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RESEARCH

Phenotypic and genetic stability of tobacco plants derived from cryopreserved seeds

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ABSTRACT

The examination of genetic stability is one of the most important issue in the evaluation of cryopreservation techniques. This research was aimed at determining if there are changes at the phenotypic and molecular level in tobacco plants derived from cryopreserved seeds. The seeds of the cultivar "Sancti Spíritus 96" were harvested 35 d after anthesis, dried to a water content of 2.1 % and kept for one year in liquid nitrogen (–196 °C) or at 5 °C. In the seedbed phase, the morphological characterization was carried out at 20, 30 and 40 d after sowing and three growth indices were calculated from the dynamics between 20 and 40 d. In the field phase, the morphological characterization was carried out 65 d after transplantation. The tobacco plants derived from each treatment did not show morphological differences, both in seedbed phase and in field phase. In addition, they did not show significant differences in the growth rates determined in the seedling phase. The genetic stability of the seedlings was evaluated by means of RAPD markers. No polymorphism was detected between the DNA from the plants derived from each treatment. In this way, both the base and duplicate collections can be conserved in liquid nitrogen, with which the tobacco germplasm will be available for long-term use by plant breeders in obtaining new cultivars.

Keywords: growth rates, morphological characterization, *Nicotiana tabacum* L., RAPD markers

RESUMEN

Estabilidad fenotípica y genética de plantas de tabaco derivadas de semillas crioconservadas. El examen de la estabilidad genética es uno de los temas más importantes en la evaluación de las técnicas de criopreservación. Esta investigación estuvo dirigida a determinar si existen cambios a nivel fenotípico y molecular en las plantas de tabaco derivadas de semillas crioconservadas. Las semillas del cultivar "Sancti Spíritus 96" se cosecharon a los 35 d después de la antesis, se secaron hasta un contenido de agua de 2.1 % y se conservaron por un año en nitrógeno líquido (–196 °C) o a 5 °C. En la fase de semillero, se realizó la caracterización morfológica a los 20, 30 y 40 d después de la siembra y se calcularon tres índices de crecimiento a partir de la dinámica entre los 20 y 40 d. En la fase de campo, la caracterización morfológica se realizó a los 65 d después del trasplante. Las plantas de tabaco derivadas de cada tratamiento no mostraron diferencias morfológicas, tanto en semillero como en campo. Además, no mostraron diferencias significativas en los índices de crecimientos determinados en la fase de semillero. La estabilidad genética de las plántulas se evaluó por medio de marcadores RAPD. No se detectó polimorfismo entre el ADN procedente de las plantas derivadas de cada tratamiento. De esta forma, tanto la colección base como la duplicada podrán conservarse en nitrógeno líquido, con lo cual el germoplasma de tabaco estará disponible para su uso a largo plazo por los fitomejoradores en la obtención de nuevos cultivares.

Palabras clave: caracterización morfológica, índices de crecimiento, *Nicotiana tabacum* L., marcadores RAPD

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Introduction

Cuban tobacco is a world leader for its quality, purity and combination of knowledge that authenticates it. This is due to the knowledge acquired through generations, to the favorable climatic and edaphological conditions for the crop and to the adequate varietal strategy [1]. The basis of this strategy is the collection of *Nicotiana* spp. present in the germplasm bank of the Tobacco Research Institute of Cuba.

Since the 1980's, cryopreservation research has been carried out worldwide as an alternative to traditional seed storage methods [2-5]. Cryopreservation techniques regularly use liquid nitrogen (LN) (–196 ºC) due to their relatively low cost. The objective is to reach temperatures below –130 ºC to achieve conditions of low molecular kinetic energy and extremely slow diffusion, so that chemical reactions are practically paused [6]. Under these conditions, extreme seed longevities are postulated.

Based on ten-year storage experiments and from the viability equation, Walters [7] predicted a longevity of 3400 years for *Lactuca sativa* L. seeds stored at –196 ºC. Therefore, cryopreservation can be very useful in the conservation of basic and duplicate collections in the seed germplasm banks, without leaving aside the classical conditions, useful for short-term storage (active collection) [8].

