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Original paper

Exposure of pineapple shoot tips to liquid nitrogen and cryostorage do not affect the histological status of regenerated plantlets

ARIEL VILLALOBOS-OLIVERA¹, LELURLIS NÁPOLES¹, JOSÉ RAMÓN MENDOZA¹,
DORIS ESCALANTE¹, JULIA MARTÍNEZ¹, OSCAR CONCEPCIÓN¹, BYRON E. ZEVALLOS²,
MARCOS EDEL MARTÍNEZ-MONTERO¹, INAUDIS CEJAS¹, FLORENT ENGELMANN³,
OSCAR VICENTE^{4,*}, JOSÉ CARLOS LORENZO¹

¹Laboratory for Plant Breeding and Conservation of Genetic Resources, Bioplant Centre, University of Ciego de Ávila, Ciego de Ávila, 69450, Cuba

²Escuela Superior Politécnica Agropecuaria de Manabí Manuel Félix López (ESPAMMFL), Campus Politécnico El Limón, Carrera de Ingeniería Agrícola, Calceta, Manabí, Ecuador

³IRD Representation in Benin, 08 BP841, Cotonou, Benin

⁴Institute for the Preservation and Improvement of Valencian Agrodiversity (COMAV), Universitat Politècnica de València, Valencia, Spain

Abstract

Conservation of pineapple (*Ananas comosus* L. Merr.) genetic resources – including cryopreservation in liquid N₂ at -196°C – is essential for future breeding programmes to develop new varieties with improved agronomic performance. However, the potentially deleterious effects of cryopreservation on subsequent plant regrowth should be evaluated before large-scale development of cryobanks is implemented. This paper describes the histological analysis of pineapple plantlets regenerated from cryopreserved shoot tips. Two controls were included in the study: *i*) conventional micropropagation-derived plantlets, and *ii*) plants from shoot tips subjected to pre-cryostorage conditioning treatments but never exposed to liquid N₂. Histological studies of roots, leaves and stems were conducted after 45 days of hardening. No statistically significant differences with the controls were observed in any of the histological parameters evaluated, which supports the practical value of cryopreservation of pineapple germplasm.

Keywords

Ananas comosus L. Merr.; cryopreservation; *ex situ* conservation; genetic resources; histology.

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✉ *Corresponding author: Dr OSCAR VICENTE, COMAV, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain; Tel. +34 96 387 78 78
E-mail: ovicente@upvnet.upv.es

Introduction

Considering their commercial importance, pineapples are some of the most valuable tropical fruits (CHEN *et al*, 2019; NATH *et al*, 2019), grown in more than 10^6 hectares worldwide, with a production of about 24.8×10^6 metric tons of fruit *per* year, and generating a gross market value of almost 9×10^9 US\$ (FAOSTAT, 2015; MING *et al*, 2015; WALLI, 2019). However, different stressful conditions, both biotic and abiotic – such as, for example, microbial pathogens and sunburn – are increasingly threatening pineapple production. There is, therefore, a strong interest to develop new pineapple varieties with improved pathogen resistance or abiotic stress tolerance, as a strategy to enhance the agronomic performance of this fruit crop and thus guarantee its future production (OGATA *et al.*, 2016; RATTANATHAWORNKITI *et al*, 2016; PAULL *et al.*, 2017; PRIYADARSHANI *et al*, 2018). In this context, conservation of pineapple genetic resources is essential for the future development of specific breeding programmes.

A large number of plant species have been cryopreserved and regenerated using shoot tips as the starting material (CHMIELARZ, 2009; ENGELMANN and RAMANATHA, 2012; GONZALEZ-ARNAO *et al*, 2014). In general, the regenerated plants have been shown to be genetically and phenotypically stable (e.g., AGRAWAL *et al*, 2014; WANG *et al*, 2014), but there are also some reports claiming that storage in liquid N₂ can cause genetic and epigenetic modifications in the recovered plants (e.g., KAITY *et al*, 2008; JOHNSTON *et al*, 2009). Therefore, the potentially deleterious effects of storage in liquid N₂ on the subsequent growth and development of the regenerated plants should be assessed before the large-scale establishment of cryobanks is attempted. We considered, as a working hypothesis, that putative cryopreservation-induced modifications of the regenerated plants could be reflected in phenotypic changes affecting histological parameters. Recently, some histological studies of cryopreserved materials have been conducted in several plant species, including *Spermacoce hispida* L. (DEEPAK *et al*, 2019), *Passiflora pohlii* Mast (SIMÃO *et al*, 2018) or a relatively large number (32) of citrus taxa (VOLK *et al*, 2017). This short communication describes an extensive histological analysis of 45-day old pineapple plantlets regenerated from shoot tips after short-term storage at ultra-low temperatures in liquid N₂, in comparison with appropriate controls not subjected to cryostorage.

