RESEARCH

Genetic stability of micropropagated banana plants (Musa spp.) with non-traditional growth regulators

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ABSTRACT

Cytogenetic and molecular markers (isozymes and DNA) techniques are very important to monitor the genetic stability of the material obtained by *in vitro* culture, because in many occasions the micropropagated plants usually produce non-normal regenerating plants or somaclonal variants of the origin cultivars. The aim of this work was to evaluate the genetic stability of banana (*Musa* spp.) plants of the 'FHIA-18' (AAAB) clone obtained *in vitro*. The cytogenetic, isoenzymatic and RAPD analyses were carried out in plants at the late acclimatization phase and *in vitro* propagated with brassinosteroids analogues (Biobras-6- ABr) or oligogalacturonides mixture with polymerization grade between 9 and 16 (Pectimorf-mOLG). Plants cultured *in vitro* without ABr or mOLG treatment, but either under indolebutyric acid (IBA), indole acetic acid (IAA) or 6-bencyl aminopurine (6-BAP), were used as controls, and field-grown mother plants of this cultivar. Two additional treatments were also used, one applied to plants cultured *in vitro* under ABr during all the developmental phases, and the other one under mOLG. The results showed that the ABr and mOLG did not induce genetic variability in the regenerants obtained, remaining constant the chromosomes number of the specie (2n = 4x = 44). Twenty-nine bands were obtained with the isozymes and twenty-seven with RAPD, all monomorphic.

Keywords: acclimatization, banana, genetic stability, growth regulators

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RESUMEN

Estabilidad genética de las plantas de banano (*Musa* spp.) micropropagadas con reguladores del crecimiento no tradicionales. Las técnicas citogenéticas y de marcadores moleculares (isoenzimas y ADN) son útiles para verificar la estabilidad genética de material obtenido por cultivo *in vitro*, pues en muchas ocasiones en las plantas micropropagadas brotan regenerantes anormales o variantes somaclonales de las variedades de origen. El objetivo de esta investigación fue evaluar la estabilidad genética de plantas de banano (*Musa* spp.) clon 'FHIA-18' (AAAB) obtenidas *in vitro*. Se cometieron análisis citogenéticos, isoenzimáticos y técnicas de amplificación aleatoria del ADN polimórfico (RAPD), luego de la fase de aclimatización de plantas propagadas *in vitro*, con un análogo de brasinoesteroides (Biobras-6-ABr) o una mezcla de oligogalacturónidos, cuyo grado de polimerización estaba entre 9 y 16 (Pectimorf-mOLG). Como control se emplearon plantas del cultivo *in vitro* que no tenían ABr ni mOLG; pero que contenían ácido 3-indolbutírico (AIB), ácido 3-indolacético (AIA) o 6-bencilaminopurina (6- BAP), y plantas madre de esta variedad provenientes del campo. También se emplearon dos tratamientos adicionales: en uno, las plantas procedían del cultivo *in vitro* con el ABr en todas sus fases, y en el otro, se empleó la mOLG. Los resultados mostraron que estos tratamientos no indujeron variabilidad genética en los regenerantes, ya que el número de cromosomas de la especie se mantuvo constante (2n = 4x = 44). Con las isoenzimas se obtuvieron 29 bandas y con los RAPD, 27 bandas; todas monomórficas.

Palabras clave: aclimatización, banano, estabilidad genética, reguladores del crecimiento

*I*ntroduction

Organogenesis is one of the plants regeneration methods using *in vitro* culture. It is widely used in micropropagation, genetic transformation and plants development studies [1]. In the last decade of the last century a great progress was made in the field of plant genetics, molecular biology, cell cycle and signal transduction of cytokinins and auxins [1, 2]. There were also advances in the knowledge of morphological and physiological aspects of *in vitro* organogenesis and also in the molecular mechanisms governing this response. Besides, the relationships between auxins cytokinins and other growth promoting substances like brassinosteroids and their analogues, as well as oligogalacturonides, are being actively although slowly investigated [2-5].