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Despite the effects on the genome are little known, it is considered that in regenerated plants of material from *in vitro* and cryopreserved cultures, a certain level of DNA polymorphism is induced due to a complete process of culture-cryopreservation-regeneration and not cryopreservation *per se* [9]. In fact, there are no references to genetic instability of plant material obtained from cryopreserved seeds. However, the analysis of the genetic integrity of plants derived from cryopreserved material is troublesome and have to be performed at phenotypic, histological, cytological and molecular levels following different analysis techniques [10, 11].

Based on these backgrounds, this work was aimed to determine the phenotypic and genetic stability of tobacco plants obtained from one year-cryopreserved seeds.

Materials and methods

Plant material

The research was carried out in the Tobacco Experimental Station of Cabaiguán, latitude 22°25'N, longitude 79°32'W and altitude of 134 m above sea level belonging to the Tobacco Research Institute of Cuba. In the study, seeds of cultivar "Sancti Spíritus 96", of wide use among the farmers of the central region of Cuba, were used for the experiments.

The seeds were collected 35 d after anthesis and with a water content of 2.1 %, and further divided into two batches of 1.5 g each. One batch of seeds was placed in 1.5 mL cryo-vials, immersed in LN and stored at –196 ºC for one year (+LN). The other batch was kept at 5 ºC for the same time (-LN). Afterwards, the seeds of both treatments were thermally bathed at 40 °C for 5 min. They were sowed in trays with 264 alveoli and containing substrate composed of 70 % cachaza (v/v), 25 % rice husk (v/v) and 5 % zeolite (v/v) [12], based on the technology of postures production in floating trays as described by García and Andino [13].

Phenotypic analyses

In seedbed , the morphological characterization was performed on ten seedlings randomly selected for each replication (30 per treatment), following the methodology proposed by Torrecilla *et al*. [14]. For each seedling, leaf color, apex shape, leaf base shape and stem color were evaluated as qualitative morphological descriptors, at 40 d after seed sowing. Stem diameter, plant height, leaves number, length of main leaf, width of main leaf and plant fresh mass were determined as quantitative morphological descriptors, at 20, 30 and 40 d after seed sowing.

Moreover, three growth rates were calculated in ten seedlings selected at random for each replication (30 per treatment), from the dynamics between 20 and 40 d, according to the methodology proposed by Hunt *et al*. [15]. They were biomass increase (ΔM), Absolute growth gate (AGR) and Relative growth rate (RGR), calculated by the following formulae:

$$\Delta M\ (g) = M_{40} - M_{20}$$

Where:
M_{40}: plant dry mass 40 d after sowing.
M_{20}: plant dry mass 20 d after sowing.

$$AGR\ (g/d) = \frac{M_{40} - M_{20}}{T_{40} - T_{20}}$$

Where:
M_{40}: plant dry mass 40 d after sowing.
M_{20}: plant dry mass 20 d after sowing.
T_{40}-T_{20}: time interval between initial measurement (20 d) and final measurement (40 d).

In the case of RGR, it expresses the amount of dry mass produced per unit of dry mass present per unit of time. It was determined by the expression:

$$RGR\ (g/\ g/d) = \frac{2\ (M_{40} - M_{20})}{(M_{40} + M_{20})(T_{40} - T_{20})}$$

Where:
M_{40}: plant dry mass 40 d after sowing.
M_{20}: plant dry mass 20 d after sowing.

T_{40}-T_{20}: time interval between initial measurement (20 d) and final measurement (40 d).

The transplant to the field was carried out 45 d after the seeds were sown, in plots of 30 plants with a completely randomized design with three replicates. The planting distance was 30 cm between plants and 90 cm between rows. Fertilization, irrigation and phytosanitary care were carried out as established in the technical instructions for cultivation [16].