Materials and Methods

Plant material

In vitro culture of pineapple (*cv.* MD-2) buds was initiated from field-grown plants as previously described (DAQUINTA and BENEGAS, 1997). Three groups of plant materials were identified and used for the histological evaluation of plantlet growth after 45 days of acclimatization: *i*) conventional micropropagation-derived plants (control 1) (DAQUINTA and BENEGAS, 1997); *ii*) plants regenerated from shoot tips that had been subjected to pre-cryostorage conditioning treatments (see below), but were never exposed to liquid N₂ (control 2), and *iii*) plants from shoot tips exposed to liquid N₂ (cryopreserved plants).

Pre-cryostorage treatments, storage in liquid N₂ and recovery of shoot tips

The droplet-vitrification method was performed as described by SOUZA *et al* (2016), except that vitrification solution PVS3 (MARTÍNEZ-MONTERO *et al*, 2012; see below) was used instead of PVS2. Shoot tips (1 mm long, ca. 5 mg) were incubated for 24 h in Petri dishes on semi-solid MS medium (MURASHIGE and SKOOG, 1962), with 2.0 M glycerol and 0.4 M sucrose. Shoot tips were then placed in 2 ml polypropylene cryovials (10 tips per vial) containing 1 ml of loading solution (1 ml of 2.0 M glycerol and 0.4 M sucrose in MS medium) and were incubated at 25°C for 20 min. Shoot tips, and the loading solution were transferred to Petri dishes, each containing filter paper wetted with 5 ml of PVS3 solution: 50% (w/v) glycerol; 50% (w/v) sucrose, pre-cooled at 0°C. Petri dishes were placed on ice for 60 min, and then the shoot tips were transferred to pieces of aluminium foil (40 mm x 5 mm x 0.05 mm; 5 tips per piece) each containing micro-drops (0.1 ml) of PVS3 solution. Aluminium foil pieces were kept on ice until transfer to 2-ml cryovials, which were then immersed in liquid N₂ and stored under these conditions for 15 h. Shoot tips were recovered at room temperature by replacing the PVS3 solution with 1 ml of modified MS medium, containing 1 M sucrose, and incubating the samples at 25°C, 20 min. The medium used to recover plantlets from shoot tips contained MS (MURASHIGE and SKOOG 1962) minerals, 100 mg l⁻¹ *myo*-inositol, 0.1 mg l⁻¹ thiamine-HCl, 30 g l⁻¹ sucrose, 4.4 µM 6-benzyladenine (BA), and 5.3 µM naphthaleneacetic acid (NAA) (DAQUINTA and BENEGAS, 1997). The complete procedure described above was used to obtain the ‘cryopreserved’ plantlets; as

mentioned before, 'control 2' material was subjected to the same treatments except for freezing and storage in liquid N₂.

Plant hardening and histological analysis

Plantlets were transferred for hardening after the *in vitro* treatments (YANES-PAZ et al, 2000). The acclimatisation trial, in a completely randomised design, included four replications (15 plants each) per treatment. After 45 days of hardening, histological studies were conducted in middle-aged roots and leaves and the stem base. Ten plantlets were randomly selected per treatment. Anatomical studies were performed according to Johansen (1940), using a Zeiss® microscope and a Canon® Power Shot A 630 digital camera. The statistical analysis of the experimental data (One-Way ANOVA and Tukey test, $p < 0.05$) was carried out using SPSS software (Version 8.0 for Windows, SPSS Inc., New York, NY).

Results

Photos of the histological samples of pineapple plantlets derived from cryopreserved shoot tips and the two controls (not shown) were used to determine a series of anatomic parameters in roots, stems and leaves. Thus, pith diameter, central cylinder diameter, parenchyma thickness, cortex thickness, epidermis thickness, and thickness of transversal root radius, were measured in roots (Table 1). Pith diameter, central cylinder diameter, epidermis thickness, and transversal radius of the stem base were determined in stems (Table 2). Similarly, the transversal thickness of leaf at the middle, adaxial epidermis thickness, abaxial epidermis thickness, adaxial cuticle thickness, abaxial cuticle thickness, the thickness of the leaf photosynthetic parenchyma, and thickness of the leaf aquiferous parenchyma were measured in leaves (Table 3). As shown in Tables 1, 2 and 3, no statistically significant differences were observed in any the histological phenotype indicators evaluated (ANOVA, $p < 0.05$), when comparing plantlets recovered from shoot tips frozen and stored in liquid N₂ and those of the controls, which were never subjected to ultra-low temperatures. Just to highlight a few examples, it can be mentioned that roots of 45-day old 'cryopreserved' pineapple plantlets averaged 53.47 µm pith diameter, whereas mean values of 52.70 µm and 51.77 µm were determined for controls 1 and 2, respectively (Table 1). The mean central cylinder diameter at the base of the stem was 409.25 µm in cryopreserved plantlets, and no significant differences were observed with the same measurements performed in micropropagation-derived plantlets (409.57 µm) or in those of control 2 (406.86

(Table 2). Regarding the transversal thickness of leaf at the middle, 771.86 µm, 774.40 µm and 772.03 µm were measured for cryopreserved, control 1 and control 2 plantlets, respectively (Table 3). Little variability was observed between replicated samples, as indicated by the calculated SE values, which were relatively low for all samples (Tables 1, 2 and 3).

