REPORT

Methodology for the cryopreservation of calli with embryogenic structures for the culture of sugarcane

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ABSTRACT

Currently, cryopreservation is the only solution for avoiding the loss of embryogenic potential during the storage of sugarcane calli with embryogenic structures. However, implementing a methodology for cryopreservation implies overcoming technological drawbacks and validating the established procedure, wich are the main objectives of this work. We present our results on the development of a simple cryopreservation methodology for the storage of sugarcane calli with embryogenic structures, based on a slow freezing process. The proposed methodology successfully preserves the regenerative capacity of the calli, supported by *ex vitro* evaluation, for 27 months. We show the phenotypic characteristics of plants regenerated from cryopreserved calli, and compare them to non-cryopreserved controls. These studies are the first detailed evaluation of the effect of long-term cryogenic storage on the survival and regeneration of sugarcane plants from calli with embryogenic structures. Furthermore, we demonstrate the importance and implications of free radicals and oxidative stress as markers for cryopreservation-induced damaged, as evaluated by determinations of the products of lipid peroxidation during sugarcane culture. These results are fundamental for the efficient use and implementation of the established technologies, based on the use of calli for the large-scale production of vitroplants.

*I*ntroduction

The scientific name of the sugarcane (*Saccharum* spp. Hybrid) is used to designate inter-species hybrids of the *Saccharum* genus. These genetic crosses are performed mainly between *S. officinarum L.* hybrids and other commercial hybrids and forms [1]. One of the distinctive features of sugarcane is the presence of physiological mechanisms for the production and accumulation of sucrose in the stem, as well as for the generation of large amounts of biomass. However, the culture of sugarcane has been characterized by an intense genetic improvement to obtain new somaclones or cultivars which efficiently use light, nutrients and water. Sugar is manufactured in approximately 120 countries around the world, and 63% of this production is based on sugarcane [2].

Cuba has favorable conditions for sugarcane growth, and sugarcane-derived products have long been a part of Cuban's economy. Currently, this crop covers about 650 000 ha, representing around 15% of the agricultural area of the country [3].

One of the main drawbacks of faced by sugarcane agriculture worldwide is the vegetative (*i.e.* asexual) nature of the propagation techniques conventionally used. The consequence of this situation is that the crop in the field must be renovated at intervals ranging from 1 to 5 years [4]; a process that is costly, tedious and time-consuming. Furthermore, if the seed is of low quality, yields decrease and more tillage is needed. The crop is exposed to the risks of natural disasters, while the propagation system leads to the systemic transmission of diseases, and natural selection and plagues also take their toll [2]. This has led to the use of *in vitro* culture technologies for sugarcane as an alternative, faster means of propagation.

In general, the *in vitro* culture technique is used for the maintenance, production or modification of plant material, and can be divided into techniques for undifferentiated or differentiated tissues [5]. Calli and cellular suspensions are examples of undifferentiated tissues which are widely used for commercial purposes and in academic research at cellular and molecular levels [6].

The culture of sugarcane calli is a very important commercial methodology. In Cuba, calli with embryogenic structures are used as the starting material for the indirect regeneration of plants through different strategies, as an alternative for increasing economic efficiency, and for the propagation of the species [7-10]. Sugarcane calli are also involved in valuable research for the selection of varieties resistant to biotic [11] and abiotic [12] factors, as well as in genetic transformation schemes [13].

However, the use of any long-term *in vitro* culture protocol with calli has associated risks, such as the loss of their regenerative potential or of their embryogenic characteristics, and disadvantages such as the high costs of continuous subcultures or in the event of diseases or technical or human errors; furthermore, using calli in this manner often requires access to a public collection [2, 4, 6]. Therefore, the use of methodologies for the safe storage of calli that can guarantee their survival and the proper conservation of their embryogenic characteristics is a better alternative than continuous subculturing.

Currently, the best technique for callus storage is cryopreservation. This methodology allows the long-term preservation of calli by *in vitro* storage at ultra-low temperatures, preferably that of liquid nitrogen (-196 °C). At this temperature, the rate of

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chemical reactions and biophysical processes is so low that no noticeable biological deterioration occurs [14]. Furthermore, cryopreservation allows the storage of plant material in a physically reduced space, protected from contaminating agents, and requiring a regime of only minimal technical maintenance, focused mainly on guaranteeing an adequate level of liquid nitrogen in the storage thermos [15]. However, the plant material has to undergo stress to be cryopreserved, such as the exposure to low temperatures, the formation of ice crystals and a process of severe dehydration [6].

Jian *et al*., Eksomtramage *et al*. and Gnanapragasam and Vasil have described the successful use of cryopreservation for sugarcane calli with embryogenic structures [16-18]. These authors have followed the strategy known as dehydration by extracellular freezing, which uses a controlled freezing regime [19]. However, this methodology requires expensive and sometimes complex programmable freezing devices, limiting its use to laboratories specializing in cryopreservation [5, 20, 21]. Furthermore, their research has been focused on the cryopreservation of sugarcane calli obtained from segments of immature leaves belonging to *in vitro* cultured plants; however, such explants are known to have a limited morphogenetic capacity [22] and it is widely acknowledged that immature embryos, as well as young inflorescences, are physiologically better explants for calli production because they retain their embryogenic capacities [23]. This explains why some technologies, for the large scale propagation of sugarcane, use immature inflorescence as the starting explant for the process [8], even though these structures are only available in a defined segment of the life cycle, and only under very specific environmental conditions for certain genotypes.

This study, using the published research described above as a starting point, shows the results of establishing a methodology for the cryopreservation of sugarcane calli with embryogenic structures obtained from immature inflorescence. The study evaluated the *in vitro* survival and regeneration (plants per 500 mg of calli) percentages for: a) three varieties (CP52-43, C1051-73, C91-301); b) explants obtained either from immature inflorescences or immature leaves from *in vitro* plants; c) calli stored for up to 16 months under liquid nitrogen, belonging to the CP52-43 variety.

The use of cryopreservation as an industrial tool not only requires methodological developments, but also their validation to guarantee the absence of side effects. There are no reports in the literature on the effect of cryopreservation on the behavior of specific parameters that signal the existence of irreversible biochemical changes for cell membranes after storage, and there is no published data on the actual field performance of sugarcane plants obtained from cryopreserved calli. Therefore, it has been difficult to predict the effect of different cryopreservation methodologies on physiological indicators or on agro-industrial yields. In this context, this work also shows the evolution of electrolyte loss, lipid peroxidation products and protein content for a period of 5 days after cryopreservation, and determines the *ex vitro* behavior of plants regenerated from cryopreserved sugarcane calli with embryogenic structures, measuring agronomic variables related to physiological markers and agricultural yields.

Materials and methods

Experimental procedures and data processing

The *in vitro* experiments were carried out at the Bioplant Center of the Ciego de Ávila University, Cuba, and the field experiments were performed at the Provincial Sugarcane Research Station of the same province. The experimental work followed the stages described below:

Selection of sugarcane plants from a donor bank

The sugarcane plants were selected from the donor bank of the Provincial Sugarcane Research Station of Ciego de Ávila. The main genotype used was CP52-43 (CP43-64 x CP38-34). The validation of the established methodology employed the C91-301 (C1616-75 x POJ 2878) and C1051-73 (B42231 x C431-62) varieties, obtained from the Hybridization Block of the National Institute for Research on Sugarcane, in Sancti Spíritus, Cuba.

Obtaining of the calli

The main experiments used calli obtained from immature inflorescence. They were collected from mature (9 to 12 months old) plants and disinfected and rinsed as described by Jiménez [7]. Then, 3.0 to 5.0 mm-long segments were selected and grown on 90 mm-diameter Petri dishes containing the Murashige and Skoog medium [24] with 3.0 $mg \cdot L^{-1}$ of 2,4-dichlorophenoxyacetic acid [8, 25], at 25 ± 2 °C for 30 days in the absence of light. The validation experiments also used calli with embryogenic structures obtained from immature leaves of plants cultured *in vitro*, again following the procedure described by Jiménez [7].

Callus maintenance

The calli were obtained transferred to the Murashige and Skoog proliferation medium [24], with 2,4-dichlorophenoxyacetic acid (1.0 $mg \cdot L^{-1}$), arginine (50.0 $mg \cdot L^{-1}$) and proline (500.0 $mg \cdot L^{-1}$). These cultures were performed in the absence of light, at 25 ± 2 °C, for 30 days [25].

Selection of calli with embryogenic structures

After the obtaining and maintenance processed described above, the calli (2 months old) were selected on the basis of morphological and cellular parameters: compact, nodular and white-yellow in color [8], formed by globular structures with mainly round meristematic cells having a dense cytoplasm and small vacuoles. For this, the calli were sectioned and examined under a stereomicroscope, choosing only calli with embryogenic structures of approximately 5 mm in diameter.

Data processing

All measurements in the laboratory experiments were performed by triplicate, using a completely randomized experimental design. The *ex vitro* culture studies used a

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randomized block design with six replicates. The data were processed wile the Statistical Package for Social Sciences software application (SPSS for Windows, ver. 8.0, Copyright SPSS Inc., 1989-1997). The assumptions of normality and homogeneity of variance were tested by the Kolmogorov-Smirnov and the Levene tests, respectively; and parametric tests (t-test, ANOVA, Tukey) were employed throughout, with a type I error probability of $p < 0.05$. The data were transformed for the analysis of the percentages of survival and of regenerated plants by the equations $x' = 2 \arcsin [(x/100)^{0.5}]$ and $x' = (0.5 + x)^{0.5}$, respectively.

Methodology for the cryopreservation of sugarcane calli with embryogenic structures

Selection of a simple freezing procedure

Maddox *et al.* and Withers [26, 27] have successfully devised uncomplicated freezing procedures for cellular suspensions of *Nicotiana* and *Musa*, respectively. Based on their results, we decided to evaluate the use of one of these procedures as an alternative to establish techniques for the cryopreservation of sugarcane calli with embryogenic structures (*i.e.*, cooling rate controlled by a computer-coupled programmable freezer).

