chimerism studies, genome composition and mutant selection. Roux *et al.* (9) used flow cytometry to follow a cytochimera dissociation process and for detection of aneuploidy in *Musa*. With this protocol had been possible to detect one single chromosome lose in *Musa* gamma irradiated mutants.

In this work we characterized with flow cytometry a population of *in vitro* plants obtained by adventitious shoots induction in high BA concentrations (20 mg/L). With this protocol for *in vitro* mutagenesis it was possible to obtain somaclones with nuclear DNA content variation as a source of new mutations for *Musa* improvement programs.

Materials and methods

The relative nucleic acid content (Rc) was estimated by flow cytometry in a population of 20 *in vitro* plants selected at random from 1,000 plants of *Musa acuminata* (AAA) cv. Williams obtained by adventitious shoots induction in 20 mg/L BA (7) at the fourth cycle of in vitro culture. A diploid *Musa acuminata* (AA) Pisang Mas (Titiaro) and tetraploid cultivar FHIA-02 (AAAA) were used as internal references to calibrate the flow cytometer in each run.

Nuclei were isolated from cells of banana leaves according to Dolezel *et al.* (8), and stained with propidium iodine (50 μg/mL) for 15 minutes. Rc was calculated measuring the propidium iodine fluorescence using a Becton Dickinson FacScan flow cytometer. The resulting histograms were generated using the ModFitLT v 3.0 software for Mac., and measurements modeled at least 5,000 to 10,000 nuclei.

Relative nucleic acid contents (Rc) were calculated using the following equation:

$$Rc = 2 (F_{xn}/F_{2n})$$

F_{xn} = maximum fluorescence of propidium iodine of the clone of unknown ploidy.

F_{2n} = maximum fluorescence of propidium iodine of the diploid reference Pisang Mas (AA).

At least two measures were performed in each plant (duplicates in two different nuclei preparations). A normal distribution test and comparison of means analysis were performed with the software Statistic version 5.5 (10).

Results and discussion

Somaclonal variation is a phenomenon that could be a very useful tool for genetic improvement. The knowledge of the genetic bases of somaclonal variation is an important issue in plant improvement by plant biotechnology. In this work was performed a nuclear DNA content analysis of *in vitro* plants that were obtained by adventitious shoots induction in sucker tips of Williams (AAA) in 20 mg/L BA. The distribution of relative DNA content (expressed as percentage of plants within statistical different intervals) was tested using the Kolmogorov-Smirnov test and found to be normal at 99% confidence level. Approximately 8% of *in vitro* plants induced in 20 mg/L BA have a relative DNA content between 3 to 3.25, 27% 3.25 to 3.5, 38% 3.5 to 3.75, 23% 3.75 to 4 and 4% 4.25 to 4.5 (figure 1 and table 1). No descendant aneuploids were detected in 20 plants analyzed selected at random from 1,000 *in vitro* plants. These results demonstrate that shoot induction in high concentration of BA (20 mg/L) from sucker tips of Williams increase the nuclear DNA content of *in vitro* plants to tetraploid levels or more(figure 1 and table 1). These results also demonstrate that it is possible to obtain tetraploid like somaclones from the triploid *Musa* (AAA) cv. Williams. A similar phenomenon was described by Giménez *et al.* (7, 11), they found variations at DNA content