Discussion

Most studies on cryopreservation of plant material have shown genetic and phenotypic stability of the regenerated plants, as in the work presented here. Several authors have reported stability in cryopreserved apices, for example, in plantain (AGRAWAL et al, 2014) or potato (WANG et al, 2014). Cryopreservation has also been used to break the dormancy of recalcitrant species, without showing genetic or phenotypic variations of the regenerated plants (Matsumoto et al., 2015). Moreover, liquid N₂ has been employed in cryo-therapy, to eliminate pathogens from plant material, without any effect of the treatment on its stability (WANG et al, 2014).

Nevertheless, contrasting with most published results, some articles claim that cryopreservation can introduce variations in the regenerated plant material. Among those reports, the works of CHANNUNTAIPAT et al (2003) in *Prunus dulcis* Mill., DeVerno et al. (1999) in *Picea glauca* (Moench) VOSS, KAITTY et al (2008) in *Carica papaya* L., and JOHNSTON et al (2009) in *Ribes rubrum* L., can be mentioned. Although these changes are probably due to the use of poorly organised tissues, such as callus or cell suspensions (HAO et al, 2002; HARDING, 2004; KACZMARCZYK, 2018), we considered it essential to evaluate the potentially deleterious effects of cryopreservation on subsequent plant regeneration.

Conclusions

Our results indicate that shoot tip exposure to liquid N₂ did not alter pineapple growth and development at 45 days of acclimation, as a relatively large number of histological parameters did not differ significantly from those of comparable control plantlets that had not been subjected to ultra-low temperatures. Although these results should be confirmed using more extended periods of cryostorage of the shoot tips, they support the use of cryopreservation as an important tool for conservation of pineapple germplasm. As far as we know, this is the first publication on histological analysis of pineapple plantlets after cryopreservation.

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Table 1. Histological effects of cryopreservation of pineapple shoot-tips on the roots of regenerated plantlets

	Origin of plantlets (treatments compared)		
	Conventional micropropagation-derived plants (control 1)	Plants from shoot tips never exposed to LN, but submitted to pre-cryostorage conditioning treatments (control 2)	Plants from shoot tips exposed to LN (cryopreserved plants)
Pith diameter (µm)	52.70 ± 0.75	51.77 ± 0.72	53.47 ± 0.73
Central cylinder diameter (µm)	67.67 ± 1.45	68.56 ± 1.45	69.21 ± 1.70
Parenchyma thickness (µm)	75.62 ± 0.67	75.50 ± 0.74	75.62 ± 0.76
Cortex thickness (µm)	90.23 ± 0.92	90.46 ± 1.05	90.82 ± 1.09
Epidermis thickness (µm)	44.54 ± 0.86	42.89 ± 1.07	42.60 ± 0.88
Thickness of transversal root radius (µm)	256.37 ± 3.02	256.22 ± 1.94	258.52 ± 2.40

Table 2. Histological effects of cryopreservation of pineapple shoot-tips on the stem base of regenerated plantlets

	Origin of plantlets (treatments compared)		
	Conventional micropropagation-derived plants (control 1)	Plants from shoot tips never exposed to LN, but submitted to pre-cryostorage conditioning treatments (control 2)	Plants from shoot tips exposed to LN (cryopreserved plants)
Pith diameter (µm)	287.90 ± 0.99	288.35 ± 1.52	288.93 ± 0.84
Central cylinder diameter (µm)	409.25 ± 1.91	409.57 ± 1.80	406.86 ± 2.38
Epidermis thickness (µm)	136.92 ± 2.08	136.21 ± 1.30	137.69 ± 1.94
Transversal radio of stem base (µm)	1339.83 ± 4.16	1338.04 ± 2.44	1339.02 ± 3.71

Table 3. Histological effects of cryopreservation of pineapple shoot-tips on the D-leaf of regenerated plantlets

	Origin of plantlets (treatments compared)		
	Conventional micropropagation-derived plants (control 1)	Plants from shoot tips never exposed to LN but submitted to pre-cryostorage conditioning treatments (control 2)	Plants from shoot tips exposed to LN (cryopreserved plants)
Transversal thickness of leaf at the middle (µm)	774.40 ± 5.90	772.03 ± 5.67	771.86 ± 6.35
Adaxial epidermis thickness (µm)	224.29 ± 2.82	223.68 ± 2.73	222.69 ± 2.52
Abaxial epidermis thickness (µm)	209.90 ± 0.27	210.39 ± 0.60	210.61 ± 0.94
Adaxial cuticle thickness (µm)	79.29 ± 2.82	78.68 ± 2.73	77.69 ± 2.52
Abaxial cuticle thickness (µm)	14.90 ± 0.27	15.39 ± 0.60	15.61 ± 0.94
Thickness of the leaf photosynthetic parenchyma (µm)	119.94 ± 1.86	119.17 ± 1.52	119.71 ± 1.94
Thickness of the leaf aquiferous parenchyma (µm)	220.27 ± 1.70	218.80 ± 1.61	218.85 ± 1.86

Histological samples were analysed at 45 days of acclimatisation. Values shown are means ± SE (n = 10). Statistically significant differences were not observed for any of the measured parameters (One-Way ANOVA, Tukey, p < 0.05).