Taking in consideration our own preliminary results and those published by Jian *et al.* and Eksomtramage *et al.* [16, 17], calli with embryogenic structures (selected as described in the preceding sections) were transferred to pieces of filter paper on top of a fresh proliferation medium in Petri dishes. After 10 days under these conditions (the post-subculture time), the calli entered and were subjected to the stages and conditions listed below:

Stage I. Cryoprotective treatment with sucrose. The calli were placed into 2.0 mL polypropylene cryopreservation vials (8 calli per vial, averaging 40 mg per callus, replicated 5 times) and transferred to an ice bath. They then received 1.0 mL of the culture medium containing 0.15 mol·L^{-1} of sucrose at the same temperature. The length of this treatment was 60 min.

Stage II. Cryoprotective treatment with dimethylsulfoxide. The vials containing the calli and the sucrose received pure dimethylsulfoxide (14 mol·L^{-1}) to a final concentration of 5% (v/v). The dimethylsulfoxide was added gradually during the first 30 min. of the stage, which had a total length of 60 min.

Stage III. Cooling. The cooling procedure essentially consisted on progressively cooling the chilled samples to a temperature of -40 °C, seeding extracellular ice crystals at a determined point along the freezing process. The factors studied for this stage were the temperature for seeding the extracellular ice crystals (the induction temperature), the time taken for their induction, and the procedure used to achieve a slow cooling rate:

Temperature for seeding ice crystals in the cryoprotective medium

The election of the optimal temperature for seeding extracellular ice crystals in the cryoprotective medium was accomplished by first determining the supercooling point and equilibrium freezing temperature of different cryoprotective sucrose mixtures, placed into cryovials in a pre-chilled (0 °C) ethanol (95%) bath. This bath was then placed in a -40 °C freezer (Figure 1b), and the of temperature was monitored using a digital thermometer with a copper-constantan thermocouple (Figure 1a). Based on the data, obtained, we decided to induce the formation of the first ice crystals in the extracellular milieu once the vials reached a temperature of -8 °C, by dipping the vial bottoms into liquid nitrogen (Figure 1c) for an induction time of 2.5 s, before reaching the equilibrium freezing temperature.

Selection of the cooling procedure and influence of the type of alcohol

The cryovials containing cryoprotected calli entered the cooling procedure, which consisted of one of the following alternatives:

a) Transfering the cryovials to a 0 °C isopropanol bath, which is then placed in a laboratory freezer at -40 °C to achieve a slow reduction of the temperature [27].

b) Inserting the cryovials into a polystyrene block, which is then placed on a laboratory freezer at -40 °C to achieve a slow reduction of the temperature [26].

Another factor which was also examined was the substitution of the alcohol used in the pre-chilled bath for the first alternative (a), by Panis *et al.* [28], who substituted methanol for isopropanol while standardizing their cryopreservation procedures. In the present study, the cryoprotected calli were placed in an alcohol bath pre-chilled to 0 °C containing either isopropanol, methanol, or Cuban-manufactured ethanol (95% v/v), before being transferred to the -40 °C laboratory freezer.

Effect of the time of induction of extracellular ice crystals

After selecting the optimal procedure for a slow cooling rate, the last parameter optimized in Stage III was the induction time of extracellular ice crystals, given its importance for the strategy of extracellular freezing in regards to the survival and regeneration of plants from cryopreserved calli. Induction periods tested were 0, 2.5, 5, 7.5 and 10 s.

The cooling rate, the time taken to reach -40 °C determined by the control vials containing the cryoprotective mixtures from the starting temperature

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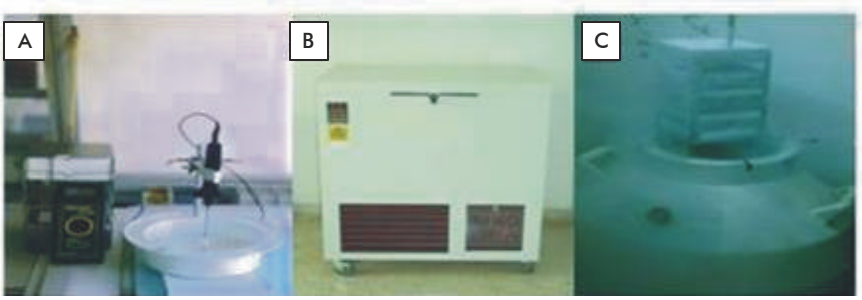

Figure 1. Accessories used for the simple cooling procedure: a) alcohol bath and digital thermometer with a copper-constantan thermocouple, submerged in a cryovial; b) laboratory freezer at -40 °C; c) Liquid nitrogen thermos for the immersion and storage of cryovials.

of 0 °C, was very homogeneous. The average cooling rate was 0.5 °C·min^{-1}, well within the range classified by Engelmann [29] as slow.

Stage IV. Pre-freezing. When the cryoprotective mixture on the cryovials reached -40 °C, the samples remained at that temperature for 1 h before the direct immersion into liquid nitrogen.

Stage V. Storage. The samples were kept on liquid nitrogen for 2 h.

Stage VI. Fast thawing. The samples were thawed by placing the cryovials in a water bath at +40 °C with constant agitation for approximately 2 min.

Stage VII. Recovery. The thawed calli were placed on a filter paper on top of the proliferation medium, to facilitate the diffusion and progressive elimination of the cryoprotective agents [Murashige and Skoog medium [24], with 2,4-dichlorophenoxyacetic acid (1.0 mg·L^{-1}), arginine (50.0 mg·L^{-1}) and proline (500.0 mg·L^{-1})], in Petri dishes with a 60 mm diameter containing 30 mL of medium per plate). The plates were incubated in the absence of light.

Stage VIII. Evaluation of survival. Survival was measured by counting the number of calli with signs of growth after 30 days in the recovery stage, as evaluated by measuring their size under a stereomicroscope. The results were expressed in percentages relative to the total number of calli used per treatment.

Stage IX. Evaluation of the regeneration of plants. After 30 days of recovery, randomly selected calli were placed into culture flasks (height 120 mm; diameter 70 mm, 500 mg of calli per flask) containing a medium that induces regeneration (proliferation medium without 2,4-dichlorophenoxyacetic acid, 25 mL per flask), and kept under a regime of 16 h light/8 h darkness with a flow of photosynthetically active photons of 40 µmol·m^{-2}·s^{-1}. After 80 days, the regeneration was evaluated as described by Eksomtramage [17], counting the regenerated plants with a height equal to or higher than 10 cm, which were later transferred to *ex vitro* culture conditions.

Effect of post-subculture time on the survival and regeneration of plants from cryopreserved calli

The physiological status of the calli to be cryopreserved is important in their survival and the later regeneration of plants from the cryopreserved material. Therefore, the effect of post-subculture time was examined after optimizing the cooling procedure for stage III. To this end, calli with embryogenic structures were selected and placed into pieces of filter paper on top of Petri dishes containing the fresh proliferation medium. After 0, 5, 10, 15, 20, 25, 30 and 35 days of post-subculture time, the calli were subjected to the cryopreservation procedure described above, using the optimized conditions established for stage III.

Additionally, since there is no available data on the growth of sugarcane calli obtained from immature inflorescence to be used for cryopreservation, and given that this parameter is important for survival after storage in liquid nitrogen [15], the growth of the calli during subculture was also followed by determining their weight throughout this period. Calli with embryogenic structures were selected and placed on pieces of filter paper on top of Petri dishes with the fresh proliferation medium, using 400 mg of calli per plate. The calli, obtained from the same batch, were divided into 5 groups, using 3 replicates per treatment. The fresh weight of the calli was determined at days 0, 5, 10, 15, 20, 25, 30 and 35 during post-subculture, by weighing them on tared Whatman No. 1 filter paper.

Effect of the concentration of sucrose of the cryoprotective medium on the survival and the regeneration of plants from cryopreserved calli

After optimizing the post-subculture period and the cooling procedure in stage III, we decided to study the effect of sucrose concentration during cryoprotection on the survival and regeneration of plants from the cryopreserved material. To this end, calli with embryogenic structures were selected and placed on pieces of filter paper on top of Petri dishes containing the fresh proliferation medium. After the optimum post-subculture period determined as described above, the calli underwent the cryopreservation process, using the optimized conditions determined for the cooling procedure of stage III but using different concentrations (0.0, 0.15, 0.30, 0.45, 0.60 mol·L^{-1}) of sucrose in the cryoprotective medium during stage I.

Additionally, the tolerance of calli to different sucrose concentrations was also evaluated. With this aim, calli with embryogenic structures were selected and post-subcultured for 15 days, after which they were placed on 2 mL polypropylene cryovials (8 calli/vial, each callus averaging 40 mg, replicated 5 times). The vials were then placed on an ice bath and received 1.0 mL of pre-chilled culture medium containing sucrose (0.0, 0.15, 0.30, 0.45, 0.60 mol·L^{-1}), and later incubated under these conditions for 60 min. The tolerance of the calli to these treatments was evaluated by determining their survival and the percentage of regenerated plants, as described in the preceding sections.

Effect of the concentration of dimethylsulfoxide of the cryoprotective medium on the survival and regeneration of plants from cryopreserved calli

The effect of the concentration of dimethylsulfoxide on the survival and regeneration of plants from the cryopreserved material was studied by using selected calli with embryogenic structures, which were placed on filter paper in Petri dishes with the fresh proliferation medium. After growing during the best post-subculture time, the calli were subjected to the cryopreservation procedure using the optimum sucrose concentration (determined as described in the section above for stage I) and the optimized cooling method for stage III, but with different concentrations of dimethylsulfoxide (0.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5% (v/v)) in the cryoprotective medium during stage II.

