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CRYOGENIC STRATEGY FOR THE ESTABLISHMENT OF A GERMPLASM BANK OF PINEAPPLE (*Ananas comosus* var. Comosus)

Estrategia criogénica para el establecimiento de un banco de germoplasma de piña (*Ananas comosus* var. comosus)

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ABSTRACT. In pineapple have been used cryopreservation protocols based on a vitrification procedure. However, the influence of some technical factors is still unknown to achieve a strategy cryogenic routine application to a wide range of genotypes. Furthermore, there has been no histological analysis to display any structural changes during a process of cryopreservation. In the present investigation different technical aspects of a cryogenic strategy are determined to increase survival levels from the apexes to immersion in liquid nitrogen. These were: type of apex, consisting of 3-4 leaf primordia with an approximate size of 2,5-3mm; preculture 0,3 mol L^{-1} sucrose; application of the loading solution 0,4 mol L^{-1}+2 0,4 mol L^{-1} mol glycerol; application temperature of the vitrification solution PVS2 at 0 °C for 420 min. The analysis of the display of structural changes in the cryopreserved apices revealed that the meristematic cells in the apical dome and leaf primordia in formation, suffered few cellular alterations and kept almost intact its morpho-physiological characteristics in the best conditions of survival. The method of vitrification was applied successfully for 9 accessions of the *in vitro* genebank of Bioplant center. This is an important step for the establishment of a gene bank for long-term cultivation of pineapple.

Key words: apices, germplasm, histology, vitrification

RESUMEN. En la piña se han utilizado protocolos de crioconservación basados en un procedimiento de vitrificación. Sin embargo, se desconoce aún la influencia de algunos factores técnicos para lograr una estrategia criogénica con una aplicación de rutina a un amplio número de genotipos. Además hasta la fecha, no se han realizado ningún análisis histológico para visualizar cambios estructurales durante un procedimiento de crioconservación. En la presente investigación se determinaron diferentes aspectos técnicos de una estrategia criogénica para aumentar los niveles de supervivencia de los ápices a la inmersión en nitrógeno líquido. Estos fueron: tipo de ápice, compuesto por 3-4 primordios foliares con un tamaño aproximado de 2,5-3mm; precultivo en 0,3 mol L^{-1} de sacarosa; aplicación de la solución de carga 0,4 mol L^{-1}+2 0,4 mol L^{-1} glicerol; temperatura de aplicación de la solución vitrificadora PVS2 a 0 °C durante 420 min. El análisis de la visualización de los cambios estructurales ocurridos en los ápices crioconservados reveló que las células meristemática presentes en el domo apical y los primordios foliares en formación, sufrieron pocas alteraciones celulares y mantuvieron casi intacta sus características morfo-fisiológica en las mejores condiciones de supervivencia. Se aplicó de manera exitosa un procedimiento de vitrificación para nueve accesiones del banco de germoplasma *in vitro* del centro de Bioplantas. Lo anterior constituye una importante etapa para el establecimiento de un banco de germoplasma a larga plazo del cultivo de la piña.

Palabras clave: ápices, germoplasma, histología, vitrificación

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INTRODUCTION

Pineapple (*Ananas comosus* var. *Comosus*) is one of the world's major fruit trees, grown to satisfy the population's nutritional needs and an important line for the production of preserves and the sale of fresh fruit (1). This fruit after banana and mango, is the most important tropical fruit, with a world production in 2012 that reached 23 333 886 tons (2). The pineapple for Cuba was one of the main plantations in Ciego de Ávila province, accounting for more than 80 % of the national production in the Spanish red variety, with low reproductive potential, although in recent years, new cultivars such as Cayenne Smooth and 'MD-2', of good performance, but are more demanding to environmental conditions (1, 2, 3).

At present, the genetic resources of pineapple are generally conserved in field collections (4). Moreover*, in vitro* culture techniques are used as an alternative to respond to the needs of gene conservation (5). To date, however, the most promising option for long-term storage is cryopreservation through the creation of a basic genebank where crops would be stored in confined spaces, protected from contamination and with limited maintenance (6, 7, 8, 9). The plant material is transferred to liquid nitrogen, in which the temperature is sufficiently low (-196 °C), to suspend metabolism and avoid processes that reduce genetic stability during storage (10,11,12) .