Occasionally, the growth regulators incorporated into the plant culture medium promote genetic or epigenetic changes. In this regard, the synthetic auxin known as 2,4-dichlorophenoxyacetic acid (2,4-D) is often employed for callus induction. Nevertheless, 2,4-D is the main responsible for genetic and epigenetic variation [6] in some tissues, so it is not recommended in plants massive micropropagation protocols. Alternative like the use of cytokinins such as zeatin, kinetin and 6-benzylaminopurine (6-BAP) may also promote chromosomal aberrations at high concentrations in the culture media [7, 8].

Therefore, brassinosteroid analogues (ABr) and the oligogalacturonides mixture (MOGs) can be used as substitutes of auxins and cytokinins in several biotechnological processes [9, 10]. These non-traditional growth regulators are conveniently used in different micropropagation steps of banana and plantain (*Musa* spp.). It has also been reported that Biobras (6-ABr) attenuated the stress to high temperatures in banana 'FHIA-18' during the acclimatization phase [11] and favored root formation *in vitro* during the rooting stage of Banana 'FHIA-21' [12]. Moreover, the

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Pectimorf (mOLG) reduced the output time of the 'Sobrino' plantain clone explants during its *in vitro* establishment [13].

The *ex vitro* growth of plants obtained by somatic organogenesis or embryogenesis results from treatments applied at the *in vitro* propagation stage [14, 15]. However, regeneration of many plant species is not usually yet an efficient process.

The application of ABR and its analogues or mOLG stimulate several plant growth processes in *ex vitro* conditions, and reduce the abiotic stress induced *in vitro* by different culture techniques [10, 11, 16]. It has been reported [15] that the success of acclimatization in plantain and banana (*Musa* spp.) depends on plants transition from heterotrophic or mixotrophic conditions (mixture of autotrophic to heterotrophic) to autotrophy, a process related to the reserves obtained during *in vitro* culture.

Identification of genetic variability in plants obtained *in vitro* by morphological markers, although the most accepted technique, is not the only one employed due to its slowness. Hence, cytogenetic [17], isozyme [18] and molecular methods such as random amplification of polymorphic DNA (RAPD) [19] have become more effective and are widely used to assess the genetic stability of the regenerants. Additionally, they are commonly used in combination to evaluate the genetic variability of several crops including plantain and banana (*Musa* spp.) [19, 20], mandarin (*Citrus reshsni* Hort. Ex Tan) [21], sweet potato (*Ipomoea batatas* L.) [22] and pineapple (*Ananas comosus* [Lindley] Coppens and Leal) var. Bracteatus [23], among others.

Since the possible impact of using nontraditional growth regulators (ABr and mOLG) at all stages of the *in vitro* plant propagation and in further *ex vitro* cultivation remains to be elucidated, this study was aimed at evaluating the genetic stability of banana plants (*Musa* spp.) cultivar 'FHIA-18' (AAAB) obtained by micropropagation *in vitro* when exposed to these nontraditional growth regulators, by combining cytogenetic, isozyme and molecular techniques.

Materials and methods

Banana plants varieties, farming techniques and treatments

For these experiments, 45-days-old banana plants of the FHIA-18' (AAAB) variety coming from the acclimation stage were used. These plants were first *in vitro* propagated at various concentrations of three traditional growth regulators (3-indole butyric acid, AIB; 3-indoleacetic acid, AIA and 6-BAP) or the nontraditional brassinosteroid analogue ABr or a mOLG of α-1,4-oligogalacturonides (Pectimorf-mOLG). Nontraditional growth regulators were only applied during the acclimatization phase.

The ABr formulation contains as active ingredients the brassinosteroid spirostanic analogue 25(R)-2α3α-dihydroxy-5α-spirostan-6-one, known as 6-ABr (Center for Natural Products Research, School of Chemistry, University of Havana, Cuba). The general formula of this compound is $C_{27}O_5H_{42}$, with molar mass of 446.606 g/mol. The mixture known as Pectimorf-mOLG with polymerization degree between 9 and 16, was obtained in the Oligosaccharines Laboratory (Department of Plant Physiology and Biochemistry, National Institute of Agricultural Sciences, Cuba). It was generated from pectin contained in bark of Persian lime (*Citrus latifolia* Tan) fruits with an average molar mass of 2042 g/mol.