Additionally, the tolerance of calli to different dimethylsulfoxide concentrations was also evaluated. Hence, calli with embryogenic structures were selected and post-subcultured for 15 days, after which they were placed on 2 mL polypropylene cryovials (8 calli/vial, each callus averaging 40 mg,

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replicated 5 times). The vials received 1.0 mL of the pre-chilled culture medium and were placed on an ice bath, then receiving progressive additions of pure dimethylsulfoxide (14 mol·L^{-1}) during the first 30 min. of the treatment until final concentrations of 0, 2.5, 5, 7.5, 10, 12.5 and 15 % (v/v) . After 60 min the tolerance to dimethylsulfoxide was evaluated by determining survival, as described earlier.

Effect of the length of the pre-freezing step on the survival and the regeneration of plants from cryopreserved calli

The effect of pre-freezing time on the survival and regeneration of plants from cryopreserved material was studied by placing previously selected calli with embryogenic structures on filter paper in Petri dishes containing fresh proliferation medium. After incubation for the best post-subculture time, the calli underwent the optimized freezing procedure, using the best concentrations of sucrose and dimethylsulfoxide during stages I and II and the optimized cooling procedure during stage III. During stage IV, the effect of incubation time (0.0, 1.0, 2.0, 3.0, 4.0 h) at the pre-freezing temperature (-40 °C) was tested.

Analytical measurement of changes induced by cryopreservation on the cellular membranes

The evaluation of cryopreservation-induced damage on cellular membranes was performed by analytical determinations for electrolyte loss, the contents of the products of lipid peroxidation, and total protein on cell membranes. These measurements were carried out on control samples of calli which did not undergo the process of cryopre-servation, comparing them with the results of the same measurements on calli which had been cryopreserved according to the stages described above, under the following conditions:

Stage I. Cryoprotective treatment with 0.3 mol·L^{-1} sucrose.

Stage II. Cryoprotective treatment with 10% (v/v) dimethylsulfoxide.

Stage III. Cooling in a container with 95% (v/v) ethanol pre-chilled to 0 °C, which was then transferred to a laboratory freezer at -40 °C for further cooling. The seeding of ice crystals was performed at -8 °C for 5 s.

Stage IV. Pre-freezing at -40 °C for 2 h.

Stage V. Storage of the cryovials for 2 h in liquid nitrogen.

Stage VI. Fast thawing.

The samples used in the assays were then taken after 0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 days during the recovery process (growth in proliferation medium, in the absence of light).

Electrolyte loss

The loss of electrolytes was calculated as described by Sun [30], with modifications. The callus samples with an approximate fresh weight of 500 mg were transferred to 50 mL beakers containing 30 mL of bi-distilled water. The conductivity of the water was measured after submersing the calli for 2 h, and then again after boiling the calli for 10 min, letting the water cool to room temperature before the second determination (which represents the maximum possible electrolyte loss). The percentage of electrolyte loss was calculated as follows:

$$\text{Percentage of electrolyte loss} = \frac{\text{Conductivity of the water after 2 h} \cdot 100}{\text{Conductivity after boiling}}$$

Preparation of the microsomal fraction to test for the presence of products of lipid peroxidation and for total protein content

The microsomal fractions for the determination of lipid peroxidation products and total protein fractions were prepared as described by Hurkman and Tanaka [31], with modifications. The calli were ground to a fine powder on liquid nitrogen. This powder (2.0 g) was homogenized on 20 mL of a cold solution, using a polytron homogenizer (the composition of the homogenizing solution was: 250 mmol·L^{-1} sucrose; 100 mmol·L^{-1} NaCl; 1.0 mmol·L^{-1} ethylenediaminetetraacetic acid; 5.0 mmol·L^{-1} ascorbic acid; polivinylpyrrolidone (1.0% (w/v); molecular weight 24.500 Da); 2.0 mmol·L^{-1} dithiotreitol; 10.0 µg·mL^{-1} of bacitracin adjusted to pH 7.2 with 10.0 mmol·L^{-1} of Tris). The crude extracts were centrifuged at 10 000 x g for 20 min at 4 °C and the pellet (containing cellular debris, the nuclear fraction and mitochondria) was discarded; the supernatant was further centrifuged at 100 000 x g for 1 h at 4 °C. The microsomal fraction was recovered by resuspending the pellet from the second centrifugation into 150 mmol·L^{-1} KCl.

Determination of the contents of lipid peroxidation products

The assay for the lipid peroxidation products was performed on the microsomal fractions, as described by Harding and Benson [32]. An aliquot (5.0 mL) of 20% (v/v) trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid was added to 0.5 mL of the microsomal fraction in an Eppendorf tube. After mixing and incubating the samples at 95 °C for 30 min, the tubes were cooled on ice and centrifuged at 10 000 x g for 5 min at room temperature. The non-specific absorbance of the reaction products was measured at 600 nm and subtracted from the maximum absorbance at 532 nm (for ma-londialdehyde [33]) or at 455 nm (for other aldehydes [34]).

Determination of total protein content

The protein concentration of the microsomal fraction was determined using the method described by Lowry *et al*. [35], modified to be used with sodium deoxycholate. Bovine serum albumin (Sigma) was used as a standard. An aliquot of 20.0 µL of 5.0% (w/v) sodium deoxycholate was added to 120.0 µL of microsomal fraction in Eppendorf tubes, followed by the addition of 60.0 µL of NaOH (1.0 mol·L^{-1}) and 800.0 µL of reagent A (1.0% copper sulfate: 1.0% potassium and sodium tartrate and 2.0% sodium carbonate) at volu-me ratios of 1:1:98 and 100 µL of Folin reagent diluted 1:2. The absorbance was measured at 720 nm after a reaction time of 30 min.

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Validation of the cryopreservation methodology-Evaluation of the influence of storage time on liquid nitrogen

Once the different parameters of the methodology for cryopreservation had been optimized as described in the preceding sections, the complete procedure was validated by evaluating the survival rate, the percentage of calli able to regenerate plants and the number of plants regenerated from 500.0 mg of fresh callus weight from calli cryopreserved by the optimized procedure and stored in liquid nitrogen for 1, 4, 12 and 16 months. The results were compared with those obtained for calli which had been kept on a monthly subculture schedule for 16 months, using a proliferation medium in the absence of light.

Validation of the cryopreservation methodology for explants of differing origins and from different varieties

After optimizing the parameters for the cryopreservation methodology as described above, the technique was validated by extrapolation to working conditions:

a) Using explants of differing origins: for calli of the variety CP52-43, obtained from immature inflorescence and immature leaves of *in vitro*-cultured plants.

b) Different varieties: comparing cryopreserved calli of the genotypes CP52-43, C91-301 and C1051-73.

In all cases, the survival rate and the number of regenerated plants were evaluated as indicated in the corresponding sections above.

Study of the *ex vitro* behavior of plants regenerated from cryopreserved calli (variety CP52-43)

This experiment was carried out in the facilities of the Provincial Sugarcane Research Station of Ciego de Ávila, on compacted red ferrallitic soil. Planting started on April, 1997. A randomized block design was used, with six replicates per treatment, and plots 7.5 m long and 6.0 m wide for each one with five rows per plot, 1.5 m apart. A density of 15 plants per row was used (Totaling 450 plants per treatment). The C266-70 variety was planted at the edges as a barrier against plagues and to compensate for edge effects. A complete formula was used for fertilizing the whole plantation (75 kg·ha^{-1} of nitrogen; 50 kg·ha^{-1} of P_2O_5 and 50 kg·ha^{-1} of K_2O).

Phytosanitary controls were performed on the plots for the most frequent diseases of sugarcane: smut (*Ustilago scitaminea* H. Sydow) and rust (*Puccinia melanocephala* H. and P. Sydow = *Puccinia erianthi* Padwick and Khan). The infection index for smut was calculated according to the following formula: Infection index = (Diseased stalks/Healthy stalks) * 100; in the case of rust, the formula was Infection index = (Ó (a·b)/n·k) * 100, where a = Number of evaluated plants, b = Average score according to the rust scoring scale, n = Total number of evaluated plants, k = 4 (Maximum value of the score).

A total of three treatments were compared: plants regenerated from cryopreserved calli, plants regenerated from calli which had not been cryopreserved, and plants obtained by vegetative propagation. Once the vitroplants reached an approximate height of 6.0 to 8.0 cm, they were transferred to the acclimatization area and underwent the procedure recommended by Rodríguez *et al*. [36]. The plants were transferred after 60 days to field culture conditions, to compare them to the plants obtained by vegetative propagation.

The vegetative propagation plants were obtained by the procedure used at the Provincial Sugarcane Research Station of Ciego de Ávila, starting from shoots isolated with a double disc saw which were first placed in a rustic pre-germinator, covered with jute fiber fabrics and nylon to raise the temperature, and then kept under greenhouse conditions for 60 days before deployment on the field [37].

The following evaluations were performed:

1. For sugar cane plants six months after starting the experiment, the usual physiological indicators were determined: five plants were randomly selected, the leaves and sheaths were separated, and the length (in meters) and diameter (in centimeters) of the stalks were measured. The number of stalks per linear meter was also determined.

2. For sugar cane plants twelve months after starting the experiment, 4 linear meters were randomly selected per plot (2 samples of 2 meters) and the usual physiological markers were measured (length (m), diameter (cm) and number of stalks per linear meter), as well as the average fresh weight of a stalk per plot, expressed in kilograms. The agricultural yield (average fresh weight of a stalk per plot multiplied by the number of stalks in a hectare) was estimated and expressed in tons per hectare, and the indicators used in the sugar industry for the composition of sugarcane juice in the stems were determined, following the methodology recommended by Spencer and Meade [38]. These were: total Brix (degrees), polysaccharides (pol) in juice, purity (%), fiber in sugar cane (%) and polysaccharides (pol) in sugar cane (%). The agro-industrial yield, expressed in ton per hectare, was calculated as pol in sugar cane x average fresh weight per stalk)/100 x number of stalks per hectare.

3. For the first ratoon 15 months after the harvest, the evaluations were similar to those performed for the sugar cane plant 12 months after starting the experiment.