In this phase, ten plants were selected at random for each plot, without considering in the selection the plants sown on the edges (marginal) . For each selected plant, flower color, corolla shape, corolla tube shape, relationship stamen stigma, leaf color, leaf base shape, leaf ripples and plant habit were evaluated as qualitative morphological descriptors. In addition, as quantitative morphological descriptors, plant diameter, plant height, time to flower, stem diameter, length of main leaf, width of main leaf, plant fresh mass, plant dry mass, flower length, flower width, capsule length, capsule width, capsule dry mass and capsule fresh mass were determined. Both qualitative and quantitative morphological descriptors were determined according to the methodology described by Torrecilla *et al*. [14].

RAPD analysis

Genomic DNA was extracted 25 d after the seeds were sown from ten seedlings selected at random by treatment. The protocol described by Doyle and Doyle [17] was developed with the modifications proposed by Valdés [18] using extraction buffer based on the cetyl trimethylammonium bromide (CTAB) protocol.

The integrity of the isolated DNA was determined by electrophoresis in 0.8 % agarose gels in TBE 1× buffer (0.04 mol/L Tris HCl; 1 mmol/L EDTA; 0.04 mmol/L H_3BO_3; pH 8.0), run at 100 V and stained with 5 mg/mL ethidium bromide. The DNA was visualized under ultraviolet light (UV; High performance Ultraviolet Transiluminator UVP) and the DNA concentration

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was visually estimated in comparison against the 100 pb ladder molecular weight marker (Promega).

RAPD primers OPA-3, OPA-7, OPA-8, OPA-10, OPA-11, OPA-16 and OPH-6 (Operon Technologies, Alameda, CA, USA) were evaluated according to the procedure described by Alfonso *et al*. [19]. Reactions were performed in a final volume of 25 µL containing 1× buffer (10×; 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8)), 0.4 mmol/L dNTPs, 1.5 mmol/L $MgCl_2$, 20 ng of genomic DNA, 0.4 µmol/L primer, (Promega) and 1 U Taq polymerase (5 U/µL) (Promega). The polymerase chain reaction (PCR) were developed in a TECHNE model TC-3000 thermal cycler, using the following amplification program: an initial cycle of 94 ºC for 5 min, followed by 5 cycles at 94 ºC (30 s), 36 ºC (30 s) and 72 ºC (1 min), with a final cycle of extension at 72 ºC for 5 min. In each amplification experiment, two PCR repeats per primer were performed.

Amplification products were separated into 1.5 % agarose gels in 0.5× TBE buffer and run at 100 V. The bands were visualized by staining with 5 mg/mL ethidium bromide under UV [20]. The size of the amplified fragments was determined by comparison with a DNA molecular mass marker of 1 kb plus (Invitrogen, USA). The number of total bands and the presence or absence of polymorphic bands were counted.

Statistical analysis

The Statistical Package for Social Sciences (Version 11.5 for Windows, SPSS Inc.) was used in the statistical processing of quantitative data. The adjustment to the normal distribution of the data for each treatment (Kolmogorov-Smirnov) and the homogeneity of the variances (Levene) were checked. Analyses for the different variables were performed through t test and one-way ANOVA as parametric analysis.

Results

The analysis of the state of the four qualitative descriptors determined at 4 d after sowing showed no morphological differences by comparing seedlings obtained from cryopreserved seeds with those regenerated from seeds preserved at 5 ºC. Similarly, during the development of seedlings at the seedling phase, no significant differences were seen in the seven quantitative descriptors determined, when comparing seedlings obtained from seeds exposed to both treatments, for each time evaluated after sowing (Table 1).

Table 2 shows the values of calculated growth rates indexes for tobacco seedlings, obtained from seeds cryopreserved for one year or stored at 5 ºC for the same period, between 20 and 40 d after sowing. There were no significant differences between treatments. Of special interest were the values obtained for RGR, suggesting similar growth intensity between 20 and 40 d, in agreement with the values of the quantitative descriptors determined.

In the field, a morphological comparison was performed between plants derived from seeds conserved by both methods. Eight qualitative descriptors and 14 quantitative descriptors were determined (Table 3). Nevertheless, as in the seedbed phase, no differences were observed between the descriptors determined for each treatment.