The following treatments were performed during *in vitro* culture: Control (setting: 0.015 μmol/L AIB + 17.77 μmol/L 6-BAP; multiplication: 3.71 μmol/L AIA + 17.77 μmol/L 6-BAP and rooting: 7.42 μmol/L AIA); ABr (setting: 17.77 μmol/L 6-BAP + 0.02-0.01 μmol/L ABr, multiplication: 17.77 μmol/L 6-BAP + 0.1-0.2 μmol/L ABr and rooting: 0.1 μmol/L ABr) and mOLG (setting: 0.015 μmol/L AIB + 0.47 μmol/L mOLG; multiplication: 17.77 μmol/L 6-BAP + 0.47-2.35 μmol/L mOLG and rooting: 2.35-4.70 μmol/L mOLG).

For culture media preparation, the previously described salts were used [24], supplemented with 0.30 μmol/L thiamine, 15 μmol/L myoinositol, 87.642 μmol/L sucrose and 6.5 g/L agar as a gelling agent (establishment and *in vitro* multiplication phases).

In the *ex vitro* acclimatization phase, the following treatments were evaluated: T_1, mother plants from the field, vigorous and without visible symptoms of viral, fungal or bacterial diseases (as controls); T_2, plants from *in vitro* culture (obtained with AIB, AIA and 6-BAP), the roots of the plantlets were immersed in 0.1 % Ridomil (commercial product Ridomil MZ 72 %, with both, systemic and contact action); T_3, plants obtained *in vitro* with ABr; and T_4, plants obtained in vitro with mOLG. The first treatment was not included in the plants cytogenetic study.

Before planting, the roots of plants treated with ABr and mOLG were immersed in 0.2 μmol/L ABr and 0.47 μmol/L mOLG solutions in T_3 and T_4, respectively, and 15 days after planting, plants were sprayed with 2 mL of the same concentrations of these growth regulators per vitroplant.

Planting was done on a 70 alveoli (dimensions: 5 × 5 × 5 cm; 125 cm^3) polystyrene seed trays containing a substrate made by volume mixture 75 % organic material (decomposed cachaza) plus 25 % of red ferralitic compacted soil. Trays were then transferred to green houses covered with a 30 % mesh polypropylene (photosynthetic photon flux density of 600 μmol/m^2s), under semi-controlled conditions. Irrigation was done by microaspersion, using a Microjet® system (2 kg/cm^2 and 120 L/h flow rate) with a frequency of 4 watering daily for 2 min each during the first 7 days (control) to achieve a 90 % relative humidity.

Radicular cytogenetic analysis

Roots karyotyping was done from 1 cm-long root tips. These were randomly selected at the beginning (day 0) and at the end (45 days) of the acclimatization phase, as described by Roman and Rodriguez Nodals [25].

Five plants were randomly selected. Chromosomes from 25 cells per plant for each treatment were counted with the use of an optical microscope (Olympus, Japan) with an attached camera (Canon, Japan). The best metaphases were photographed with a 1000× magnification.

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Isoenzymic analysis

Extracts preparation for the electrophoretic analysis of carbonic anhydrase (CA), esterases (Est), peroxidases (POX), and polyphenol oxidases (PPO) isozymes was performed according to Roman [26]. Leaves (5 g) were macerated in liquid nitrogen using 0.1 M sodium bicarbonate, pH 7.2, as the extraction buffer.

Samples were then centrifuged at 6000 rpm for 15 min, at 4 °C in a refrigerated Eppendorf centrifuge 5804 R (Eppendorf, Germany). Subsequently, supernatants were filtered through double gauze and were finally packed in 1.5 microcentrifuge tubes mL and stored at -20 °C until use.

Electrophoretic runs for isozyme systems were performed in a discontinuous polyacrylamide gel system [27]. For this purpose, a 10 % Tris-HCl 1.5 M, pH 8.8, separator gel, and a 4 % stacking gel buffer at 0.125 M Tris-HCl, pH 6.8, were used.

The electrophoresis running buffer was 0.025 M Tris, 0.019 M glycine, pH 8.3. The run time was determined by the displacement of the Kolrhauch band to about 6 cm from the starting electrophoresis point. The electrophoresis device was a SE 260 Mini-Vertical (Hoefer ®, Pharmacia Biotech, Germany) at a constant current Intensity of 20 mA per electrophoresis run. On each run, extract samples were applied at 15 µL per well.

