Storage of Pineapple Shoot Tips in Liquid Nitrogen for Three Years Does Not Modify Field Performance of Recovered Plants

Ariel Villalobos-Olivera
University of Ciego de Avila
Ysmel Entensa
Bioplant Centre, University of Ciego de Avila
Julia Martínez
Bioplant Centre, University of Ciego de Avila
Doris Escalante
Bioplant Centre, University of Ciego de Avila
Nicolás Quintana
University of Ciego de Avila
Fernanda V. D. Souza
EMBRAPA
Marcos Edel Martínez-Montero
Bioplant Centre, University of Ciego de Avila
Elliosha Hajari
Agricultural Research Council
José Carlos Lorenzo Feijoo (jclorenzo@bioplantas.cu)
Laboratory for Plant Breeding and Conservation of Genetic Resources, Bioplant Centre, University of Ciego de Ávila, Ciego de Ávila, 69450, Cuba. https://orcid.org/0000-0003-3610-1789

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Abstract

Pineapple is the third most important tropical fruit traded after banana and mango. In recent years, pineapple producers have faced production challenges due to unpredictable weather patterns as a consequence of climate change. In order to develop new genotypes with improved tolerance to these biotic and abiotic stresses, it is necessary to have access to a diverse gene pool, however, the germplasm currently stored in field gene banks are at risk from prevailing environmental conditions. Therefore, there is a need to develop and implement supplementary strategies to conserve plant germplasm under controlled conditions in the laboratory and cryopreservation represents the most viable option for this. To date, methods have been developed for cryopreservation of pineapple shoot tips but there is a lack of information on whether extended periods of storage in liquid nitrogen (LN) has any effect on the regeneration capacity and field performance of cryostored germplasm. Hence, the current study investigated the field performance of pineapple shoot tips after storage for 24 h, one, two and three years. Results were collected following nine months for plant morphological characteristics and 14 months for evaluation of fruit and nutritional characteristics. The results indicated that storage of pineapple shoot tips for up to three years did not have any adverse effects on field performance of plants or on fruit characteristics. This provides evidence that cryopreservation offers a suitable tool for the long term storage of germplasm.

Introduction

The cultivation of tropical and subtropical fruit makes a valuable contribution to the Gross Domestic Product in many developed and developing countries. In terms of volume, pineapple (*Ananas comosus* L. Merr.) represents third most traded tropical fruit after banana and mango (Leal and d'Eckenbrugge 2018). In 2019, global production of pineapples was approximately 28.18 metric tons with Costa Rica, the Philippines and Brazil being the leading producers (Shahbandeh 2020). Pineapples are consumed primarily as fruit and juice (fresh and canned). The flesh is rich in vitamins, antioxidants and phenolic compounds with notable anti-inflammatory benefits (Gil et al. 2006; Montero-Calderón et al. 2010; Poiroux-Gonord et al. 2010). Successful cultivation of pineapple is dependent upon suitable environmental conditions which have an influence on fruit set, growth and development. In recent years, climate change has emerged as a global environmental concern with impacts predicted in many sectors including agriculture. Unpredictable weather patterns mean that annual rainfall events are no longer occurring as normal and water scarcity is becoming a major problem. In addition, global warming leading to higher temperatures mean that fruit are expose to higher temperatures for extended periods of time leading to deterioration in fruit quality parameters, for example sunburn, changes in texture, nutritional components, etc. (Bindi et al. 2001). Secondary effects include changes in the migration patterns of pests, increased incidence of both pests and diseases, changes in weed pressure, etc. (Prasad and Chakravorty 2015). Hence, climate change is threatening the sustainability of production systems and efforts need to be focused towards the development of mitigation strategies to improve the resilience of agricultural systems (reviewed by Parajuli et al. (2019)). This includes the adoption of a holistic approach

encompassing the development of improved management practices coupled with the production of superior genotypes capable of withstanding the imposed stresses.

In order to generate improved genotypes, it is necessary to have access to a diverse gene pool. With regard to pineapple, germplasm is stored in field gene banks, greenhouses or as *in vitro* cultures (Souza et al. 2007). Brazil is considered one of the centers of origin for pineapple and houses the Pineapple Active Germplasm Bank of *Embrapa Mandioca e Fruticultura* (AGB-Pineapple) containing more than 700 accessions. Additional collections are maintained by the USDA-ARS in Hawaii, CIRAD-FLOHR in Martinique and the Bioplantas Centre in Cuba (Yanes-Paz et al. 2012; da Silva et al. 2021). However, field gene banks are expensive to maintain and are at risk from prevailing environmental conditions such as adverse weather, pest and disease outbreaks (particularly *Fusarium subglutinans* and *Phytophthora nicotinae*), etc. (Villalobos-Olivera et al. 2019b). While *in vitro* storage overcomes some of the disadvantages of field gene banks, cultures remain at risk from microbial contamination, equipment failure and loss of genotypes through somaclonal variation as a result of extended storage durations (Souza et al. 2016). Therefore, there is a need to have valuable germplasm stored in a supplementary system to provide a back-up in the event of loss of material from field gene banks, i.e. using cryopreservation (Uchendu et al. 2011).