Table 1. Quantitative morphological descriptors determined 20, 30 and 40 d after sowing in tobacco seedlings obtained from seeds collected 35 d after anthesis, dried to 2.1 % water content and cryopreserved for 1 year (+LN) or stored at 5 °C for the same period (–LN)*

Descriptors	20 d		30 d		40 d		ET
	+LN	–LN	+LN	–LN	+LN	–LN	
Stem diameter (cm)	0.80 d	0.88 d	2.47 c	2.52 bc	2.71 a	2.64 ab	0.06
Plant height (cm)	8.10 c	9.18 c	42.57 b	44.59 b	110.84 a	111.43 a	2.91
Leaves number (cm)	5.05 b	5.31 b	6.55 a	6.68 a	6.80 a	6.88 a	0.01
Length of main leaf (cm)	2.04 c	2.46 c	8.32 b	8.48 b	12.25 a	11.98 a	2.77
Width of main leaf (cm)	1.46 c	1.66 c	4.40 b	4.46 b	5.53 a	5.51 a	1.15
Plant fresh mass (g)	0.092 c	0.133 c	1.130 b	1.222 b	2.603 a	2.634 a	0.08
Plant dry mass (g)	0.002 c	0.003 c	0.047 b	0.050 b	0.128 a	0.133 a	0.004

* Descriptors were selected according to Torrecilla *et al*. [14]. Different letters in the same row stand for statistically significant differences. One-way ANOVA, $p \leq 0.05$, n = 30. For statistical processing, data from variable leaves number were transformed according to $y' = SQRT(y)$. ET, total standard error. All the descriptors were statistically significant.

Table 2. Growth rates indexes calculated between 20 and 40 d after sowing in tobacco seedlings obtained from seeds collected 35 d after anthesis, dried to 2.1 % water content and cryopreserved for 1 year (+LN) or stored at 5 °C for the same period (–LN)*

Treatments	Biomass increase (ΔM; g)	Absolute growth rate (AGR; g/d)	Relative growth rate (RGR; g/ g/d)
+LN	0.1262	0.0063	0.0964
–LN	0.1300	0.0065	0.0952
ET	0.0055	0.0003	0.0002

* One-way ANOVA, $p \geq 0,05$, n = 30. ET: Total standard error. The three parameters showed no statitical significance.

Table 3. Quantitative morphological descriptors determined in field phase in tobacco plants obtained from seeds collected 35 d after anthesis, dried to 2.1 % water content and cryopreserved for 1 year (+LN) or stored at 5 °C for the same period (-LN)*

Descriptors	Treatments		ET
	+LN	–LN	
Plant diameter (cm)	75.82	76.30	1.14
Plant height (cm)	129.68	130.70	1.27
Time to flower (d)	62.18	61.23	0.04
Stem diameter (cm)	1.91	1.93	0.03
Length of main leaf (cm)	43.11	43.69	0.51
Width of main leaf (cm)	24.38	21.55	0.44
Plant fresh mass (g)	103.14	106.23	2.93
Plant dry mass (g)	14.08	14.10	0.37
Flower length (cm)	6.57	6.54	0.05
Flower width (cm)	2.72	2.68	0.04
Capsule length (cm)	2.12	2.06	0.02
Capsule width (cm)	1.40	1.43	0.01
Capsule dry mass (g)	3.72	3.83	0.06
Capsule fresh mass (g)	1.10	1.12	0.02

* Descriptors were established according to Torrecilla et al. [14]. Student's t test, $p \leq 0.05$, n = 30. Only for statistical processing the data Time to flower were transformed according to $y' = SQRT(y)$. ET, total standard error. None of the descriptors showed statistical significance.

RAPD profiles were determined for seedlings regenerated from cryopreserved seeds to evaluate their genetic fidelity, and compared with those of seedlings obtained from seeds preserved at 5 ºC (Figure A and B). The seven primers produced up to 39 strong, clear and reproducible bands. The number of bands for each primer ranged from 3 to 9, for an average of 5.6 bands per primer. No polymorphic bands were detected when comparing plants regenerated from cryopreserved seeds with those obtained from seeds preserved at 5 ºC.