In cryopreservation of species propagated vegetatively, organized tissues such as apexes or meristems are preferred for their genetic stability (13, 14). In addition, these are recommended as the ultimate explant to store germplasm because virus-free plants (cryotherapy) regenerated directly from these cryopreserved materials can be obtained (15, 16). In the case of pineapple, protocols based on a vitrification process have been used (17, 18). In recent years the micro-drip/vitrification technique has been implemented in pineapple apices where it has been possible to increase the survival of apices (19, 20). However, the influence of some technical factors to achieve a cryogenic strategy with a routine application to a large number of pineapple genotypes is still unknown. In addition, little research has been done to visualize the structural changes at the cellular level during the different stages of a cryopreservation procedure. Therefore, the objective of this work was to determine different technical aspects of a cryogenic strategy that increase survival in liquid nitrogen for apices of pineapple vitroplants, to visualize the structural changes by histology during cryopreservation and to apply the vitrification procedure for nine accessions of the bank *in vitro* of pineapple germplasm.

MATERIALS AND METHODS

The research was carried out in the Laboratory of Genetic Improvement of the Center of Bioplants of the University of Ciego de Avila, using as plant material *in vitro* plants of pineapple (*Ananas comosus* var. Comosus) 'MD-2' from a germplasm Bank *in vitro* micropropagates according to the established protocol (21).

Stage I. conditioning of donor vitroplants: vitroplants were used after four subcultures. Multiplicated in liquid culture medium with salts MS + 100 mg L^{-1} of Myoinositol + 2,1 mg L^{-1} BA + 0,3 mg L^{-1} ANA +1,0 mg L^{-1} Thiamin + 30 g L^{-1} sucrose (0,08 mol L^{-1}), with a pH of the medium set at 5,67 according to Skoog (22). The plants were subcultured every 30 days and grew at 27 ± 2 °C and photoperiod of artificial light from 18 h day^{-1}, between 1500 and 2000 lux of illumination.

Stage II. Pre-cultivation of apices: Two types of apices were used: apices type I (2,5-3 mm in length) with a small apical base and three to four leaf primordia that covered it; and apexes with the small apical base with one or two leaf primordia (apexes type II) (0,8-1,3 mm in length), to determine the most appropriate apex type before and after immersion in liquid nitrogen for a time of (0-30 min). Dissecting the explant was performed under a stereo microscope (ACUS SCOPE) in a laminar flow cabinet from vitroplants. The apices were cultivated for two days, five petri dish-tips containing semi-solid MS culture medium supplemented with 100 mg L^{-1} of Myoinositol 1,0 mg L^{-1}, Thiamine 30 g L^{-1} and 0,08 mol L^{-1} sucrose. The effects of preculture were then determined on sucrose supplementing 0,1; 0,3; 0,5 and 0,7 mol L^{-1} sucrose, to improve the survival of the cryopreserved apices. Then the effect of the loading solution was determined, taking into account the best treatments from the previous experiment, where the explants precultured in sucrose were subjected to 1mL of the loading solutions (0,4 mol L^{-1} sucrose + 2,0 mol L^{-1} glycerol, 0,75 mol L^{-1} sucrose + 1,0 mol * L^{-1} glycerol) in cryovials with a total capacity of 2 ml for 25 min at 25 °C.

Stage III. Dehydration with the PVS2 vitrification solution: After two days of preculture the apices were transferred to the cryovials (2mL total volume) containing 1,5 mL of PVS2 solution at room temperature ± 25 °C. The plant material

was maintained for a period of time (0-30 min) at 25 °C, to determine the dehydration time in PVS2. The time of the PVS2 hydration in the material was then determined over a period of time (0-180 min) at 25 °C according to the application of the loading solution before immersion in liquid nitrogen and after immersion in liquid nitrogen. In addition, it should be noted that the total number of OH groups was evaluated by the following formula: Molarity * NA * [OH] where, NA - Avogadro constant 6,022 x 10^{23} mol^{-1}, [OH] OH groups per molecule.

Afterwards to improve the survival of the apices during cryopreservation the effect of the temperature was evaluated taking into consideration the best treatments of the previous sections with the following modifications, where the vegetal material was maintained during a period of time (0-540 min) At 25 °C and 0 °C.

Stage IV. Immersion in liquid nitrogen: Subsequently the cryovials were immersed in liquid nitrogen and held for 2 h under these conditions.

Stage V. Heating: The heating of the cryovials was carried out in a water bath at (+) 40 °C for 2-3 min.

Stage VI. Detoxification (flushing solution): The PVS2 vitrification solution of the cryovials was once replaced by 1 mL of a solution of 1,2 mol L^{-1} sucrose and maintained for 30 min at 25 °C.

Stage VII. Recovery: The apexes of the different treatments were passed filter paper covering the surface of Petri dishes containing micropropagation medium but devoid of growth regulators.

Stage VIII. Survival evaluation: At a week were transferred to petri dishes containing micropropagation medium with identical initial conditions 27 ± 2 °C and artificial light photoperiod of 18 h day-1, between 1500 and 2000 lux of illumination, for three weeks. Survival evaluation was performed according to Martinez-Montero (23), considering the percentage of survival, the number of apices that showed symptoms of regrowth at five weeks of recovery by measuring their size. The percentages of survival were expressed with respect to the total of apices used per treatment.