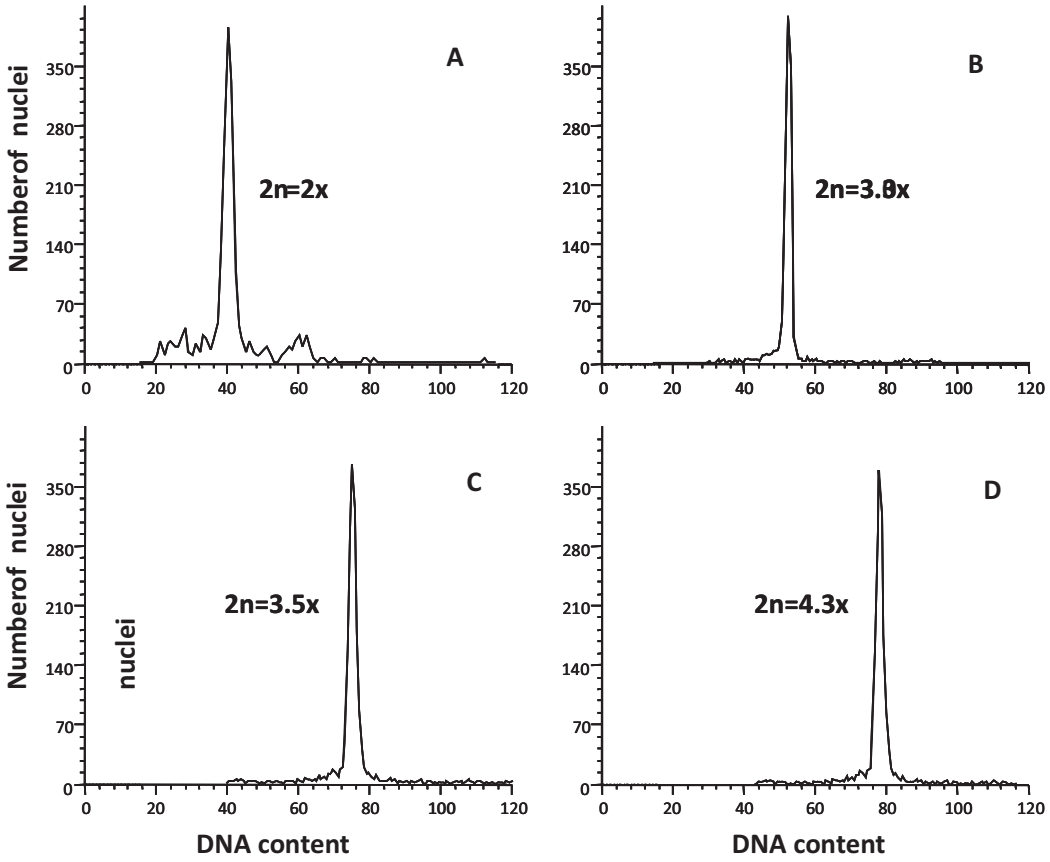

Figure 1. Flow cytometry histograms of relative nuclear DNA content. A: *Musa acuminata* (AA) cv. Pisang Mas 2n = 2x, B: *Musa acuminata* (AAA) cv. Williams 2n=3x, C: *Musa acuminata* somaclonal variant of cv. Williams 2n = 3.5x, D: *Musa acuminata* somaclonal variant of cv. Williams 2n = 4x. Acquired events: 5,000 -10,000 nuclei, % CV: 2.37- 4.0.

Table 1
Relative DNA Content of 20 plants selected at random from a population of 1,000 of *in vitro* plants, obtained from the triploid, *Musa acuminata* (AAA) cv. Williams cultivated in BA 20 mg/L Intervals Obtained by the Kolmogorov-Smirnov Test at 99% of confidence level

	Relative DNA content intervals				
	3-3.25	3.25-3.5	3.5-3.75	3.75-4	4.25-4.5
Number of plants	3	4	6	4	3
Mean± SE	3.2 ± 0.07	3.4 ± 0.03	3.7 ± 0.02	3.9 ± 0.03	4.3 ± 0.03
Percentage	8%	27%	38%	23%	4%

and sequence mutations in plants micropropagated from *Musa* (AAA) cultivar Williams, using 15 and 20 mg/L BA, with flow cytometry and selective amplification of microsatellite polymorphic loci (SAMPL) markers respectively. In Cotton, flow cytometry studies reveal a relative stable DNA content but high mutation rates in plants propagated with a combination of 2,4 Dichlorophenoxyacetic acid (2,4D) and Kinetin evaluated with single sequence repeats (SSR) and random amplified polymorphic DNA (RAPD) markers (12). However, Lakshmanan *et al.* (13), did not found any somaclonal variations evaluated by RAPD and SSR markers in the dessert banana cv. Nanjanagudu Rasabale (AAB) micropropagated with 10 mg/L of BA. Fusheng *et al.* (14), report similar results in micropropagated medicinal plant *Anoectochilus formosanus*, by axillary branching induced with 0.5 mg/L naphtalenacetic acid (NAA) and 1 mg/L BA.

Shepherd and Dos Santos (1) reported that some growth regulator such as cytokinins and auxins can affect the chromosomes number in *Musa* spp. The auxins NAA and 2,4D had been reported as polyploids inductors (15) and in banana the cytokinin BA is reported as an agent that causes aneuploids cells (16,1). Additionally, Shepherd and Da Silva (6) reported the presence of aneuploids and minichromosomes in triploids bananas propagated by conventional methods, and mosaic tissues with different chromosome numbers, descendent (32 chromosome) and ascendant aneuploids (34, 35, 36 and 37 chromosomes) but never a tetraploid like somaclone. These mosaic tissues are common in different triploid bananas and the percentage of cells with abnormal chromosome number increase when they are propagated by tissue culture in a media with 5 mg/L BA (1). Van Duren *et al.* (17) performed a comparison between tetraploid induction with oryzaline and colchicine from diploid *Musa acuminata*. Mutant characterization and selection were done with flow cytometry. In this report, they performed a flow cytometry analysis and the authors find mixoploids (2n=2x-4x), (2n=2x-6x), (2n=4x-8x) and solid normal and polyploidy plants (2n=2x), (2n=4x), and (2n=8x).