Results and discussion

A methodology for cryopreservation, through a simple cooling procedure, of sugarcane calli with embryogenic structures obtained from immature inflorescence

Selection of the cooling procedure

Effect of the cooling procedure and of the type of alcohol

Table 1 shows the differences in survival after cryopreservation for calli which have gone through different cooling procedures. The best results are obtained with a pre-chilled alcohol bath, with no statistically significant differences between the use of ethanol, methanol or isopropanol (Table 2). This implies that it is possible to use 95% (v/v) ethanol as an equivalent substitute for the solvents most often used in the literature.

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Table 1. Effect of the cooling procedure on the survival of cryopreserved sugarcane calli with embryogenic structures, belonging to variety CP52-43

Cooling procedure according to:	Survival (%)
Maddox [26]	5.5 b
Withers [27] with isopropanol	20.1 a

Means with different letters are statistically different (t-test, $p < 0.05$). The data were transformed before the analysis using $x' = 2 \text{ arcsine } [(x/100)0.5]$. Standard Error of the Mean (SEM) = 0.89.

The fact that there was detectable survival after storage in liquid nitrogen for both cooling procedures implies the existence of a protective dehydration process that allows the vitrification of some cells without the formation of intracellular ice crystals. From a practical point of view, seeding ice crystals is much more difficult when using the procedure proposed by Maddox *et al.* [26]. Taking this into account, together with the higher survival rates afforded by the procedure of Withers [27], we decided to use the latter method for all subsequent experiments, using an ethanol bath pre-chilled to 0 °C.

Effect of the induction time of extracellular ice crystals

Table 3 shows the range of induction periods tested for seeding extracellular ice crystals in the cryoprotective medium. The data suggests an optimal induction time of 5 s, which affords higher survival rates and more regenerated plants per 500 mg of calli.

Apparently, seeding extracellular ice crystals for only 2.5 s is not enough to stabilize the first extracellular ice nucleation centers when transferring the vials from the liquid nitrogen back to the alcohol bath. On the other hand, using induction periods of 7.5 and 10 s lead to sudden, unwanted freezing of the samples and, therefore, to cell death.

In general, the 'classical' cryopreservation protocols provide insufficient detail on this important step [38]. For instance, although the analysis of this parameter must have been unavoidable for the development of the protocols of Jian *et al*. and Gnanapragasam and Vasil [16, 18], these analyses are not described in their manuscripts; and even Eksomtramage *et al.* [17], who first mentioned the need for this step when freezing sugarcane calli, provide little detail on its implementation. Therefore, there were no published precedents, before this paper, on the effect of induction time of ice crystals within the context of a cryopreservation procedure for sugarcane calli.

Table 2. Effect of the type of alcohol on the survival of cryopreserved sugarcane calli with embryogenic structures, belonging to variety CP52-43

Type of alcohol	Survival (%)
Isopropanol	21.3
Methanol	22.6
Ethanol (95%, v/v)	20.7

There were no statistically significant differences as determined b ANOVA. The data were transformed before the analysis using $x' = 2 \text{ arcsine } [(x/100)^{0.5}]$. Standard Error of the Mean (SEM) = 0.95.

Table 3. Effect of induction time of extracellular ice crystals on the survival and regeneration of plants from cryopreserved sugarcane calli with embryogenic structures, belonging to variety CP52-43

Temperature (°C)	Induction time (s)	Survival (%)	Regeneration (plants per 500 mg of calli)
-8	0	0 d	0 d
	2.5	20.5 b	10.5 b
	5	35.5 a	30.5 a
	7.5	3.0 c	4.0 c
	10	0 d	0 d
Standard Error of the Mean (SEM)		0.320	1.35

Means with the same letter are not statistically different (ANOVA, Tukey, $p < 0.05$). The data were transformed for the analysis of survival percentages using $x' = 2 \text{ arcsine } [(x/100)^{0.5}]$; using instead $x' = (0.5 + x)^{0.5}$ for the analysis of regeneration.

As mentioned above, our results show that the optimal induction time for seeding extracellular ice crystals at -8 °C, based on survival and plant regeneration data, is 5 seconds, and this induction time was selected for all further experiments. However, the highest survival rates obtained in our conditions (approximately 35%) are still low when compared to those achieved by Jian *et al.* [16] and Eksomtramage *et al.* [17], which were around 90%. This suggests that our procedure needs further optimization in other parameters, to establish an efficient methodology for cryopreservation.

Effect of post-subculture time on the survival and regeneration of plants from cryopreserved calli

Figure 2A shows that post-subculture time has a marked effect on the survival levels of cryopreserved calli. The best survival rates were achieved using a post-subculture time of between 15 to 20 days, and similar conclusions were reached when using the number of regenerated plants per 500 mg of calli as a selection criterion of the optimal post-subculture time (Figure 2B). It should be noted, however, that the best values for survival percentage were never higher than 50%.

Our results partially coincide with those reported by Jian *et al.* [16] during the cryopreservation of sugarcane calli obtained from *in vitro* plants. These authors found that survival after cryopreservation is associated with the selection step during the post-subculture period, reaching a maximum at 10 days post-subculture. However, they did not relate this finding to the physiological state of the calli before cryopreservation. Therefore, and in order to further clarify this point, we measured the growth of the calli by following the evolution of weight with time during post-subculture, and the results are shown in figure 3.

Figure 3 shows a lag phase that extends into the fifth day, after which the calli started growing following an approximately linear rate until day 25, with a value

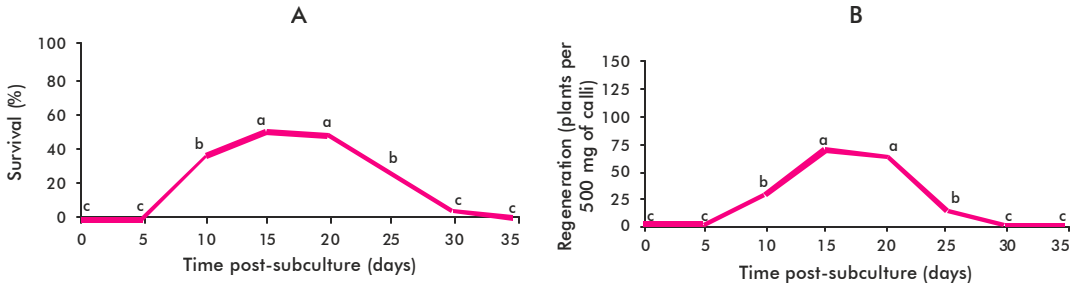

Figure 2. (A) Effect of post-subculture time on the survival of cryopreserved calli with embryogenic structures, belonging to the variety CP52-43. Means with the same letter are not statistically different (ANOVA, Tukey, $p < 0.05$). The data were transformed for survival analysis using the function $x' = 2 \text{ arcsine } [(x/100)^{0.5}]$. Standard Error of the Mean (SEM) = 0.221 (B) Regeneration of plants from cryopreserved calli with embryogenic structures belonging to the variety CP52-43. The data were transformed for the regeneration analysis with the function $x' = (0.5 + x)^{0.5}$. Standard Error of the Mean (SEM) = 1.31.

of 100 mg/day. From day 25 on, the calli enter a stationary phase.

These results are the basis for a rational selection of the material to be cryopreserved, since several authors have shown that there is a correlation for different species between the phase of active growth of the calli and its performance upon cryopreservation [6, 27, 39]. It has been proven that the morphology of the cultured cells has a marked influence on cryotolerance. In most species, only small cells with a highly dense cytoplasm, usually found in small cellular aggregates in the periphery of the callus, survive after cryopreservation; whereas large, vacuolated cells are damaged during freezing [27, 40]. Based on is background and the results presented here, we therefore decided to use a post-subculture time of 15 days for the optimized cryopreservation methodology.

Effect of sucrose concentration in the cryoprotective medium on the survival and regeneration of plants from cryopreserved calli (variety CP52-43)

Figure 4A shows that the concentration of sucrose also has a significant effect on the survival levels of the cryopreserved calli. The best results, close to 65%, were achieved using a sucrose concentration of 0.3 mol·L^{-1}. Further increasing the concentration of sucrose to 0.60 mol·L^{-1} was, however, detrimental for the survival rate, which decreased to 20%. As shown in figure 4B, the concentration of sucrose is also affecting when analyzing the number of regenerated plants. In full coincidence with the results obtained using the survival rate as a criterion, the number of regenerated plants peaks around a sucrose concentration of 0.3 mol·L^{-1}, and decreases with further increases in this parameter. The decrease in survival and regeneration when using sucrose concentrations higher than 0.3 mol·L^{-1} is due to the lower tolerance of the calli to these conditions.

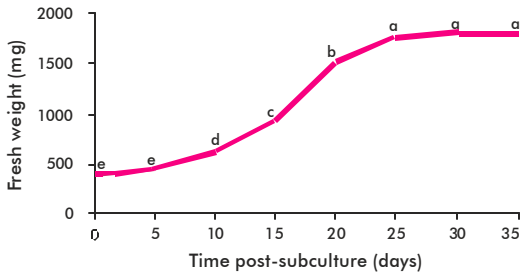

Figure 3. Effect of post-subculture time on the fresh weight of sugarcane calli with embryogenic structures, before cryopreservation. The calli belong to the CP52-43 variety. The values of fresh weight with the same letter are not statistically different (ANOVA, Tukey, $p < 0.05$). Standard Error of the Mean (SEM) = 4.51 mg.

The importance of sucrose tolerance within this setting is determined by the role of this disaccharide in the regulation of the hydric potential of the cells [41]; sucrose has also occasionally been considered an inducer of cellular division and differentiation [42]. Furthermore, there is evidence suggesting that sucrose functions as a genetic regulatory signal for genes coding for enzymes and proteins involved in transport and storage [43].

