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CROP SCIENCE

Morphoanatomical aspects of the starting material for the improvement of pineapple cryopreservation by the droplet-vitrification technique

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Abstract: Cryopreservation of pineapple shoot tips has been established from various protocols, including droplet vitrification. Thus, this work aimed to evaluate the morphoanatomical conditions of the starting material over different times (30, 45 and 60 days) of culture before freezing and its correlation with the survival percentage of the cryopreserved shoot tips. Four accessions, *Ananas comosus* var. *comosus* (BGA-009); var. *bracteatus* (BGA-119); var. *parguazensis* (BGA-376), var. *erectifolius* (BGA-750) from the Pineapple Active Germplasm Bank (BGA Pineapple) and two hybrids from the Embrapa Genetic Breeding Program, FIB-ROX1 (var. *bracteatus* X var. *erectifolius*) and FIB-ROX2 (var. *erectifolius* X var. *bracteatus*), recently introduced in the field from *in vitro* storage, were used. Histological sections before freezing and the percentages of survival after freezing were obtained taking into account the different times of cultivation of the donor plants. The results showed a significative interaction between genotypes (accessions and hybrids) and the culture period. The accessions BGA-009 and BGA-119 showed the highest survival rates, with 95% and 90% respectively for the 30-day culture time. Different results were obtained for each genotype, showing the need for improvements in the standardization of starting material, which would allow better repeatability of the protocol.

Key words: *Ananas comosus* (L.) Merril, *Ex situ* conservation, Growing time, Starting material, Regeneration.

INTRODUCTION

Advances in biotechnology have provided new options for short- and long-term multiplication and conservation of plant biodiversity, using *in vitro* culture techniques. Cryopreservation in liquid nitrogen at -196 °C is a technique that has a good cost-benefit relation for long-term conservation of many plant species (Reed 2008, Cruz-Cruz et al. 2013). The nearly total interruption of the plant's metabolism avoids the need for renovation, minimizing the risks of somaclonal variation and significantly reducing the maintenance costs.

The cryopreservation of pineapple shoot tips has been accomplished with different protocols, through the techniques of encapsulation-vitrification (Gamez-Pastrana et al. 2004), vitrification (González-Arnao et al. 1998, 2000, Martinez-Montero et al. 2005, 2012), and more recently, droplet vitrification (Souza et al. 2016, 2018). This last technique has produced satisfactory results for cultivated

and wild pineapple varieties, with regeneration percentages between 40% and 90%. Exposure to the PVS2 (plant vitrification solution) for 45 minutes promoted the best performance for the majority of genotypes, although some responded better to exposure times of 30 and 60 min. This variability demonstrates the need to consider the effect of genotype for successful cryopreservation of pineapple (Souza et al. 2016) and can be a barrier to the repeatability of the protocol for different cultivated or wild varieties.

The success of the cryopreservation protocol requires the explants to pass through a series of steps (pre-culture, treatment with a cryoprotectant, immersion in liquid nitrogen and thawing), without losing viability and potential for regeneration (Sakai et al. 2008). Among the factors that can influence the final result of cryopreservation of pineapple plants, the choice of the starting explants is one of the most important. Since the shoot tips are obtained from plants grown *in vitro*, the incubation conditions of these plants can substantially alter the cell conditions of the structure to be cryopreserved, such as the ease of water removal from the tissues and the mechanisms to protect the cell membranes (Engelmann 2011).

According to Panis et al. (2011), depending on the target species, the development of a suitable protocol can take several years of investigation. The use of cryoprotective solutions is extremely important, because they determine the cell dehydration and number of components that will permeate the cells (Chen et al. 2011). The main success factor of a cryopreservation protocol is its applicability to a wide range of cultivars, so the problems associated with genotype variability must be overcome (Jeon et al. 2015).

The droplet vitrification technique has been shown to be efficient for cryopreservation of shoot tips of various pineapple genotypes (Souza et al. 2016, 2018). It is inexpensive and easy to perform compared with other techniques. However, additional tests have revealed the need to evaluate and standardize the plants used to obtain the shoot tips for cryopreservation, considering the culture time of the donor plants and morphoanatomical conditions of the shoot tips excised from these plants.