Roux *et al.* (18), report the use of DNA flow cytometry for quality monitoring of banana cell suspension cultures. Embryogenic suspensions from Williams tissues induced in 20 mg/L BA were very unstable. They found groups of cells with different nuclear DNA content; however they did not report tetraploids plants from these mutant cells suspensions.

More recently, Roux *et al.* (19) develop a protocol for rapid detection of aneuploidy in *Musa* using flow cytometry. In this report they were able to detect aneuploids with one or two chromosome less.

An average chromosome in a triploid banana represents 3% of the genome size

(19). This means that a DNA content increase of 33% could represent a chromosome number increase of approximately 11 chromosomes (from 33 to 44 chromosomes). Giménez *et al.* (7) reported an increment in the chromosome number from 33 to 44, in the somaclonal variant of Williams CIEN BTA-03 obtained with the same strategy used in this report but using 15 mg/L BA. However Lysák *et al.* (20) report that large variations in genome size (8.8%) were found among different triploid *Musa* accession without any chromosome number changes. Then, it is necessary to perform a chromosome count for these somaclones to make a correlation between nuclear DNA increase and chromosome increment.

In conclusion, the adventitious shoots induction from sucker tips of Williams (AAA) in 20 mg/L BA, promotes high levels of somaclonal variation, with high frequencies of somaclones with an increased relative DNA content even similar to tetraploid cultivars. This propagation protocol applied to Williams (AAA) cultivar could be useful to increase the narrow genetic variability of triploid commercial bananas for *Musa* improvement.

References

1. SHEPHERD K., DOS SANTOS J. ***Fruits*** 51: 5-11. 1996.
2. ROWE P. ***Plant Breed Rev*** 2:135-155. 1984.
3. NOVÁK F., AFZA R., VAN DUREN M., OMAR M. ***Trop Agr*** 67: 21-28. 1990.
4. VUYLSTEKE D., SWENNEN R., DE LANGHE E. ***Fruits*** 46: 429-439. 1991.
5. SANDOVAL J., COTE F., ESCOUTE J. ***In Vitro Cell Dev B*** *32:* 14-17. 1996.
6. SHEPHERD K., DA SILVA K. ***Fruits*** *51:* 99-103. 1996.
7. GIMÉNEZ C., GARCÍA E., XENA N., BLANCA I. ***In Vitro Cell Dev B*** 37(2): 217-222. 2001.
8. DOLEZEL J., DOLEZELOVÁ M., NOVÁK F. ***Biol Plant*** *36*: 351-357. 1994.
9. ROUX N., DOLEZEL J., SWENNEN R., ZAPATA-ARIAS F. ***Plant Cell Tiss Org Cult*** 66: 189-197. 2001.
10. STATSOFT, Inc., 1999. STATISTICA for Windows [Computer program manual]. Tulsa, OK: StatSoft, Inc., 2300 East 14th Street, Tulsa, OK 74104, email: info@statsoft.com, WEB: http://www.statsoft.com
11. GIMÉNEZ C., PALACIOS G., COLMENARES M., KAHL G. SAMPL. ***Plant Biol Rep*** 23: 263-269. 2005.
12. SHUANGXIA J., RAMESH M., HUAGUO Z., LILI T., ZHONGXU L., YANXIN Z., XIANLONG Z. ***Plant Cell Rep*** 27(8): 1303-1316. 2008.
13. LAKSHMANAN V., SREEDHAR R., NEELWARNE B. ***Plant Growth Regul*** 51:193-205. 2007.
14. FUSHENG Z., YALI L., HAILING D., SHUNXING G. ***Plant Biol Pharm Bull*** 33(3) 384-388. 2010.
15. D'AMATO F. ***Applied and fundamental aspects of plant cell, tissue and organ culture*** (Eds. Reinertard and Bajaj YPS) Springer Verlag, 150-180. 1977.
16. HESLOP-HARRISON J., SCHWARZACHER T. ***Ann Bot London*** 100: 1073-1084. 2007.
17. VAN DUREN M., MORPURGO R., DOLEZEL J., AFSA R. ***Euphytica*** 88: 25-34. 1996.
18. ROUX N., ALKHIMOVA O., STROSSE H., TOLOZA A., PANIS B., SAGI L., DOLEZEL J., SWENNEN R. ***3rd International Symposium on Molecular and Cellular Biology in Bananas***. 9-11. Leuven (Belgium) 2002. http://www.promusa.org/publications/publications.htm.
19. ROUX N., TOLOZA A., RADECKI Z., ZAPATA F. ***Plant Cell Rep*** *21*: 483-490. 2003.
20. LYSAK M., DOLEZELOVA M., HORRY J., SWENNEN R., DOLEZEL J. ***Theor Appl Genet*** *98*: 1344-1350. 1999.