Showed a relationship between vitrification and survival for somatic embryos of oil palm upon storage in liquid nitrogen, mediated by a reduction in the amount of freezable intracellular water; sucrose plays an important role in this phenomenon. Additionally, Ausborn *et al.* and Turner *et al.* [44, 45] detected that sucrose stabilizes the lipid bilayers on the membranes by forming disaccharide-lipid hydrogen bonds, whereas Niu *et al.* [46] found that the right amount of intracellular sucrose can protect a number of enzymes from ion-mediated toxicity. In all, it is evident that sucrose has an important and multiple role in cryoprotection.

Based on the results and the literature described above, we decided to adopt a sucrose concentration of 0.30 mol·L^{-1} for our cryopreservation methodology and all subsequent experiments.

Effect of the concentration of dimethylsulfoxide in the cryoprotective medium on the survival and regeneration of plants from cryopreserved calli

Figures 5A and 5B show the dependence of survival and plant regeneration from the cryopreserved material, respectively, on the concentration of dimethylsulfoxide in the cryoprotective medium. The best results were achieved using 10% (v/v) dimethylsulfoxide; further increases on the concentration of this agent were detrimental, as

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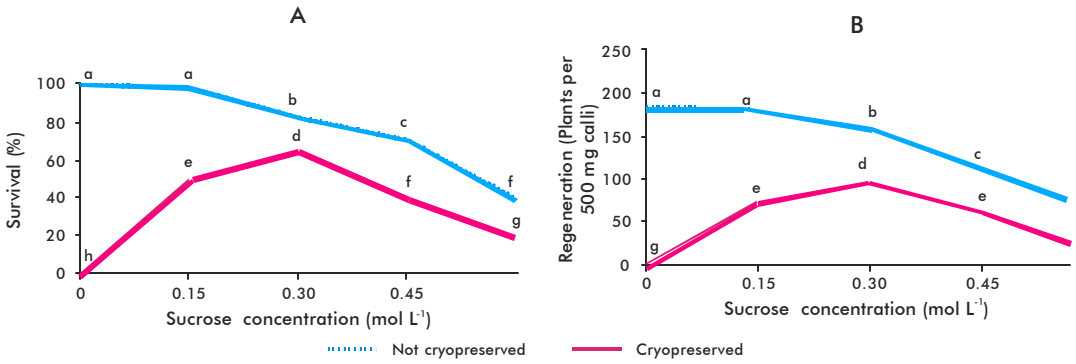

Figure 4. Effect of varying sucrose concentrations during cryoprotection on survival (A) and plant regeneration (B) from cryopreserved and non-cryopreserved calli with embryogenic structures from variety CP52-43 (cryoprotected only with sucrose). Means with the same letter are not statistically different (ANOVA, Tukey, $p < 0.05$). The data were transformed for the survival analysis using $x' = 2$ arcsine $[(x/100)^{0.5}]$ (Standard Error of the Mean (SEM) = 0.210), using $x' = (0.5 + x)^{0.5}$ instead for the analysis of regeneration (Standard Error of the Mean (SEM) = 1.28).

assessed by both criteria. This result was mirrored in the experiment studying the tolerance of non-cryopreserved calli to dimethylsulfoxide, which showed decreased survival and plant regeneration percentages at concentrations higher than 10%.

According to our results, the sugarcane calli did not survive the cryopreservation procedure when dimethylsulfoxide was omitted from the cryoprotective mixture; and both the survival and plant regeneration percentages rose steadily with increasing dimethylsulfoxide concentrations, up to 10%. However, in clear contrast with the results obtained when testing different amounts of sucrose, there are no differences in survival between the cryopreserved and non-cryopreserved samples at concentrations higher than the optimum (10% v/v in this case), and the contrast is even starker when comparing plant regeneration rates, where the cryopreserved material performs even better than the non-cryopreserved calli. These results agree with those of Finkle *et al.* [47] for rice cells, who concluded that the effects achieved by using dimethylsulfoxide are paradoxical, since although this substance is clearly toxic, it also inhibits the growth of ice crystals during cryopreservation.

The data obtained during the experiment for dimethylsulfoxide tolerance are coherent with the reports for other biological systems, associated to the high degree of toxicity of dimethylsulfoxide [48, 49]. Kartha *et al.* [50] detected that dimethylsulfoxide produces an inhibition of 35 to 42% on the growth of embryogenic cultures of white spruce when used at a concentration higher than 5% (v/v), and Klimaszewska *et al.* [51] reported a 28% reduction in the growth of embryogenic tissues from black spruce when treated with 15% (v/v) dimethylsulfoxide; this effect, according to the microscopic observations of these authors, is due to the induction by this substance of a strong plasmolytic effect at the cellular level.

However, and in spite of these findings, dimethylsulfoxide has been, and still is used as a cryoprotectant during storage at ultra-low temperatures. According to Engelmann [52], this apparent paradox is due to the fact that dimethylsulfoxide is always used as part of a cryoprotective mixture, rather than individually. Arakawa *et al.* [47] have provided evidence that the toxicity of dimethylsulfoxide in isolated proteins is mediated by hydrophobic interactions, which are favored at increasing temperatures; in this context, this effect is minimized by the use, during preculture, of sucrose at 0 °C, which induces the biosynthesis of proteins that neutralize the toxic effects of this agent (caused by its interaction mainly with lysine residues) [51, 53, 54].

Although the exact cryoprotective mechanism of dimethylsulfoxide at ultra-low temperatures remains unknown, it is widely acknowledged that it depends on the colligative properties of this penetrating compound; that is, dimethylsulfoxide affects the formation of ice crystals by decreasing the equilibrium freezing point of the solution, in direct dependence on its molar concentration [55, 56]. Dimethylsulfoxide, as a cell-penetrating agent, also decreases the intracellular concentration of toxic electrolytes on unfrozen cells [47].

Anchordoguy *et al.* [53] suggest, furthermore, that there is another, not colligative mechanism for dimethylsulfoxide-mediated cryoprotection, which involves ionic interactions between the oxygen atom from this molecule and phospholipid bilayers. Such a mechanism would stabilize the cell membranes during the freeze-thaw cycle.

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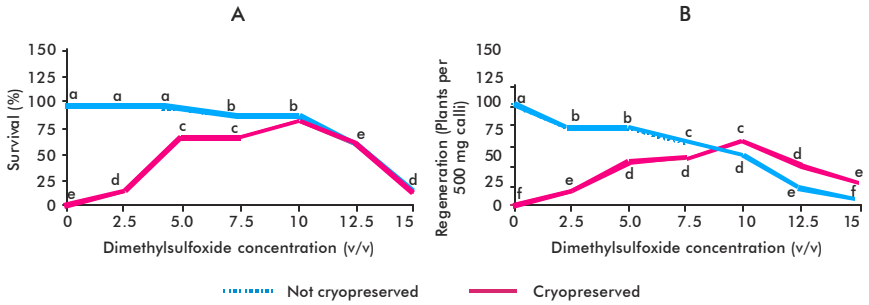

Figure 5. Effect of the concentration of dimethylsulfoxide during cryoprotection on the survival (A) and regeneration of plants (B) from calli with embryogenic structures cryopreserved or not (cryopreserved only with dimethylsulfoxide), belonging to variety CP52-43. Means with the same letter are not statistically different (ANOVA, Tukey, $p < 0.05$). The data were transformed before the analysis using $x' = 2$ arcsine $[(x/100)^{0.5}]$. Standard Error of the Mean (SEM) = 0.201.

Our findings of better plant regeneration percentages from cryopreserved calli as compared to calli which had not been cryopreserved are similar to those of Aronen *et al*. [57] in embryogenic cultures of *Abies cephalonica*. These authors found that the storage in liquid nitrogen eliminates a high proportion of cells which had been previously damaged by dimethylsulfoxide, since only small, meristematic cells survive this treatment. Furthermore, the use of cryoprotective mixtures containing other agents greatly minimizes the inherent toxicity of dimethylsulfoxide.

On the other hand, it is recognized that the process of apoptosis (programmed cell death) is not circumscribed to animals, but also occurs in plants, where it is used for the selective elimination and suicide of unwanted cells [58]. According to Joyce *et al*. [59], among the cells undergoing this process are those which have sustained high levels of *in vitro* stress, which can compromise their physiology. Such a mechanism might, therefore, be involved in dimethylsulfoxide-mediated toxicity. However, the mechanistic details of apoptosis have not been completely worked out in plant cells, and are supposed to be associated to the intracellular accumulation of toxic substances without subsequent turn-over.

Finally, based on our results and the available data on the specialized literature described above, we decided to use a dimethylsulfoxide concentration of 10% (v/v) for our cryopreservation methodology and for all further experimentation.

Effect of the pre-freezing time on the survival and the regeneration of plants from cryopreserved calli (variety CP52-43)

Figure 6 shows that the optimal pre-freezing time is of 2 to 3 hours at -40 °C. There were no statistically significant differences between the mean of survival rate (89%) and the number of regenerated plants per 500 mg of calli (147) between both time points.

After using the simple freezing procedure proposed in this study, it was determined that, apparently, the best dehydration levels are reached by the sugarcane calli when kept for 2 or 3 hours at -40 °C. Survival rates did not increase with longer pre-freezing times, probably due to excessive dehydration of the material. Studies based on the use of nuclear magnetic resonance spectroscopy in *Catharanthus roseus* cells [60], vegetative apple buds [61] and different tissues from *Rhododendron japonicum* [62] have determined that the optimum pre-freezing time for a specified pre-freezing temperature depends on the amount of water still remaining inside the cells. Tyler *et al.* [61] proved the need for the pre-freezing step when they showed that the incubation of samples at an intermediate negative temperature before immersion in liquid nitrogen would result in a better performance of the cryopreserved material after thawing.