Histological analysis during cryopreservation of pineapple apices

The histological analysis was performed from samples of dehydrated and cryopreserved apices of the MD2 cultivar, which were submitted for 0, 180, 300, 420 and 540 min in the PVS3 vitrification solution at 0 °C. A similar procedure was followed for all samples. These were fixed in F.A.A (formaldehyde - acetic acid - ethyl alcohol) solution at 5-5-50 % (v / v), respectively. They were then washed in tap water for 24 h to remove excess fixative. After washing, the tissue was dehydrated in serial concentrations of ethyl alcohol (30, 50, 70, 80 and 90 %) and three times with absolute ethyl alcohol for 2 h each pass. After the dehydration process the samples were rinsed in alcohol-Bensol (50 % v / v). The dehydration was then continued in three passages of Bensol for 1 h and finally embedded in a series of three paraffin passages with a period of impregnation an hour per pass. Subsequently, paraffin blocks with 20 µm thick slices were placed on a vertical sliding hand microtome. The staining technique used was Flemming staining (24). A Carl Zeiss microscope to which a Canon Power Shot A620 digital camera was attached was used to observe the samples with which all the images were taken. After the vitrification procedure, it was validated in nine accessions of the national pineapple germplasm bank, taking into consideration the best treatments of the previous sections (Table I), in addition to the evaluation of survival the dehydrated and cryopreserved apices, respectively at six weeks of incubation.

Statistical treatment of data

It was performed monofactorial experiments with more than two levels, with five replications and applied a completely randomized design. In the statistical processing of the data, the SPSS utility for Windows was used (25). In all cases, the normal distribution of the data was verified through the test of homogeneity of the variances through the Levene test. Arametric tests (ANOVA and Tukey) were performed $p < 0,05$. In some cases the data transformation was required as x '= 2 arcosen $((x / 100)^{0.5}$.

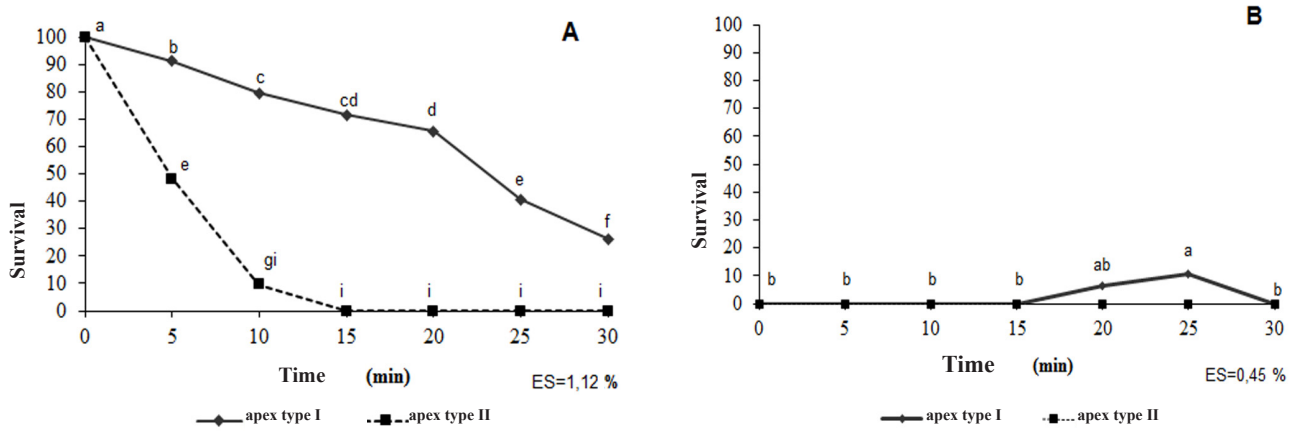
RESULTS AND DISCUSSION

In Figure 1 a greater tolerance was observed with significant differences for the apices of type I (Figure 1A) with respect to the apices of type II (Figure 1B). Type I apices supported PVS2 dehydration levels three times longer, up to 30 min exposure.

When an observation was made under an optical microscope, differences were also observed among the types of apices used. Figure 2 shows the longitudinal section of the two types of apexes. The apex type I (Figure 2A) consists of 3 to 4 leaf primordia, which guarantee a degree of protection to the meristem, and a type II apex (Figure 2B) composed of one or two leaf primordia where the meristem is practically uncovered, which were more susceptible to dehydration treatments.