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Discussion

Due to its economic relevance, the cultivation of tobacco in Cuba occupies a fundamental place and is one of the lines that contributes the most foreign currency to the country [21]. Given the new prevailing climatic conditions, it is paramount to preserve the variability of the genus in the long term, as to introduce genes into commercial varieties of *N. tabacum*. In this context, cryopreservation can be of great use in the long-term conservation of plant germplasm.

Phenotypic and genetic stability are among the most important concerns in plants regenerated from cryopreserved material [11, 22]. There is little information on phenotypic variation due to cryopreservation and it only refers to *in vitro* cultivation.

Fukai *et al*. [23] reported phenotypic alterations in the flower coloration of *Dendranthema grandiflorum* (Ramat.) Kitam after regenerating 106 cryopreserved apexes. Similarly, Medina *et al*. [24] found differences in the yield of *Fragaria ananassa* Duch fruits when studying 50 plants obtained from cryopreserved apexes. However, for species such as *Dioscorea floribunda* Mart. and Gal. [25], *Saccharum* sp. [26], *Carica papaya* L. [27, 28], *Argyranthemum* [29] and *Chrysanthemum morifolium* 'Hangju' [30] regenerated plants after cryopreservation showed no modifications at phenotypic level.

Cejas *et al*. [31] studied the effects of cryopreservation on several indicators in the first stages of development of *Phaseolus vulgaris* L. seedlings. These authors did not observe phenotypic variations when comparing seedlings originated from cryopreserved seeds for two weeks with seedlings preserved for the same period at 4 ºC. Nevertheless, at the biochemical level, they identified changes such as a decrease in protein content and an increase in the concentration of malondialdehydes in the stem, the latter an indicator of oxidative stress [32]. Furthermore, they detected a reduction in the concentration of phenolic compounds in the roots and identified this organ as the most affected when comparing the different biochemical parameters.

On the other hand, Zevallos *et al*. [33] observed an increase in germination of *Solanum lycopersicum* Mill in seeds cryopreserved for two weeks, five days after the start of the trial, despite these differences were not significant after seven days. Moreover, significant differences were found in biochemical parameters due to exposure to LN in different organs of the plant, more accentuated in roots. The biochemical alterations in both *P. vulgaris* and *S. lycopersicum*, product of exposure to LN, could have been caused by the seed cryopreservation conditions. Both studies do not delve into the conditions of maturity at the time of collection. In addition, *P. vulgaris* seeds were stored at a relatively high water content (12.0 %) [34, 35]. The present study considered these conditions prior to cryogenic storage when developing the protocol of cryopreservation of tobacco seeds, together with the demonstrated presence of regions with water content higher than the average determined for the seeds [36-38]. Therefore, this could have had a differential impact on the biochemical indicators determined for the different organs of plants derived from cryopreserved seeds.