Once the electrophoretic separation concluded, phytochemical stains were performed specific for each of the evaluated isozyme systems: CA (EC 4.2.1.1) [28], Est (EC 3.1.1.1) [29], POX (EC 1.11.1.7) [30] and PPO (EC 1.10.3.1) [31].

RAPD molecular analysis

The RAPD technique was performed on plant material from five randomly selected treatments. The last leaf of each plant was used at the end of the acclimatization phase, selected by measuring from the base of the pseudostem, and the cigar leaf of field plants with no visible disease symptoms. DNA extraction was performed according to Dellaporta *et al*. [32].

DNA quality was determined by 0.8 % agarose gel electrophoresis in TBE 1× buffer (45 mM Tris-Borate, 1 mM EDTA, pH 7.0), stained with ethidium bromide (5 mg/mL) and observed in a transilluminator (Bioblock Scientific, France). Final DNA concentration was estimated by measuring the optical density at 260 nm in an Ultrasepec Plus Spectrophotometer (Pharmacia LKB, England) spectrometer. The amplification reaction was carried out in 25 mL as final volume containing: 10 mM Tris- HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.001 % gelatin, 100 µM each dNTP, 5 pmoles of primer (Kits OPA and OPF, Operon Technologies Inc., Alameda, California), 50 ng of genomic DNA and 1 U of Taq DNA polymerase (Promega). Six arbitrary primers were used: OPA-04 (5'-AATCG GGCTG-3'), OPA-10 (5'-GTGATCGCAG-3'), OPA-11 (5'-CAATCGCC GT-3'), OPA-13 (5'-GGCTGCA GAA-3'), OPF-04 (5'-GGTGATCAGG-3') and OPF-13 (5'-GGCTGCA GAA-3') [20, 33].

DNA was amplified using a Progene (Techne, USA) thermocycler programmed for 45 cycles of 1 min at 94 °C , followed by 1 min at 36 °C and 2 min at 72 °C , and one cycle of 10 min at 72 °C. PCR products were electrophoresed in 1.5 % agarose gels in TBE 1× buffer, further stained with ethidium bromide (5 mg/mL) and visualized in UV transilluminator (Bioblock Scientific, France).

Resulting PCR amplified bands from donor plants and those coming from *in vitro* culture plants were binary evaluated for their presence (1) or absence (0). Results were expressed in percentage of monomorphic bands.

Results and discussion

Radicular cytogenetic analysis

It was verified during the karyotyping studies that the use of ABr and mOLG kept constant chromosome number: 2n = 4x = 44, in all micropropagation stages of banana clone 'FHIA-18' (AAAB) (Figure 1).

Ploidy variations are not usually evident for more than 20 *in vitro* cultivation cycles for two years by combined cytogenetic and flow cytometry studies. This was evidenced when the number of chromosomes (22) remained constant in banana varieties 'Kluai Sa' (AA) and 'Leb Mue Nang Kluai' (AA) [34].

Chromosomal changes have been observed in *Fragaria* spp. cultured *in vitro*, especially in plants from callus and *in vitro* cells maintained under long term storage [35]. Variations in the ploidy level of *in vitro* culture may result from the relationship between the genetic constitution of the cultured species and culture medium composition [36].

After using the growth regulators mOLG and ABr in all micropropagation stages, no chromosomal mosaics were detected, either 2n = 2x = 22 chromosomes (diploid) or 2n = 3x = 33 chromosomes (triploid). These chromosomal mosaics have been described in the accelerated micropropagation clones of *Musa* spp. [37] and cause genetic instability.