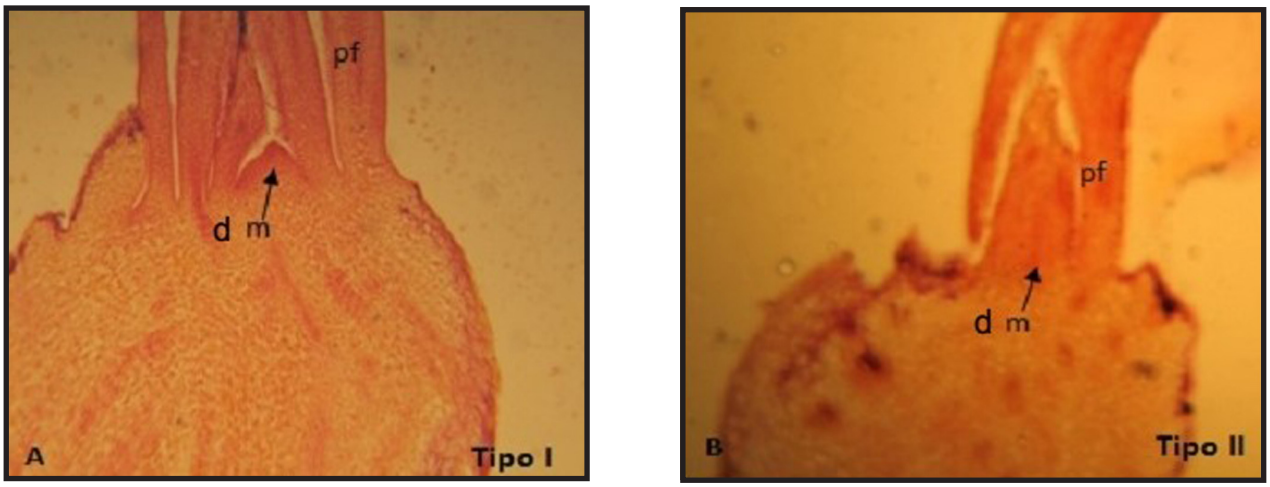
Table I. *In vitro* Bank accessions of pineapple germplasm that were used in the validation of the cryogenic strategy

Number	Genre	Species	Horticultural group	Cultivar
1	*Ananas*	*comosus*	Española	Cabezona
2	*Ananas*	*comosus*	Española	Española Roja P3R5
3	*Ananas*	*comosus*	Española	Española Roja del Caney
4	*Ananas*	*comosus*	Cayena	Cayena lisa Serrana
5	*Ananas*	*comosus*	Cayena	MD2
6	*Ananas*	*comosus*	Cayena	Cayena de Puerto Rico
7	*Ananas*	*comosus*	Maipure	Perolera
8	*Ananas*	*comosus*	Pernambuco	Piña blanca
9	*Ananas*	*comosus*	Genre in order	Piñuela Karata



Means with equal letters do not differ (ANOVA, Tukey, p <0.05). The data were transformed for the analysis as x'= 2 arcsen ((x / 100) $^{0.5}$

Figure 1. Survival of apex in survival before immersion in liquid nitrogen (A) and after immersion in liquid nitrogen (B)

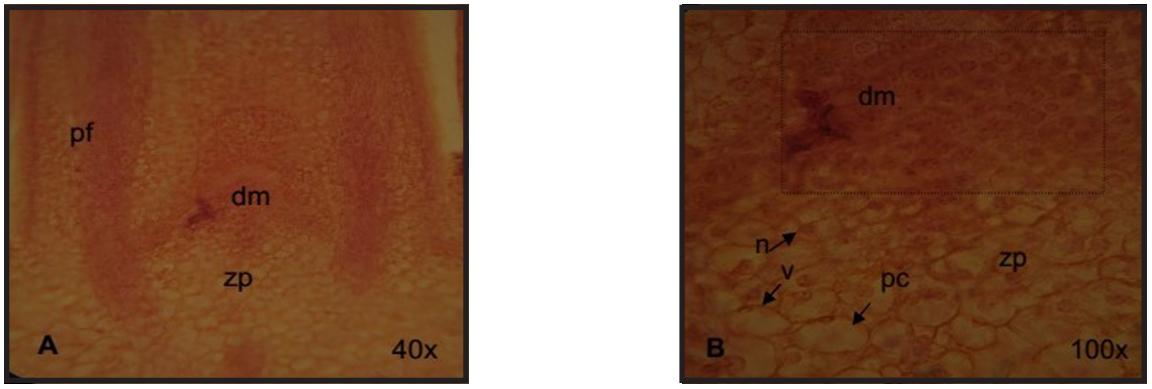


A: apex type I with developing leaf primordia that serve as protection for the apical meristem
B: apex type II devoid of leaf primordia