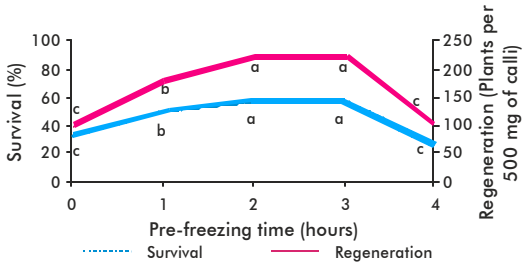

Figure 6. Effect of the pre-freezing time on the survival and regeneration of plants from cryopreserved sugarcane calli from the variety CP52-43. Identical letters for the means of each time point (independent between the lines for survival and regeneration) represent the absence of statistically significant differences (ANOVA, Tukey, $p < 0.05$). The data were transformed prior to survival analysis using $x' = 2 \text{ arcsine } [(x/100)^{0.5}]$ (Standard Error of the Mean (SEM) = 0.215); using instead $x' = (0.5 + x)^{0.5}$ before analyzing the regenerative capacity (Standard Error of the Mean (SEM) = 1.29).

Based on the results described above and the literature, we decided to use a pre-freezing exposure of 2 hours at -40 °C for our cryopreservation methodology and for all subsequent experiments.

It should be noted that the survival percentages achieved in this experiment are comparable to the best values obtained by Jian *et al*. and Eksomtramage *et al.* [16, 17]. Therefore, the results described in this section conclude the optimization procedure and finally establish the methodology for cryopreservation that is one of the objectives of this work.

Analytical measurement of changes induced by cryopreservation on cell membranes

During all the experiments described so far (including those using the wholly optimized cryopreservation procedure) there were significant differences between the performance of the cryopreserved material and that of the non-cryopreserved controls, as defined by the survival percentages and the number of plants regenerated per weight unit of calli. These findings agree with those previously published by Eksomtramage *et al.* [17], and underline the fact that cryopreservation induces damage at the cellular level. However, and with sugarcane, there are no published reports on the determination, using analytical techniques, of the damage induced by cryopreservation on biological membranes. Since such knowledge would serve as a guide for the design of new, improved strategies for cryopreservation, we measured certain parameters to quantify and characterize this damage:

Electrolyte loss

Figure 7 shows the behavior of electrolyte loss for the samples with time. The cryopreserved samples show a greater loss of electrolytes at the first day of the experiment, which tapers down gradually afterwards until matching that of the control treatment on the third day. From there on, the behavior of the samples from both treatments remained constant and undistinguishable until the fifth and last day of the experiment.

The high levels of electrolyte loss (22%) at time zero after cryopreservation are probably due to the lysis of some cells in the cryopreserved calli. Based on the hypothesis put forward by Mazur [63], it is expected that during cryopreservation, small ice crystals will appear inside those cells which are not well dehydrated. The size of these crystals may increase during thawing, leading to cell lysis and, therefore, the loss of semipermeability in the plasma membrane. On the other hand, it should be pointed

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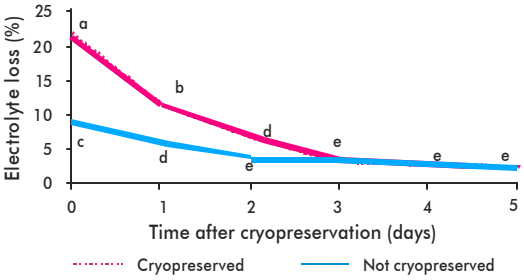

Figure 7. Changes in electrolyte loss after cryopreservation of sugarcane calli with embryogenic structures, belonging to variety CP52-43. Means with identical letters are not statistically different (ANOVA, Duncan, $p < 0.05$). The data were transformed before the analysis using $x' = 2$ arcsine $[(x/100)^{0.5}]$. Standard Error of the Mean (SEM) = 0.10.

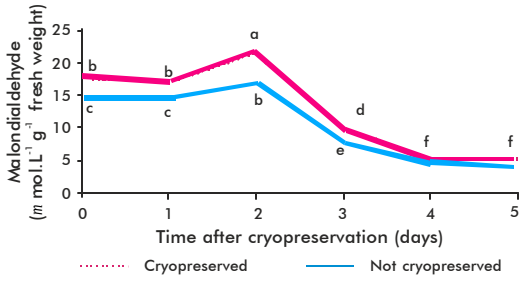

Figure 8. Changes in malondialdehyde concentration after cryopreservation of sugarcane calli with embryogenic structures, variety CP52-43. Means with identical letter are not statistically different (ANOVA, Duncan, $p < 0.05$). Standard Error of the Mean (SEM) = 0.15 mol.L^{-1}.g^{-1} fresh weight.

out that an excessive dehydration is also detrimental, since it leads to an increase in the intracellular concentration of toxic compounds, pH changes, and disorders in enzyme activity.

The gradual decrease in the amount of electrolyte loss, which stabilizes at the third day after cryopreservation, suggests a possible gradual recovery of the plasma membranes. Studies with cryopreserved wheat cells evidence that some membranous material can become detached from the plasma membrane, forming small endocytotic vesicles in the cytoplasm [64, 65]. These vesicles are kept intact, if vitrified, during storage in liquid nitrogen, and there is evidence supporting their slow reincorporation to the plasma membrane during thawing and rehydration to repair the damaged membranes [66]. A recent confirmation showed that vesicle traffic does indeed take place in plants, using a mechanism of vesicular transport for endomembranes.

There were variations in electrolyte loss also during the first five days for the material which had not been cryopreserved, after transferring of the callus to the fresh culture medium. These, however, are mainly attributable to mechanical damage in the membrane, caused by the sectioning of the calli when selecting the starting material [67].

Products of lipid peroxidation

Figure 8 shows the behavior of the contents of products of lipid peroxidation (malondialdehyde) during the first 3 days of the experiment. The malondialdehyde concentration peaks at the second day, and the highest values are observed for the cryopreserved calli. This parameter varies with time both for the cryopreserved calli and the non-cryopreserved controls.

Understanding these results requires acknowledging the fact that biophysical injury to the plasma membrane is not the only cause of a loss in cellular viability, since an important role in this phenomenon is also played by purely biochemical changes. Currently, there is evidence that suggest the presence of free radicals (which are mediators of oxidative stress) during different stages in the cryopreservation process [67]. In other words, membranes that are sensitive to dehydration will be attacked by free radicals, which will in turn lead to lipid peroxidation and the consequent release of malondialdehyde and related compounds. In the light of these facts, the high values for the concentration of malondialdehyde that are observed at time zero after cryopreservation are due to the tolerance mechanisms that are triggered by the cell membranes during the dehydration produced by the slow freezing process.

According to Wolfe and Bryant [69], the dehydration process by slow freezing and desiccation in an atmosphere with low relative humidity share a number of common characteristics, due to the high losses of water by osmosis (approximately 10%) in plant cells before reaching the state of vitrification. From a molecular point of view, both types of stresses are convergent. This has been proven by sequence analysis of most genes induced during desiccation or exposure to low temperatures, which has revealed the presence of a number of highly conserved repetitive motifs [70]. Therefore, a thorough knowledge of the tolerance to desiccation mechanisms is vital for the interpretation of the results obtained with cryopreserved plant cells [71].

The study of the tolerance to desiccation mechanisms in plant cells led Hoekstra *et al.* and Oliver *et al.* [71, 72] to argue that the partition of amphiphilic metabolites (when their cytoplasmic concentration increases due to water loss) from the aqueous phase of the cytoplasm to the lipid phase of the membranes, produces structural perturbations in the latter. These perturbations might lead to the metabolic uncoupling of the electron transport chain, and therefore, to an increase in the generation of reactive oxygen species [73].

Some published research supports the existence of a link between a reduction in metabolism (expressed as a reduction in the rate of respiration) and survival to desiccation [74] or cryopreservation [68]. However, the exact nature of this presumptive passive regulation of metabolism remains unknown. Hoekstra *et al.* [72] hypothesize the existence of a coordinated control for metabolic energy in tolerance to desiccation, which would be essential to avoid the conditions of oxidative stress and/or the accumulation at toxic concentrations of secondary metabolites.

Therefore, the absence of variation in the contents of the products of lipid peroxidation during the first day after cryopreservation might be due to a decrease in cellular metabolism and the restructuring of the cryopreserved material. The peak observed at the second day must correspond to the metabolic activation of the cells, which would trigger an increase in the concentration of oxygen-reactive species and,

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subsequently, extensive lipid peroxidation of the membranes which have not yet recovered.

Another important observation is that the values for the products of lipid peroxidation were also high in the control samples. This result is associated to mechanical damage to the membranes, as previously observed for electrolyte loss. Confirming this, Fleck *et al.* [67] described an increase in the products of lipid peroxidation after sectioning filaments from algae. Additionally, it should be noticed that the transfer of the material to a fresh medium constitutes in itself another source of stress that might lead to an increase in the concentration of reactive oxygen species [75, 76].

The contents of malondialdehyde decreased as of the third day, stabilizing at the fifth day for both treatment groups (cryopreserved and non-cryopreserved calli). This decrease on the third day might have been caused by the activation of anti-oxidative defense mechanisms and free radical scavengers [77]. There are anti-oxidative molecules in plant cells, as well as scavenger systems such as ß-carotenes, tocopherol isomers, ascorbic acid, glutathione, and an enzymatic system for processing free radicals that includes the enzymes superoxide dismutase and glutathione reductase, as well as catalases, peroxidases and others [76, 78, 79]. These anti-oxidative systems can be directly activated by oxidative stress and, as a consequence, may lead to a decrease in the levels of reactive oxygen species [76, 77].

Proteins

Figure 9 shows the total protein levels of the microsomal fraction, which are higher during the first three days after thawing for the cryopreserved samples. The values decrease on the fourth day, matching then those of the untreated controls.

The presence of statistically significant differences at time zero between the total protein contents of the microsomal fractions of the cryopreserved samples and the untreated controls must be associated to the induction of a variety of genes which are expressed upon exposure to low temperatures and dehydration in different plant species. According to Shinozaki *et al.* [80], the induced genes participate, among other roles, in the protection of cell membranes against stress through the production of important metabolic proteins.