Therefore, the objective of this study was to assess the morphoanatomical aspects taking into account different culture times of donor plants of the starting material (shoot tips) on the efficiency of cryopreservation and regeneration rate of pineapple plants by the droplet vitrification technique.

MATERIALS AND METHODS

Plant material

We used four accessions from the Pineapple Active Germplasm Bank (BAG Pineapple) and two hybrids from the Pineapple Breeding Program of the Embrapa Cassava and Fruits research unit (*Embrapa Mandioca e Fruticultura*). The accessions came from different botanical varieties: *Ananas comosus* var. *comosus* (BGA-009); *A. comosus* var. *bracteatus* (BGA-119); *A. comosus* var. *parguazensis* (BGA-376); and *A. comosus* var. *erectifolius* (BGA-750); and two hybrids, denominated FIB-ROX1 (*A. comosus* var. *bracteatus* X *A. comosus* var. *erectifolius*) and FIB-ROX2 (*A. comosus* var. *erectifolius* X *A. comosus* var. *bracteatus*).

Starting material and extraction of shoot tips

The accessions/hybrids were first multiplied in culture medium composed of MS salts and vitamins (Murashige and Skoog 1962) supplemented with 3.0% (m/v) of sucrose, 2.4 g L^{-1} of Phytagel®, 0.5 mg L^{-1} of 6-benzylaminopurine (BA) and 0.02 mg L^{-1} of 1-naphthaleneacetic acid (NAA). The culture dishes were placed in a growth

room at 27 ± 1°C, photoperiod of 16 hours and photon flux density of 22 µmol m^{-2} s^{-1}, to obtain a sufficient number of plants, considering three culture times of the donor plants (30, 45 and 60 days) (Fig. 1a-c). Shoot tips of these plants were excised with approximate length of 0.5 mm and submitted to cryopreservation (Fig. 1d-f).

Pre-culture of the shoot tips

Shoot' tips from the plants after different culture times (30, 45 and 60 days) were grown in Petri dishes containing a pre-culture medium composed of MS salts and vitamins supplemented with 3.0% (m/v) of sucrose and 2.4 g L^{-1} of Phytagel® and incubated for 48 hours in a growth room at 27 ± 1°C, photoperiod of 16 hours and photon flux density of 22 µmol m^{-2} s^{-1}.

Vitrification: exposure to the PVS2 solution

After the pre-culture period, the shoot tips were transferred under aseptic conditions to aluminum foil sheets containing five to ten droplets (volume of 4 µL) of PVS2 vitrification solution (15% dimethyl sulfoxide – DMSO; 30% glycerol; 15% ethylene glycol; and 0.4 M sucrose), with one shoot tip placed in each droplet. The tips were exposed to the PVS2 for 45 minutes, over ice to maintain the temperature near 0°C. Then the aluminum foil sheets containing the shoot tips were placed directly in liquid nitrogen before being inserted in 2 mL cryotubes, which were immersed in nitrogen inside the cryogenic tank and maintained for 24 hours.

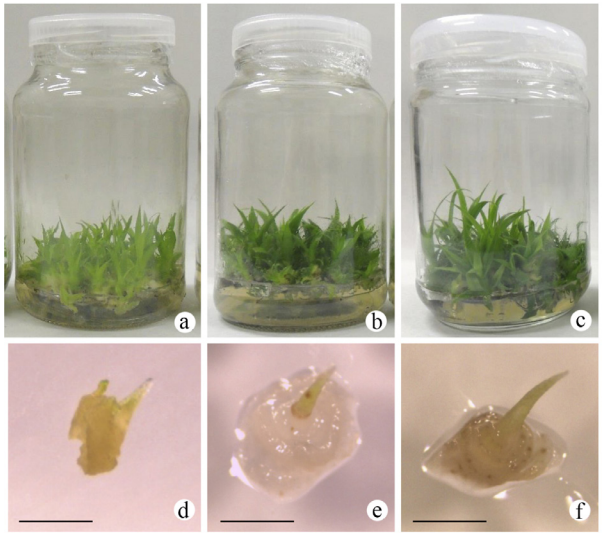

Figure 1. a-b) Plants grown *in vitro* of *Ananas comosus* var. *parguazensis* (BGA-376). d-f) Shoot tips of the same accession cultured for 30 (a, d) 45 (b, e) and 60 days (c, f) after multiplication in culture medium. Bar: 0.5 mm.