Figure 2. General view of plant material used in cryopreservation experiments

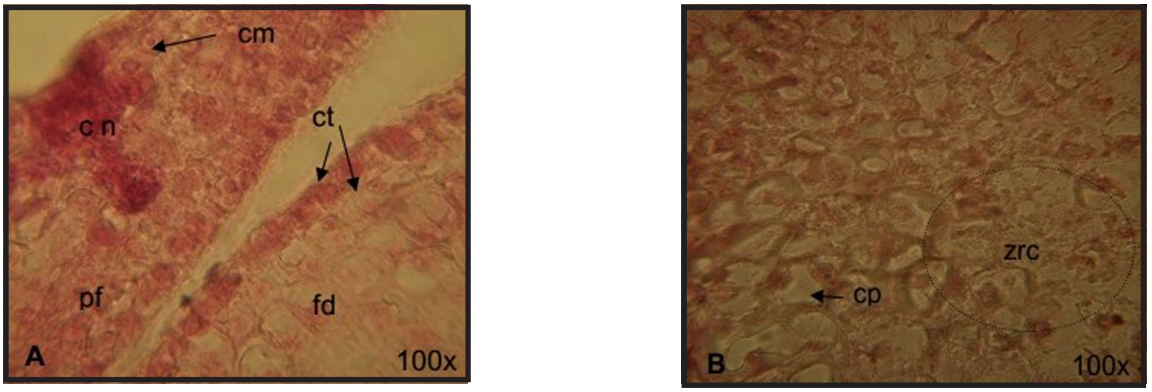
Generally, several authors accept that for a successful cryopreservation of the apices, these should consist of a meristematic dome plus one or two leaf primordia with a size of 0,5-2 mm in length, depending on the species (26). However, it is recommended that appropriate selection of the size and development status of the starting material is necessary as an essential factor in achieving high recovery rates after heating (27). For the above, type I apices were selected to continue the cryogenic strategy in the following experiments.

In contrast to the tolerance results obtained above only 5,3 % survival was observed for the type I apices when at 25 min they were dehydrated by the PVS2 vitrification solution, they were cooled in liquid nitrogen. When evaluating the coloring of the tissue, it was observed that in all cases, if they were kept deep green, they could be recreated at 45 days of evaluation. However, if they changed from green to pale yellow they could not grow, and therefore died. On the other hand, and to compare the behavior of the selected apexes in Figures 3 and 4, the histology performed on type I apices without cryoprotective treatments (time 0) before (Figure 3A and B) and afterwards (Figure 4A and B)) of the immersion in liquid nitrogen. The explants were formed by the meristematic dome or apical meristem (Figure 3A and 4A) and a subapical zone of parenchyma cells (Figure 3B and 4B).



A. foliar primordium (pf), meristematic dome (dm) and subapical parenchyma zone (zp). B. enlargement of the parenchyma zone with large vacuolated cells (v), slightly thickened cell walls (pc) and more or less centric nuclei (n)

Figure 3. Microphotographs of shoot apexes in the MD2 variety without cryoprotective treatments, before immersion in liquid nitrogen



A. Dome flank (fd) and leaf primordium (pf). Observe necrotic cells (cn), meristematic cells with abnormally thickened (cm) and very vacuolated cell walls and layers of the multiseriate tunic (ct). B. Subapical parenchyma zone where zones of cellular rupture (zrc) with rest of cell walls and dispersed nucleo plasmatic content are observed. Note the presence of parenchyma cells with much thickened cell walls and attached nucleus (cp)

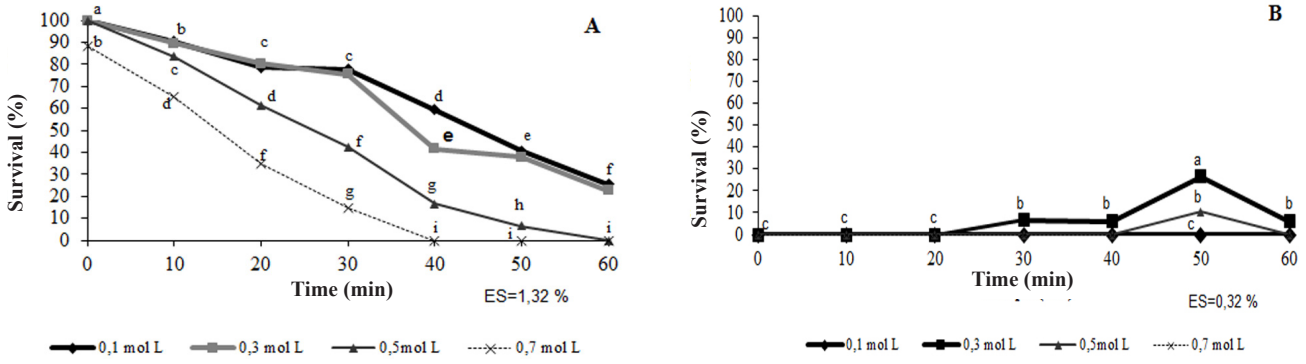
Figure 4. Microphotographs in vegetative apices of the MD2 variety without cryoprotective treatments, after immersion in liquid nitrogen