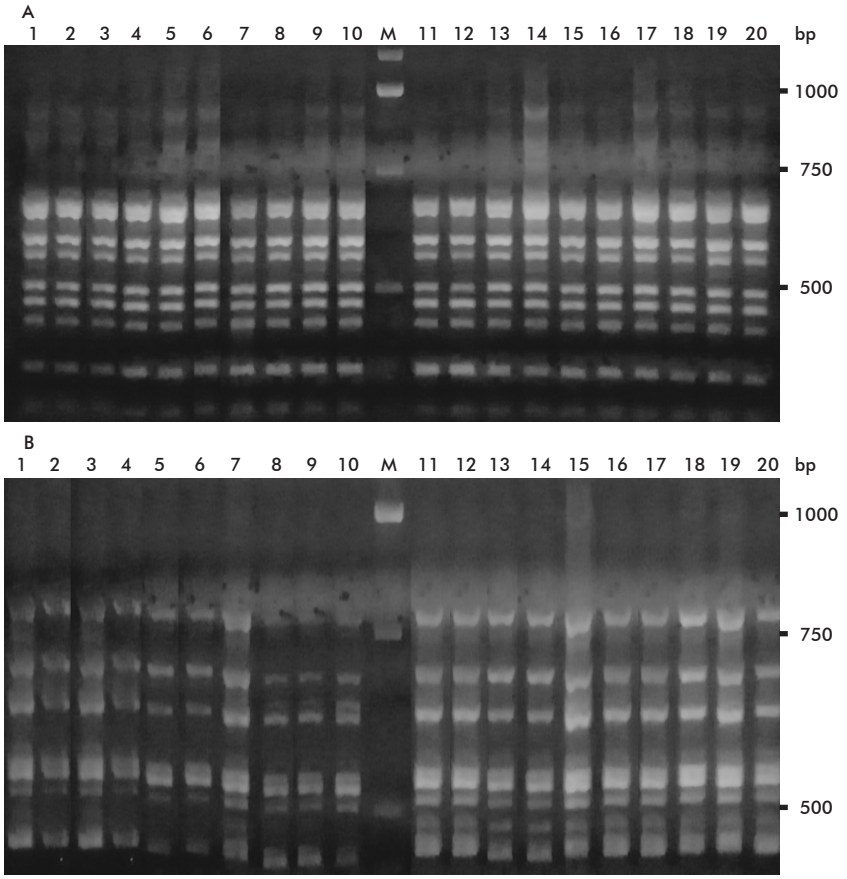

Figure. Patterns of RAPD bands in 25-day-old tobacco seedlings obtained from seeds collected 35 days after anthesis, dried to 2.1 % water content and cryopreserved for 1 year (+LN) (lanes 1-10) or stored at 5 °C for the same period (-LN) (lanes 11-20). A) OPA-08 primer combination. B) OPA-16 primer combination. M: molecular weight marker 100 bp ladder (Promega).

Successful cryopreservation is often judged not only by the survival of plant tissue and its ability to regenerate into whole plants [22], but also by the genetic stability obtained after cryopreservation [39, 40]. Restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), methylation-sensitive amplification polymorphism (MSAP) and random amplified polymorphism (RAPD) markers have been used as DNA fingerprints for cryopreserved samples [22, 40-43]. The RFLP, AFLP, and MSAP analyses are very reliable, but the procedures are troublesome and time-consuming. In comparison, RAPD analysis requires less technical skills and time, although its lower reproducibility and reliability compared to other methods constitute its main disadvantage [19, 44].

In accessions of the genus *Nicotiana*, RAPD markers have been used mainly in the detection of quantitative trait loci (QTL) and their mapping to specific genomic regions [45, 46] and in phylogenetic and diversity studies [18, 47]. There are no references to analysis of genetic stability after conservation in accessions of this genus.

However, the absence of polymorphic bands for each of the RAPD markers evaluated here (Figure) was in complete agreement with the phenotypic

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evaluation (Table 1, 2 and 3). Considering factors such as the maturity and water content of the seeds before cryopreservation and the rate of thawing during the extraction of the seeds from LN, the damage was avoided. This corroborated the hypothesis that only a certain level of DNA polymorphism is induced as a result of a complete culture-cryopreservation-regeneration process and not cryopreservation *per se* [22].

Previously, the superiority of the established cryopreservation protocol over traditional conservation methods (5 ºC) for seeds of the cultivar "Sancti Spíritus 96" was demonstrated through the evaluation of physiological and biochemical indicators [48, 49]. In the present study, we conclude that the desiccation to water content of 2.1 % of tobacco seeds collected 35 d after anthesis, and subsequent cryopreservation for one year, did not cause any visible phenotypic differences in the characteristics studied between plants derived from cryopreserved and non-cryopreserved seeds. Moreover, no changes were detected at the DNA fragments profile level.

Nevertheless, the protocol established for the cultivar "Sancti Spíritus 96" must be validated in a greater number of *Nicotiana* accessions, prior to its incorporation as a daily procedure for handling seeds in the germplasm bank at the Tobacco Research Institute of Cuba. Thus, both, the base and the duplicate collection, could be preserved in NL, making germplasm available in the long-term for its use by breeders to obtain new cultivars.

In summary, this work proposes a novel approach for the conservation of plant germplasm in the form of seeds, both in Cuba and internationally, which can be extended to other species.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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