Svensson *et al.* [81] have found low temperature response proteins, which fall within the group known as dehydrins. Most of the members of this family have been found in maize embryos, where they are associated to the endomembranous system of the cytoplasm [82]. On the other hand, the increase in total protein contents in the microsomal fraction during the first and second day after cryopreservation may be associated to the ongoing repair of the plasma membrane, as evidenced above by the electrolyte loss determinations. Supposedly, such a repair must increase vesicle trafficking through the endomembranous system, and this is achieved by protein-membrane fusions which are the base mechanism of vesicular transport. Furthermore, the synthesis of proteins, such as peroxire-doxin, is increased in response to membrane stress mediated by oxygen-reactive species [71].

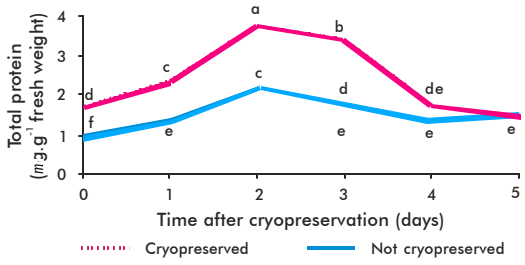

Figure 9. Changes in total protein content of the microsomal fraction after cryopreservation in calli from variety CP52-43. Means with identical letters are not statistically different (ANOVA, Tukey, $p < 0.05$). Standard Error of the Mean (SEM) =0.15 $\mu g.g^{-1}$ fresh weight.

The decrease in protein contents from day 2 onwards may be caused by a rise in the concentration of known geno- and cytotoxic agents, such as malondialdehyde and related molecules. These agents can covalently modify the structure of proteins and nucleic acids, and have been shown to inhibit DNA synthesis [83-85]. In other words, proteins can be at the same time either signaling molecules for a response to oxidative stress, or the target for oxidative stress-mediated damage, as has been shown during the expression of small heat shock proteins in plants [86]. Some of the proteins induced during the freeze-thaw process may also play an important role in decreasing the endogenous levels of malondialdehyde and related aldehydes, in addition to the functions detailed earlier.

The three analytic techniques used in this study are a vital source of information which demonstrate the possible causes of damage to cell membranes during cryopreservation, while suggesting the possible nature of the repair processes that help minimize of this damage and, lead, to the success of cryopreservation. Hence, the methodology proposed in this study may be a paradigm for further studies on the mechanisms for tolerance to dehydration and freezing in the plant cell.

Validation of the cryopreservation methodology-Evaluation of the influence of storage time in liquid nitrogen

Table 4 shows the effect of storage in liquid nitrogen, studied during the validation of the cryopreservation methodology established. It is noteworthy that there are no published reports on the validation of a cryopreservation strategy regarding the survival rate and regenerative capacity of cryopreserved sugarcane calli with time.

The calli that were maintained by monthly subculturing experienced a marked decrease in regeneration capacity as of the eighth month, in contrast with the cryopreserved material, which maintained high rates of survival and regenerative capacity throughout the experiment. There were no statistically significant differences between the values of the evaluated parameters at different timepoints for the cryopreserved samples.

The loss of the regenerative capacity in calli which are preserved subculturing is one of the main

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Table 4. Validation of the cryopreservation methodology. Behavior of the survival, the percentage of calli regenerating plants, and the number of regenerated plants per unit weight of calli, of cryopreserved sugarcane calli (variety CP52-43) with embryogenic structures, according to storage time in liquid nitrogen. (+NL): Cryopreserved calli; (-NL): Non-cryopreserved controls

	Time (months)	Survival (%)	Calli regenerating plants (%)	Regeneration (plants per 500 mg of calli)
-NL	1	100 a	100.0 a	225.2 a
	4	98.6 a	100.0 a	224.3 a
	8	96.7 ab	66.7 b	77.0 c
	12	97.5 ab	7.1 c	19.1 d
	16	11.5 c	0.0 d	0.0 e
+NL	1	90.6 b	96.7 a	149.3 b
	4	87.6 b	97.8 a	142.9 b
	8	88.0 b	93.3 a	135.1 b
	12	86.4 b	97.0 a	141.7 b
	16	90.0 b	97.5 a	140.3 b
Standard Error of the Mean (SEM)		0.189	0.253	1.312

Means with the same letters are not statistically different (ANOVA, Tukey, $p < 0.05$). The data were transformed using $x' = 2$ arcsine $[(x/100)^{0.5}]$ for the analysis yielding results in percentages; using instead $x' = (0.5 + x)^{0.5}$ for the analysis of regeneration.

problems of plant biotechnology [6], and one of the aims of this paper. This may be caused by losses of cellular totipotence due to adaptation of the cells to the hormones used in the culture medium. It is acknowledged that the cells in calli can switch to a status of partial or complete independence upon adaptation to an exogenous supply of auxins or cytokinins, which in turn leads to an irreversible decrease or loss of regenerative capacity [76].

Another possible cause is a change in the concentration of products of lipid peroxidation, triggered by reactive oxygen species when transferring the calli from auxin-containing to hormone-free media [68, 77]. There is a correlation between the proliferative capacity and aging of the calli with an increase in the concentration of Schiff bases, together with the activities of anti-oxidative systems [87].

A study on the comparative behavior with time of the concentration of reactive oxygen species, malondialdehyde, and related anti-oxidative systems between calli preserved by subculturing or cryopreservation also supports their important role in the cryopreservation methodology established in this work.

The survival and regeneration capacity after storage in liquid nitrogen, using our cryopreservation method, guarantee the long-term (up to 16 months) availability of embryogenic material. Our data proves that the calli maintained through periodic subculturing, which are often used in embryogenesis programs, show a gradual loss of regenerative capacity and are therefore, an efficient solution only for short-term goals.

Validation of the cryopreservation methodology for sugarcane calli of differing origins or from diverse explants

Type of explant

Table 5 shows the validation of the proposed methodology for the cryopreservation of sugarcane calli with embryogenic structures obtained either from immature leaves of *in vitro* plants or from immature inflorescence. The results were significantly different, depending on the nature of the starting explant. The highest survival rates and regenerative capacities were obtained with calli from immature inflorescence.

The specialized literature has dealt only with the cryopreservation of sugarcane calli obtained from immature leaves of *in vitro* plants [16-18]. This is, therefore, the first report describing the cryopreservation of calli obtained from segments belonging to immature inflorescences, comparing their performance to that of cryopreserved calli from immature leaves.

Morrish *et al.* [88] have shown that the embryogenic capacity of calli is influenced among other impor-tant factors, by the origin of the starting explant and the genotype. There is a wealth of data in the literature supporting this conclusion. For example, Khrishnaraj and Vasil [22] consider the genotype as an important parameter in the behavior of *in vitro*-cultured tissues, although they acknowledged that this behavior is also highly related to the physiological state of the explant. Castillo [8] showed that calli from segments belonging to immature inflorescence often outperform those obtained from explants of immature leaves from plants cultured *in vitro*. Benson and Roubelakis-Angelakis [87], on the other hand, also demonstrated differences in the behavior of explants from different sources *in vitro* culture, resulting in different levels of oxidative stress. The latter

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Table 5. Validation of the methodology for cryopreserving sugarcane calli with embryogenic structures, obtained from immature leaves of *in vitro* plants or immature inflorescence, according to the survival rates and regenerative capacity (variety CP52-43)

Explant origin	Survival (%)	Regeneration (plants/500 mg of calli)
Immature inflorescence	90.3 a	150 a
Immature leaves from *in vitro* plants	71.4 b	82 b
Standard Error of the Mean (SEM)	0.23	1.85

Means with identical letters are not statistically different (t-test, $p < 0.05$). The data were transformed using $x' = 2$ arcsine $[(x/100)^{0.5}]$ for the survival analysis, using $x' = (0.5 + x)^{0.5}$ instead for analyzing the regenerative capacity.

authors concluded that the proper selection of the explant is essential for successfully establishing competent *in vitro* cultures.

However, in all the cases the physiological state of the callus is ultimately the most important factor in the success of a cryopreservation strategy. Therefore, the survival and regenerative capacity results do not only depend on the type of explant, and the results can be improved if the physiological state of the callus is taken into account duringat the initial selection process.

Influence of the variety

Table 6 shows the validation of the cryopreservation methodology developed in the preceding section, evaluating its performance on different sugarcane varieties. The use of the proposed methodology led to survival rates ranging from 22.2 to 89% after cryopreservation in all cases. However, both the survival rate and the regenerative capacity obtained with calli from varieties C91-301 and C1051-73 were significantly lower than those obtained for the variety CP52-43.

The results of these experiments show that the proposed cryopreservation methodology can be used for sugarcane calli from other varieties. Obtaining the highest survival rates and regenerative capacities would perhaps entail making smaller adjustments in each case for the parameters optimized in the preceding sections, such as the post-subculture time, and the concentrations of sucrose and dimethylsulfoxide. However, the higher gains will be achieved by setting up an efficient system of *in vitro* propagation of sugar cane calli for each variety to be later processed with cryopreservation techniques, since the latter imply the use of identical tissue culture protocols both when obtaining and regenerating embryogenic cultures [5].

Study of the ex vitro behavior of sugarcane plants regenerated from cryopreserved calli

Table 7 compares the diameter and length of the stalks, as well as the number of stalks per linear meter, under field culture conditions for the plants regenerated from cryopreserved samples. The table contains the data collected after six months of growth, and the controls include plants regenerated from non-cryopreserved calli and plants obtained through traditional vegetative propagation (variety CP52-43). There were statis-tically significant differences between the treatments for the three variables; however, there were no statistically significant differences between the plants from cryopreserved or non-cryopreserved calli.

The differences (or their absence), therefore, seem to be caused by the effect of the *in vitro* culture and not by the cryopreservation procedure, since the number of stalks per linear meter is statistically higher when the plants have been obtained *in vitro* and, conversely, the diameter and length of the stalks are larger for the plants obtained through conventional vegetative propagation.