The histology of the apices without cryoprotective treatments prior to immersion in liquid nitrogen showed that the cells of the subapical zone or parenchyma cells (Figure 4B), presented larger with more or less thickened cell walls, larger vacuoles in comparison to the Meristematic zone. There is no clear definition as to the formation of meristem in a row (rib meristem) as is clearly observed in adult plants grown *ex vitro*.

In the histology of the apices without cryoprotective treatments after immersion in liquid nitrogen, it was observed that in the apical area (Figure 4A) there was cellular heterogeneity, where some cells retained their meristematic characteristics, in the case of strata Cells corresponding to the younger cells of the meristematic dome, necrotic cells, meristematic cells with abnormally thickened cell walls and small vacuoles or eryplasmatic spaces were observed, and the layers of the tunic were shown to be multiseriate. While in the subapical parenchyma zone (Figure 4B), the cells were more vacuolated, some were damaged, showing areas of cell rupture, cell wall remnants and dispersed plasma nucleus content. Note the presence of parenchyma cells with much thickened cell walls and attached nucleus. Finally it was observed that a number of cells existed with the cytoplasm contracted in the cell wall.

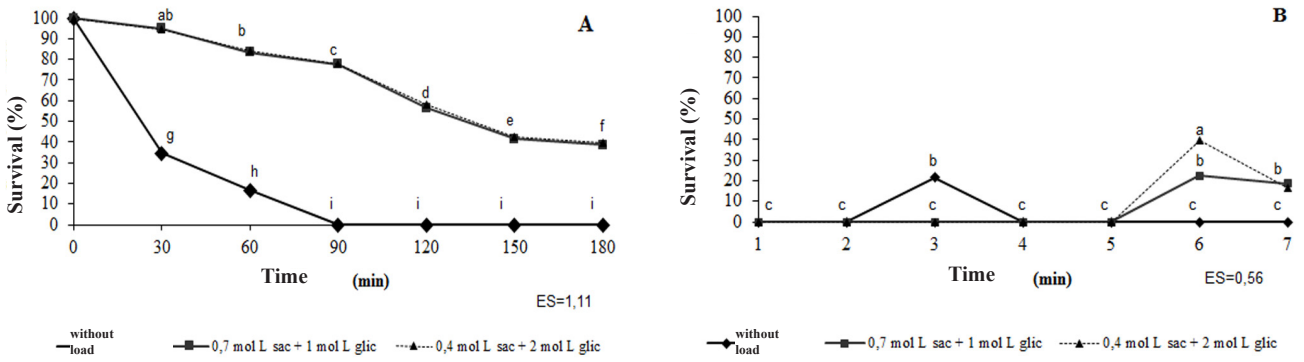
Similar observations were made (28) in meristems of *Musa* spp, where in the unrefrigerated meristematic the cells maintained more or less regular shape and no vacuoles were distinguished in their cytoplasm. However, after freezing the samples below -30 °C, they observed that as the cells were less meristematic there was an increase in the number and size of the vacuoles present in them. As expected for a sucrose concentration of 0,3 mol L^{-1}, the best survival levels for the apices after liquid nitrogen immersion and heating were attained (Figure 5B). Therefore, this sucrose concentration was selected for further experimentation. The results of different research groups are varied in the sense of standardizing appropriate concentrations of sucrose during cryoprotection. For each material to be stored, the appropriate concentration must be found, since this varies according to the species or explant (4, 13, 28).

When sucrose is used, the appropriate concentration tolerated by the cells must be determined before initiating the cryopreservation process, based on two fundamental aspects: osmotic dehydration and toxicity to cells (4). The most important results of this experiment were selected from the sucrose concentration of 0,3 mol * L^{-1} to continue the experimentation. In Figure 6 it was observed that the levels of tolerance to dehydration by the PVS2 solution increased markedly when using the treatments of the loading solution up to 180 min (Figure 6A). Cryopreservation survival levels also increased up to 40 %, with significant differences at 150 min dehydration in PVS2 in favor of the cryoprotective mixture of 0,4 mol * L^{-1} sucrose and 2 mol glycerol * L^{-1} (Figure 6B), so that the latter will be the loading solution to be used in the following experiments. However, it was shown that it was not possible to obtain an increase in apex survival even though a longer incubation time was used. This coincides with the published results regarding the use of pre-treatment time in the concentrations of glycerol and sucrose by the vitrification procedures (9, 12). This indicates that it is the right time to activate the necessary mechanisms involved in cell tolerance to different stressful moments in a cryopreservation strategy.