Thawing and regeneration

For thawing, the shoot tips were removed from the cryotubes and immersed quickly in a wash solution composed of MS salts and vitamins supplemented with 1.0 mol L^{-1} of sucrose for 20 minutes. Then the tips were cultured in MS medium supplemented with 3.0% (m/v) of sucrose, 2.4 g L^{-1} of Phytagel® and 0.5 mg L^{-1} of BAP, and incubated in the growth room under the same conditions as for the pre-culture step. The regeneration percentages were determined 30 days afterward.

Experimental design

The experimental design was completely randomized with a 3 x 6 factorial scheme (3 donor plant culture times x 6 genotypes) with 10 replications per treatment, where a replication consisted of one shoot tip. The controls consisted of recently removed shoot tips (absolute control), shoot tips that only underwent the pre-culture step and tips that were exposed to the PVS2 but were not frozen in liquid nitrogen (LN -).

The regeneration percentage data were transformed to arc sin ($\sqrt{x}/100$) before statistical analysis. The transformed data were submitted to analysis of variance by the F-test ($p \leq 0.01$) and the means were grouped by the Scott-Knott test ($p \leq 0.01$) for genotype and the means were compared by Tukey test ($p \leq 0.05$) for the time

variable. All the statistical tests were run with the SAS Institute v. 9.1.3 (2010).

Effect of genotypes and time of culture in the morphology of the shoot tips

To characterize the morphology, three shoot tips with approximate length of 1 mm, submitted to each of the culture times (30, 45 and 60 days), were fixed in modified Karnovsky's solution (Karnovsky 1965) [glutaraldehyde (2%), paraformaldehyde (2%), $CaCl_2$ (0.001 M), sodium cacodylate buffer (0.05 M), at pH 7.2] for 48 hours and then dehydrated in an ethyl series (35-100%). The samples were then dried to critical point with liquid CO_2 and mounted on metal supports and sputtered with gold. The images were obtained with a scanning electron microscope with variable pressure (LEO 435 VP, Carl Zeiss, Jena, Germany).

For anatomical characterization, five tips under the same conditions were collected and fixed in the same modified Karnovsky's solution (Karnovsky 1965) for 48 hours, infiltrated and embedded in resin using the Historesin kit (hydroxyethyl methacrylate, Leica, Heidelberg, Germany). The resin was polymerized at room temperature for 48 hours and then serial histological sections (4-5 µm) were obtained with a Leitz model 1516 rotary microtome, placed on slides and stained with acid fuchsin (0,1% p/v), followed by toluidine blue (0.05% p/v) (Feder and O' Brien 1968). The slides were observed and photographed with a fluorescence microscope system (B x S1, Olympus Latin America Inc.).

RESULTS AND DISCUSSION

Effect of genotype and time of the culture in the regenaration rate

The analysis of variance revealed a significant effect of genotype alone as well as the genotype x culture time interaction on the regeneration rate of the shoot tips in the control group (LN -) and the group only immersed in liquid nitrogen (LN +) (Table I). These results corroborate the findings of Souza et al. (2016) that worked with sixteen different accessions and cultivars, where the effect of genotype was also significant.

The results obtained for the controls only exposed to PVS2 (for 45 minutes) of the different genotypes were highly variable, ranging from 10% regeneration of BGA-750 tips cultured for 45 days to 100% for BGA-119 cultured for 30 days (Table II). PVS2 can be extremely toxic to cells, due to the presence of glycerol which can cause physicochemical changes and lead to the rupture of the membrane (Reed 2008). So it is important to test controls to distinguish the effect caused by freezing from other factors of the cryopreservation process (Volk & Walters 2006).