These differences agree with the results obtained by other authors when comparing *in vitro*-regenerated sugarcane those with plants obtained by conventional means. Some researchers conclude that tissue culture reduces parameters such as the weight and stalks diameter, while increasing the number of stalks per linear meter [11, 89-92].

The effect of *in vitro* culture on the number of stalks per linear meter could be explained by rejuvenation *in vitro*-multiplied tissues, which is absent in the material propagated vegetatively. Therefore, plants regenerated from *in vitro* cultures would be in a more favorable physiological state generating more stalks. The diameter and the length of the stalks, on the other hand, are inversely proportional to the competition established between neighboring stalks, and the latter increases with increasing stalk density (up to 25%); these results are coherent with this rationale. In spite of this, the further evaluation of agricultural yield indicators for the sugar cane plant (table 8) and the first ratoon (table 9) revealed no statistically significant differences between the plants obtained through *in vitro* culture and those obtained by vegetative propagation.

The results indicate that the growth of plants obtained from cryopreserved calli is no different from that of plants obtained from non-cryopreserved calli or through vegetative propagation after 12 months in the field for the sugar cane plant or 15 months, for the first ratoon. However, it was necessary to wait another 6 months in field conditions for the initially observed differences to disappear.

Table 6. Validation of the methodology for the cryopreservation of sugarcane calli with embryogenic structures (varieties C91-301 and C1051-73), evaluating the survival percentage and the regeneration of plants. (+NL): Cryopreserved samples; (-NL): Non-cryopreserved controls

	Varieties	Survival (%)	Regeneration (plants per 500 mg of calli)
-NL	CP52-43	98.8 a	230 a
	C91-301	69.5 c	72 c
	C1051-73	44.1 d	55 d
+NL	CP52-43	89.0 b	150 b
	C91-301	38.8 d	42 e
	C1051-73	22.2 e	25 f
Standard Error of the Mean (SEM)		0.190	1.421

Means with identical letters are not statistically different (t-test, $p < 0.05$). The data were transformed using $x' = 2 \text{ arcsine } [(x/100)^{0.5}]$ for the survival analysis, using $x' = (0.5 + x)^{0.5}$ instead for analyzing the regenerative capacity.

Table 7. In-field comparison of plants obtained from cryopreserved calli in embryogenic structures with plants regenerated from non-cryopreserved calli or obtained through conventional vegetative propagation, after six months of growth in the field

Evaluation	Origin of the plant			Standard Error of the Mean (SEM)
	Cryopreserved calli	Non-cryopreserved calli	Isolated shoots	
Stalk diameter (cm)	1.51 b	1.45 b	1.82 a	0.12
Stalk length (m)	0.57 b	0.49 b	0.85 a	0.10
Number of stalks (stalk/m)	5.20 a	5.45 a	4.22 b	0.35

Means with identical letters are not statistically different (ANOVA, Tukey, $p < 0.05$).

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Table 8. Comparison between plants obtained from cryopreserved sugarcane calli and non-cryopreserved controls (variety CP52-43) after 12 months of growth in the field

Evaluation	Origin of the plant			Standard Error of the Mean
	Cryopreserved calli	Non-cryopreserved calli	Isolated shoots	
Stalk diameter (cm)	2.58	2.51	2.66	0.09
Stalk length (m)	1.85	1.90	1.70	0.11
Fresh weight of the stalk (kg)	1.37	1.33	1.49	0.09
Number of stalks (stalks/m)	9.80	10.55	9.32	1.50
Agricultural yield (t/ha)	111.88	116.93	115.72	2.62
Brix on juice (°brix)	22.07	21.22	24.69	1.87
Pol on juice (%, m/m)	19.17	18.37	19.35	1.93
Apparent juice purity (%)	86.86	86.57	86.47	1.33
Fiber on cane (%)	11.55	11.23	11.52	0.43
Pol on cane (%, m/m)	16.95	16.30	17.12	1.90
Agro-industrial yield (t/ha)	18.96	19.06	19.81	1.51
Agricultural yield / agro-industrial yield	5.89	6.13	5.84	0.68

There were no statistically significant differences between the treatment groups (ANOVA).

The results agree with published research evidencing a low probability of somaclonal variation or of reversion to the original phenotypic traits after the vegetative propagation of sugarcane on the field. This reduces the potential of the use of tissue culture for obtaining new cultivars. Some researchers [2, 89, 90, 93-96] indicate that the differences are significantly reduced and revert to the original phenotype during growth on the field and with the first clonal multiplication, suggesting that, as observed here, the variations after tissue culture are only transitory.

Our results confirm those of other authors, reporting a high stability of the plant material after cryopreservation [29, 97]. The originality and importance of our experiments for Cryobionomics lies in the number of samples used per treatment (more than 400 plants), the considerable length of the experiment (27 months on the field, with two culture cycles) and the use of statistical analyses on agricultural parameters. So far, the only comparisons of the stability of cryopreserved material in *ex vitro* conditions have been carried out in greenhouses, after a short growth period of the vitroplants, and have been mainly based on qualitative observations, without a rigorous statistical analysis [57, 98-102].

The study that is most similar to our methodological proposal is that of Cote *et al.* [103]. These authors reported in the behavior of banana plants under field conditions, obtained by regeneration from cryopreserved cellular suspensions together with their corresponding controls, and evaluating different descriptors for the vegetative development of this culture. Although there were differences for two out of the eleven descriptors used in the study during the first culture cycle, these became statistically irrelevant during the second cycle.

The initial phenotypic variation in regenerated potato, banana or sugarcane plants appears to be originated by a persistent, and still unknown residual effect of *in vitro* culture. Some authors [97, 104] describe the somatic inheritance of traits and the reduction of this *in vitro* culture effect after several cycles of vegetative growth, suggesting that this initial variation is not inherited in successive somatic generations, as observed here for the plants obtained from cryopreserved samples. It has been affirmed that this variation is unstable, that is, epigenetic in nature [77]. Furthermore, it is argued that epigenetic regulation is a necessary phenomenon in plants, in order to maintain their genomic plasticity during development in constant interaction with the environment [59].

As for the phytosanitary controls on the different treatment groups, there were no statistically significant differences for the main diseases of sugarcane. For smut (*Ustilago scitaminea* H. Sydow) the average infection index was lower than 5%, mainly evidenced in the appearance of whips. In the case of the rust (*Puccinia melanocephala* H. and P. Sydow) the average infection index was lower

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Table 9. Comparison between plants obtained from cryopreserved calli having embryogenic structures with controls regenerated from non-cryopreserved calli or obtained by conventional vegetative propagation, after 15 months of growth of the first ratoon in the field

Evaluation	Origin of the first ratoon			Standard Error of the Mean
	Cryopreserved calli	Non-cryopreserved calli	Isolated buds	
Stalk diameter (cm)	2.62	2.59	2.65	0.10
Stalk length (m)	1.93	1.98	2.04	0.16
Fresh weight of the stalk (kg)	1.50	1.51	1.54	0.21
Number of stalks (stalks/m)	9.77	9.65	10.09	2.20
Agricultural yield (t/ha)	122.10	121.42	129.48	5.12
Brix on juice (°brix)	23.22	23.02	22.98	1.79
Pol on juice (%, m/m)	20.45	20.07	19.95	1.85
Apparent juice purity (%)	88.07	87.71	86.81	1.34
Fiber on cane (%)	12.9	12.17	12.5	0.35
Pol on cane (%, m/m)	17.81	17.62	17.45	1.80
Agro-industrial yield (t/ha)	21.75	21.35	22.59	1.51
Agricultural yield / agro-industrial yield	5.61	5.67	5.73	0.11

There were no statistically significant differences between the treatment groups (ANOVA).

than 15%, with the main symptoms being chlorotic and necrotic spots with small protuberances in the back of older leaves.

Integrated methodology for the cryopreservation of sugarcane calli with embryogenic structures, obtained from immature inflorescence

The results of these studies have led to the development of comprehensives methodology for the cryopreservation of sugarcane calli with embryogenic structures, obtained from immature inflorescence (Figure 10). This methodology integrates all the relevant stages, including callus selection, cryoprotection stages, a simple procedure for slow cooling to -40 °C, storage in liquid nitrogen, thawing the calli, recovery, plant regeneration, acclimatization of the regenerated plantules, the evaluation of physiological markers, and the determination of the agricultural and agro-industrial yields during a period of 27 months, spanning 2 cycles of culture.

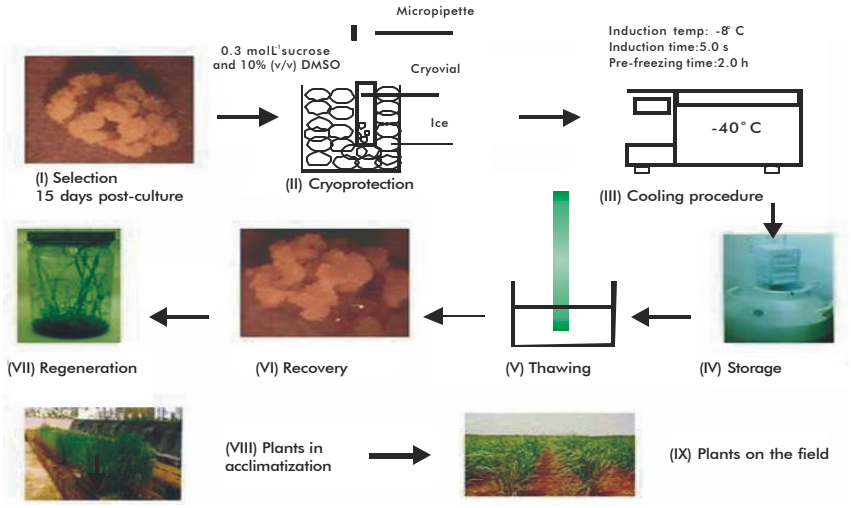

Figure 10. Integrated methodology for the cryopreservation of sugarcane calli with embryogenic structures.

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