Isoenzyme analysis

The isoenzyme study for the evaluated systems using ABr or mOLG showed monomorphic patterns and did

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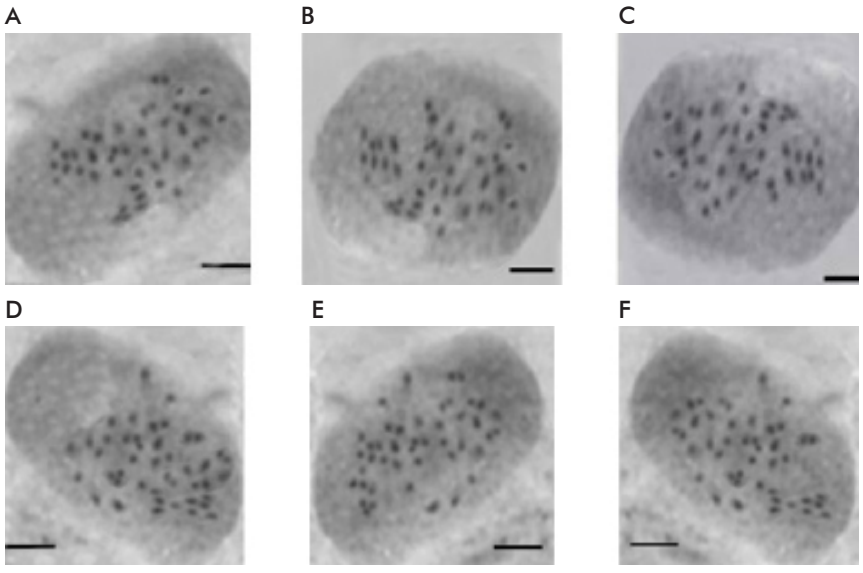

Figure 1. Cells from banana plants (*Musa* spp.) clone 'FHIA-18' (AAAB) in mitotic metaphase in the control treatment (start: A and end: D), under ABr treatment (starting: B and end: E) at all stages and mOLG (star: C and end: F) during acclimatization, with chromosome number 2n = 4x = 44. The bar represents 25 microns (1000×).

not induce any variations relative to the control plants, suggesting that these growth regulators did not influence the genetic stability of micropropagated materials (Figure 2).

In the CA and PPO systems, eight bands were visualized each. There were six bands for the POX system (bands of the second and sixth genetic system were thicker), and seven in the PPO system. These bands are characteristic of the clone 'FHIA-18' (AAAB), genus *Musa*, characteristics of plants predominantly with an *acuminata* genome [26].

Additionally, Est and PPO isozymes are among the most commonly used for plant species characterization, since they display a high degree of reproducibility and polymorphism, particularly in *Musa* spp. genus cultivars. However, in this study no polymorphism was observed in isozyme systems.

Other authors found complete stability in potato plants (*Solanum tuberosum* L.) with the POX CA and acid phosphatase isozyme systems [18], obtained from callus and cultured at different concentrations of the brassinosteroids analogues ABr-6 MH-5-6 and Pectimorf-mOLG.

The results obtained for the isozyme systems (CA, Est, POX and PPO) for banana (*Musa* spp.) clone 'FHIA-18' showed no genetic variability, since the 29 analyzed bands were monomorphic, corroborating its correct use to determine the genetic variability and stability on this genus, as some authors have recommended [26]. Furthermore, the results of the cytogenetic study were corroborated.

Molecular analysis using RAPD

From the two analyzed kits, four primers were selected based on the quantity, quality and reproducibility of the amplified bands. The most informative were the OPA-10 and OPF-13 that amplified eight bands each, followed by OPA-04 with seven and OPA-11 with four amplified bands.

These four arbitrary sequence primers amplified 27 bands ranging about 7 bands per primer. All bands matched perfectly with the DNA of the field donor plant previously cultured *in vitro*, and for all the 'FHIA-18' hybrid regenerants obtained after treatment with growth regulators (ABr and mOLG) at all micropropagation stages. The detected bands were 100 % monomorphic, indicating that the use of ABR and the mOLG in all micropropagation phases caused no variation in the hardened plants of this banana genotype.

Molecular studies with RAPD in *Musa* spp. are currently used for variety identification, genetic mapping of useful clones and location of genes conferring resistance to major diseases that affect these crops [19, 33]. In this research, RAPD was used to detect *Musa* spp. somaclonal variants caused by tissue culture. Other groups (for example, Harirah and Khalid [38]) have obtained monomorphic profiles, so concluded that the micropropagation of *Musa acuminata* cv. Berangan from male flowers did not cause somaclonal variation.