For the accessions BGA-009, BGA-119 and BGA-376, the exposure to PVS2 for 45 minutes did not result in a toxic effect, but was not uniformly efficient for the vitrification process, as can be noted from the post-freezing results (Table II). The absolute control group had high regeneration percentages (80 to 100%), as did the pre-culture control (MS+ sucrose). Sucrose did not have a deleterious effect on these shoot tips, indicating it can be used to protect the tissues of these accessions.

These three accessions presented better results after cryopreservation compared to accession BGA-750 and the hybrids FIB-ROX1 and FIB-ROX2, both in the PVS2 control and cryopreserved groups, with the exception of the culture time of 30 days for accession BGA-376 and 45 days for BGA-009, where the regeneration rates were extremely low (16.67%).

For BGA-750, a deleterious effect of sucrose was observed, with drastic reductions of the regeneration percentages, meaning that most of the shoot tips lost viability in this step. The

Table I. Analysis of variance of the regeneration rate after cryopreservation of pineapple accessions/hybrids in function of different culture times of the starting material.

Source of variation	Degree of freedom	Mean square[2]	
		LN⁻ (Control)	LN⁺
Genotype	5	1.5223**	1.3462**
Times	2	0.1246^{ns}	0.0029^{ns}
Genotype x Times	10	0.1496**	0.4298**
Error	33 (30[1])	0.0424	0.0440
Coefficient of variation (%)		19.86	36.55
Average		67.4510	35.4167

****significant at 1% probability by the F-test. ^{ns}not significant at 5% probability. [1]related to cryopreservation. [2]data transformed to arc sin √X/100.**

absence of complete regeneration after freezing is a result of the effect of sucrose combined with an additional effect of PVS2. The hybrids also presented declines in the regeneration rates after the pre-culture step, but much less pronounced than observed for BGA-750. Sucrose is an important cryoprotective agent used in processes involving freezing in liquid nitrogen (Reed 2008). Its action involves removal of intracellular water, to prevent the formation of ice crystals, besides protecting the cell membrane and cytoplasm from the rapid entry of cryoprotective substances (Woelders et al. 1997, Joo et al. 2014). Sugars have been found to stabilize the phospholipid bilayer, acting as external osmotic agents (Joo et al. 2014).

The hybrids FIB-ROX1 and FIB-ROX2 achieved regeneration percentages ranging from 13 to 60% for the PVS2 control group (LN -) after different culture times. The shoot tips of FIB-ROX2 cultured for 60 days had the highest regeneration rate after freezing, of 45%. For FIB-ROX1, the average rates were low, 20% (30 days), 17% (45 days) and no regeneration after 60 days. These results are similar to those in the PVS2 control groups of both hybrids, indicating the importance of the starting material for the efficiency of the cryogenic protocol.

As can be noted from the pre-culture control plants, sucrose had an effect on the shoot tips, with much lower regeneration percentages than the absolute control group. The evaluation regarding PVS2, in turn, should consider the toxicity, efficiency in altering the physical state of the water and protection of the cell membrane. The cryoprotective agent should quickly penetrate the cytoplasm and form hydrogen bonds with the water molecules to prevent their crystallization (Aye et al. 2010). This did not consistently occur in the materials studied here, since the regeneration rates of the PVS2 controls (LN -) varied from 0 to 100%.

Morphology of the shoot tips

The plants of accessions BGA-009, BGA-119 and BGA-376 presented normal morphology, with leaves arranged in spirals and good development. The plants of BGA-750 and the hybrids FIB-ROX1 and FIB-ROX2 presented various lateral buds, without precise definition of the shoot tip (Fig. 2a), making it hard to remove them, irrespective of the culture times of the donor plants (30, 45 and 60 days). This uneven morphology was most evident in BGA-750. The presence of lateral buds, in turn, suggests loss of apical dominance, which can have various causes and can affect

Table II. Regeneration rate of pineapple shoot tips in function of genotype and culture time in different treatments.