A: before immersion in liquid nitrogen B: after immersion in liquid nitrogen.
Means with equal letters do not differ (ANOVA, Tukey, p <0.05)
Data were transformed for analysis according to x '= 2 arcsen ((X / 100) 0.5

Figure 5. Effect of dehydration time on PVS2 on apex survival (according to the preculture in sucrose)



A:before immersion in liquid nitrogen B: after immersion in liquid nitrogen
Means with same letters do not differ (ANOVA, Tukey, p<0,05)
The data were transformed for analysis according to x'= 2 arcsen ((x/100)0,5

Figure 6. Effect of dehydration time in PVS2 survival of the apexes (depending on the application of the load solution)

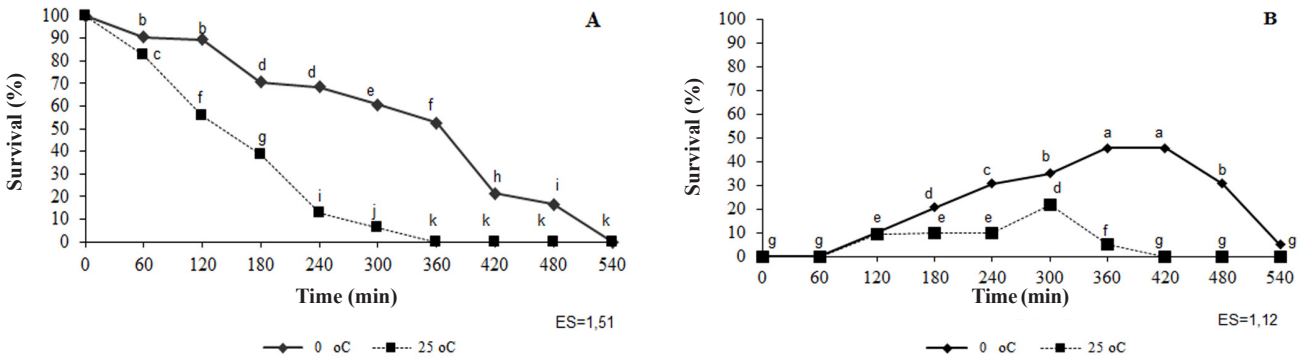
When the total number of OH groups per molecule of the mixtures used (sucrose + glycerol) was determined to compare the survival results with statistical differences obtained in Figure 6. For the case of the mixture 0,4 mol * L^{-1} sucrose + 2,0 mol * L^{-1} glycerol yields 55,402 x 1023 total OH groups per molecule while the mixture 0,75 mol * L^{-1} sucrose + 1,0 mol L^{-1} glycerol yields 54,198 x 1023 total OH groups per molecule. This is why the significant differences are evident. The rearrangement of hydroxyl groups of molecules of different sugars and poly-alcohols constitutes an important factor for effective cryopreservation. Figure 7 shows the results by decreasing the application temperature of PVS2. An increase in the tolerance time was observed up to 480 min (Figure 7A) and the maximum cryopreservation survival values were increased up to 45 % at 420 min of PVS2 exposure (Figure 7B). Therefore, in the continuation of the cryogenic strategy the temperature will be lowered to 0 °C to apply the PVS2.

According to Takagi (29) in the cryopreservation of shoot apices of tropical species, the survival of *Dioscorea alata* to cryopreservation from 47 to 91 % decreasing the temperature from 25 ° C to 0 ° C during PVS dehydration2). At present there is evidence of studies of Differential Scanning Calorimetry where several authors hypothesize about this (15). The authors suggest that the mechanism of protection of PVS2 is not only related to the non-formation of ice crystals but also is based on the restriction of molecular mobility and the disorganization of the structure of ice crystals.

In Figure 8 it was observed that there was clear statistical evidence where the PVS2 vitrification solution (Figure 8A) is less effective than PVS3 (Figure 8B) for the case of pineapple apices used. This is manifested in the higher values of survival from the 180 min of the vitrification solutions applied during the dehydration of the apices. In the case of the survival of the cryopreserved apices also the maximum levels are reached with the PVS3 vitrification solution.