The combination of two molecular techniques to study variations generated by *in vitro* culture techniques, allow the evaluation of a greater percentage of genomic loci. In this regard, some researchers have described the use of RAPD markers and intersimple sequence repeats (ISSR) to examine genetic variation in plants of banana (*Musa* spp. var. 'Nanjanagudu Rasabale' AA) micropropagated for 10 years [20]. They obtained uniform band patterns, and samples were devoid of polymorphic bands in spite that morphologically different plants were included.

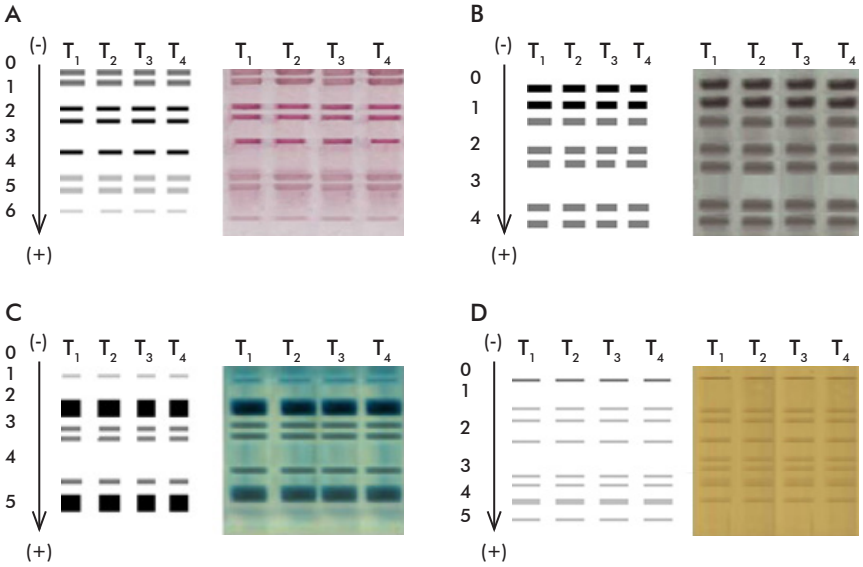

Figure 2. Isoenzymatic evaluation of banana plants (*Musa* spp.) clone 'FHIA-18' (AAAB) obtained with the use of brassinosteroid analogue (ABr) and oligogalacturonides mix (mOLG) by isozyme systems during the acclimatization phase. A) Carbonic anhydrase. B) Esterases. C) Peroxidases. D) polyphenoloxidases. The following treatments were applied: - T1, control of mother plants from field; T2, plants grown *in vitro* - control seedling without dipping the roots for 15 min before planting (I) or no foliar spray during 15 days after planting (AF); T3: plants obtained *in vitro* with ABr (0.2 µmol/L) and I + AF; T4, plants obtained *in vitro* with immersion + mOLG (0.47 µmol/L) AF.

However, other authors have found a high polymorphism in regenerants from *in vitro* culture with the single use of RAPD in *Musa* spp. [19]. Also, a high percentage of monomorphism or complete genetic stability has also been described on crops such as *Ipomoea batatas* (L.) Lam. clone 'INIVIT B 93-1' [22], *Ananas comosus* var. 'Bracteatus' (Lindley) Coppens and Leal [23], and *Centaurea ultreiae* Silva Pando [39]. Also with this technique, a high rate of polymorphism (93.19 %) in medicinal plants has been reported [40].

Results with RAPD molecular markers for the analyzed primer combinations indicated no intra- or inter-population genetic variation in the four studied populations of 'FHIA-18' banana plants, regardless its origin (mother plants from the field, cultured *in vitro* with AIB, AIA and 6-BAP, or generated *in vitro* and treated *ex vitro* with ABr or mOLG, respectively).

Conclusions

The genetic stability of banana (*Musa* spp.) 'FHIA-18' was corroborated in regenerant clones after using ABr and mOLG during the *in vitro* and *ex vitro* plant propagation. It was confirmed that the chromosome number of the specie remained constant and they were stable, as determined by the cytogenetic, isozymes and RAPD techniques. This was verified by the identical band patterns displayed by progenitor

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