Genotype	Culture time (days)					
	30	45	60	30	45	60
	Absolute control (*)			Pre-culture control		
BGA-009	100	100	100	100	80	100
BGA-119	100	100	100	100	100	100
BGA-376	90	90	90	90	80	100
BGA-750	90	90	100	30	10	30
FIB-ROX1	80	100	100	60	60	80
FIB-ROX2	100	90	80	70	40	80
	PVS2 control (LN-)			LN+		
BGA-009	97 aA	90 aA	97 aA	95 aA	17 cC	63 bB
BGA-119	100 aA	83 aA	100 aA	90 aA	57 bB	57 bB
BGA-376	73 bB	93 aAB	100 aA	17 bB	100 aA	100 aA
BGA-750	20 cAB	10 cB	47 bA	0 cA	0 cA	0 cA
FIB-ROX1	55 bA	53 bA	13 cB	20 bA	17 cA	0 cA
FIB-ROX2	57 bA	35 bB	60 bA	30 bA	37 bA	45 bA

(*) Culture of shoot tips without pre-culture and PVS2 exposure; Means followed by the same lowercase letters in the column within the same factor (LN $^{-}$ and LN $^{+}$) belong to the same group by the Scott-Knott test at 5% probability, and those followed by the same uppercase letters in the rows do not statistically differ by the Tukey test at 5% probability.

the development of the shoot tips (Usman et al. 2013).

The best morphological traits of shoot tips for use as starting material are length of approximately 0.5 mm and presence of two or three primordial leaves (Wang & Valkonen 2009). This was the case of BGA-119 tips (Fig. 2b-c). In contrast, tips without a meristematic dome region can be considered unsuitable for extraction, as observed for BGA-750 (Fig. 2d-e).

The histological observations of the plants after different culture periods confirm the anatomical difference among the genotypes and the relationship with the regeneration rates measured (Fig. 2f-h). In accession BGA-009, the shoot tips from the plants cultured for 30 days presented a meristematic dome formed by isodiametric cells, dense cytoplasm, well-defined nuclei, few vascular bundles and two primordial leaves in perfect state, meaning tips ideal for cryopreservation (Fig. 2f). These traits were not observed in the tips of the plants cultured for 45 days (Fig. 2g), where we observed the presence of cells with large vacuoles and bigger intracellular spaces, indicating room for a greater volume of water and thus greater damage from freezing, explaining the lower regeneration percentage (16.67%). A change in the cell volume is an important factor related to the possibility of mechanical damage such as membrane rupture (Benson et al. 2007). The anatomy of the tips of plants cultured for 60 days (Fig. 2h) was intermediate between those cultured for 30 and 45 days, with the presence of cells with dense cytoplasm, but also cells with larger vacuoles and intercellular spaces.

Accession BGA-119 (Fig. 2i-k) presented primordial leaves with regular cells, dense