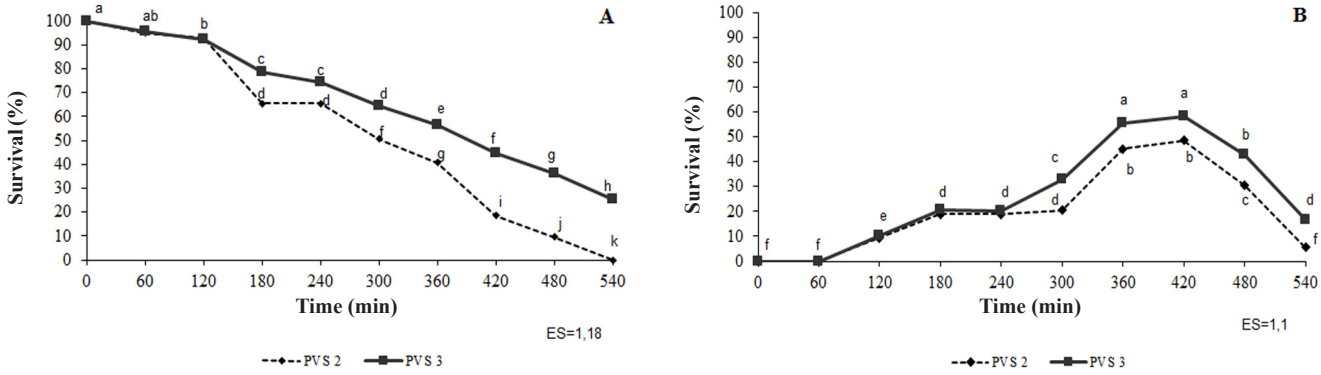
The above could be explained by the fact that the PVS2 solution uses the chemical compound dimethylsulfoxide which must be well investigated for its use because it has negative physiological and mutagenic effects for some plant materials. The negative tolerance results coincide with that reported for other biological systems and it is associated with the high toxicity of dimethylsulfoxide (9) accessions, it can be seen that the plant material maintained a survival rate between 6.3-65 %. In relation to the above, Table II shows the application of the cryogenic strategy to nine accessions of the in vitro germplasm bank, where it was possible to verify the varied response of the different genotypes in the survival after cryopreservation. The plant material during the cryopreservation process may suffer from several stresses including pH changes, mechanical changes, dehydration (osmotic damage), rehydration damage, oxidative stress and low temperature stress (30). The percentages of survival achieved by cryopreserved pineapple apices could be influenced by some of these stresses.

[A] Thinh, N. T. *Cryopreservation of germplasm of vegetatively propagated tropical monocots by vitrification*. Doctoral Paper, Kobe University, Faculty of Agriculture, Japan, 1997.



A: before immersion in liquid nitrogen B: after immersion in liquid nitrogen
Equations with the same letters do not differ (ANOVA, Tukey, p <0,05)
The data were transformed for the analysis according to x '= 2 arcsen ((x / 100) 0.5

Figure 7. Effect of dehydration time on PVS2 on apex survival (depending on the application temperature of the vitrification solution)



A: before immersion in liquid nitrogen B: after immersion in liquid nitrogen
Means with equal letters do not differ (ANOVA, Tukey, p <0.05)
Data were transformed for analysis according to x '= 2 arcsen ((X / 100) 0,5

Figure 8. Effect of dehydration time on apex survival (according to the vitrification solution employed)

Table II. Percentage of survival that was obtained with the application of the cryopreservation process by vitrification in nine accessions of the *in vitro* pineapple germplasm bank

Cultivar	Survival (%)	
	Not cryopreserved	Cryopreserved
Cabezona	61,5	27,9
Española Roja P3R5	53,1	20,0
Española Roja del Caney	45,5	12,1
Cayena lisa Serrana	50,3	25,3
MD2	80,1	60,2
Cayena de Puerto Rico	80,2	65,5
Perolera	49,9	33,8
Piña blanca	57,9	24,7
Piñuela Karata	33,1	6,3

CONCLUSIONS

Different technical aspects of a cryogenic strategy were determined to increase apex survival levels to immersion in liquid nitrogen. These were: apex type composed of 3-4 leaf primordia with an approximate size of 2,5-3 mm; preculture in 0,3 mol L^{-1} of sucrose; application of the loading solution 0,4 mol L^{-1} of sucrose + 2 mol L^{-1} of glycerol; application temperature of the PVS3 vitrificator solution at 0 °C for 420 min. While in the visualization of the structural changes occurring in the cryopreserved apices, it was observed that the meristematic cells present in the apical dome and the leaf primordia in formation, suffered little cellular alterations and maintained almost intact their morpho-physiological characteristics in the best conditions of survival. A vitrification procedure was successfully applied for 9 accessions from the *in vitro* germplasm bank of the Bioplant Center.

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Note:

During the editing process it was not possible to access the work of retouching and improvement of images, so they have been inserted with the same quality as the ones sent by their authors.

The editorial

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