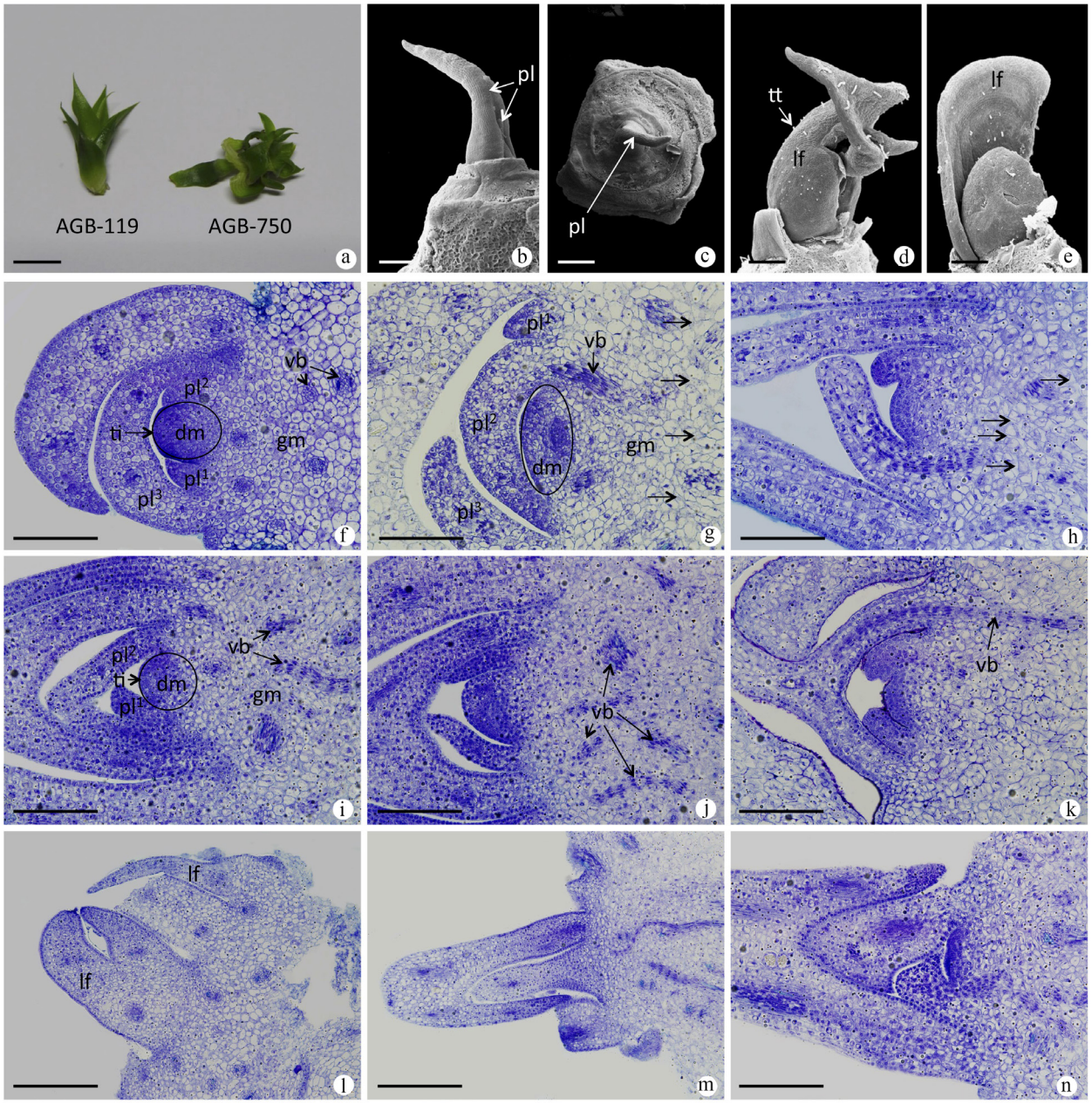

Figure 2. a) Pineapple plants after multiplication for 45 days in MS culture medium, showing ideal morphology (BGA-119) and unsuitable morphology (BGA-750) for excision of shoot tips b-e) Shoot tips observed by scanning electron microscopy. b, d, e) Shoot tips in transversal and c longitudinal views (BGA-119 and BGA-750) respectively. f-n) Shoot tips observed by light microscopy. f-h) Shoot tips of *A. comosus* var. *comosus* (BGA-009). i-k) *A. comosus* var. *bracteatus* (BGA-119) and (l-n) *A. comosus* var. *erectifolius* (BGA-750) after 30 days (f, i, l) 45 days (g, j, m) and 60 days (h, k, n) of multiplication in MS culture medium. dm = apical dome cells, gm = secondary shoot tip cells, pl = (1, 2 and 3) primordial leaves, tt = tector trichomes, vb = vascular bundles, ti = tunica. Arrows indicate large intracellular spaces. Bars: a = 1 cm, b-e = 200 µm, f-n = 100 µm.

nuclei, meristematic dome with well juxtaposed cells and a large number of vascular bundles in the region of secondary cells of the shoot tip. In the basal region of the tip, there were various traces of a procambium with elongated cells and cytoplasm with low density. The anatomical differences within the same genotype after different culture times explains the different regeneration rates of the tips from explants grown for 30 days, with excellent performance (90%), in contrast to those from plants cultured for 45 and 60 days, with regeneration rates around 57%. The sequence of images shows reduction of dense cells with increasing culture time, as well as modification in the meristematic dome area of tips from plants grown for 60 days. This is closely related to the start of budding in this genotype, probably denoting the start of loss of apical dominance.

Accession BGA-750 and hybrids FIB-ROX 1 and FIB-ROX2 presented in the most sectioned apices for anatomy, absence of an organized meristematic dome, but with the presence of primordial leaves with unistratified epidermis, vascular bundles distributed in a well-vacuolated fundamental parenchyma and large presence of intracellular spaces (Fig. 2l-n).

The causes of cell damage after cryopreservation can be related to the protoplasm, due to a larger volume of water and inefficiency of the dehydration/vitrification process. More vacuolated cells are subject to a higher level of plasmolysis when treated with sucrose during the cryoprotection and vitrification processes, and may not survive the osmotic stress imposed (Sakai et al. 2008). This appears to have happened in this study, where the results for these genotypes obtained for the control groups already indicated failure after freezing. This was particularly evident in BGA-750, with extremely low regeneration rates in the pre-culture control and PVS2 control, and no regeneration after freezing.

The existing anatomical studies have been focused on injuries after cryopreservation (Volk & Walters 2006, Ganino et al. 2012, Wang et al. 2014, Jeon et al. 2015, Souza et al. 2016), without considering the anatomical condition of the starting explant. The morphological study of Souza et al. (2016) was the first involving botanical varieties of the genus *Ananas*, and demonstrated that damages mainly occurred in cells that were already differentiated and had low density. Our observations corroborate those findings, since the starting material that presented these characteristics did not survive cryopreservation.

This study is the first to assess shoot tips as starting material and the correlation of genotypes with regeneration rates and cryoinjuries after freezing. It also raises relevant questions about the *in vitro* treatment of the material until the process of excising the shoot tips.

The germplasm of the genus *Ananas* is composed of accessions of approximately 700 different botanical varieties. Finding protocols that are adjusted to this diversity is crucial for the success of cryopreservation of the genus. In this respect, further studies are needed regarding the condition of the starting material for the success of cryopreservation, a task that is daunting due to the large number of genotypes.

In this study, we removed the shoot tips after the plants had been cultured for 30, 45 and 60 days, which provided information on the conditions of the starting material, since the incubation conditions can substantially alter the cellular conditions and affect the removal of water from the cells and the mechanisms that protect the cell membrane (Engelmann 2011).

Although the droplet vitrification protocol led to significant variation in the regeneration

rates among the accessions/hybrids, it should be noted that rates higher than 40% are considered satisfactory (Souza et al. 2016).

The results of this study show that if the starting material is in good cellular condition, the culture time is not a major determinant, since good results were obtained with shoot tips of plants cultured during all three periods analyzed, as can be observed in Table II and the significant interaction observed.

Nevertheless, we did observe modification as the culture time increased. This can be related to the start of loss of apical dominance and the start of budding, since regeneration medium used contains a cytokinin in its formulation (0.5 mg L^{-1} of BAP). Another observation is that two distinct aspects need to be considered regarding PVS2, as mentioned previously: whether the solution is toxic to the cells, and whether it is performing a protective role.

Finally, our findings confirm that the droplet vitrification technique is efficient to preserve shoot tips of the accessions BGA-009 (*A. comosus* var. *comosus*), BGA-119 (*A. comosus* var. *bracteatus*) and BGA-376 (*A. comosus* var. *parguazensis*), although adjustments in the protocol are necessary to increase the survival and regeneration rates.

CONCLUSIONS

This is the first study on the relationship between the anatomical structure of starting explants for cryopreservation considering different varieties and culture periods of the donor plants of shoot tips. The results show the importance of the starting material for successful cryopreservation, since it is directly affected by the freezing in liquid nitrogen and is strongly influenced by the genotype.

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