Cryopreservation technologies have developed from initial conception of the technique in the 1970s to the establishment of various methods during the 2000s to the present day implementation in cryobanks (reviewed by Normah et al. (2019)). The type of explant selected for cryopreservation is critical and it is widely accepted that shoots tips are the explant of choice, particularly when the goal is the maintenance of clonal fidelity (Engelmann 2013). To date, hundreds of plant species have been cryopreserved using shoot tips (Engelmann and Ramanatha 2012; Gonzalez-Arnao et al. 2014; Ochatt et al. 2021; Senula and Nagel 2021) with vitrification being one of the most commonly used techniques in the 21st century (Normah et al. 2019). The various steps of the vitrification protocol necessitate exposure to highly concentrated solutions during preculture, loading and immersion in the vitrification solution followed by exposure to LN. These steps impose various stresses on plant material; therefore, it is critical to evaluate the performance of cryostored plants in the field prior to large-scale implementation in cryobanking facilities. In previous studies, we evaluated the field performance of pineapple plants following: (1) conventional micropropagation (Daquinta and Benegas 1997); (2) exposure to cryopreparative stages (but not immersed in LN); and (3) immersion in LN for 15 h (cryopreserved plants). The results showed that exposure of shoot tips to cryogenic temperatures did not alter the field performance of pineapple plants. This observation supports the assertion that cryopreservation is an important tool for the conservation of pineapple germplasm (Villalobos-Olivera et al. 2019b). However, that study did not consider the effect of longer term exposure to LN. This short communication reports on cryopreservation of pineapple shoot tips for up to three years and subsequent field performance of adult plants.

Materials And Methods

Pineapple buds (cultivar MD-2) from field-grown plants were initiated *in vitro* as per Daquinta and Benegas (1997). For cryopreservation, the droplet vitrification technique was performed as outlined by Souza et al. (2015), however, PVS3 was used in place of PVS2, as suggested by Martínez-Montero et al. (2012). For the preculture step, shoot tips (1 mm long weighing approximately 5 mg) were incubated in Petri dishes with semi-solid MS salts and vitamins (Murashige and Skoog 1962), 2 M glycerol and 0.4 M sucrose for 24 h. Shoot tips were then transferred to 2 mL polypropylene cryovials (10 shoot tips per vial) containing 1 ml of loading solution comprised of MS medium with 2 M glycerol and 0.4 M sucrose. Loading was performed at 25 ± 2°C for 20 min.

For exposure to the vitrification solution, shoot tips were transferred to Petri dishes with filter paper moistened with PVS3 solution [50% glycerol (m:v) and 50% sucrose (m:v)] at 0°C. There was 5 mL of PVS3 supplied to each Petri dish. The Petri dishes were placed on ice for 60 min. Following this, shoot tips were transferred to strips of aluminum foil (4 x 0.5 x 0.005 cm with 5 shoot tips per foil strip) containing micro-drops (0.1 mL) of PVS3 solution. The aluminum foil strips were maintained on ice until transfer to cryovials which were subsequently immersed in LN for 24 h, one, two or three years.

Shoot tips were recovered at room temperature as follows: PVS3 solution was discarded and replaced with 1 mL of MS medium supplemented with 1 M sucrose for 20 min. For re-growth after cryostorage, shoot tips were cultured on MS salts and vitamins, 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). Plants from shoot tips that were not immersed in LN but were exposed to the cryopreparative stages were used as a control. To conduct simultaneously the acclimatization and field growth of the five groups of plants, *in vitro* procedures were not performed at the same time.

All *in vitro* plantlets were hardened-off using the method outlined by Yanes-Paz et al. (2000). Following six months of hardening-off, the plants were planted in a field trial at the Bioplant Centre Field Experimental Station (Ciego de Avila, Cuba). The field trial was set up as a completely randomized design and was performed in compliance with the regulations stipulated by the Cuban Ministry of Agriculture. The trial was designed with four replicates (15 plants each) per treatment. Rows were separated from each other by 1 m. Each row was comprised of 15 plants which were 30 cm apart.

Plant phenotypic traits were evaluated following nine months of growth while fruit characteristics were assessed after 14 months, as suggested by others, e.g. Py et al. (1987). Five plants were randomly selected per replicate for evaluation (5 plants / rep * 4 reps = 20 plants evaluated). Five fruit randomly selected per replicate were also evaluated (5 fruits / rep * 4 reps = 20 fruit evaluated). Border plants were not evaluated to exclude potential edge effects. Data were analyzed using SPSS (Version 8.0 for Windows, SPSS Inc., New York, NY) by performing One-Way analysis of variance ($p = 0.05$).

Results And Discussion

The current study reports on the field performance of shoot tips of pineapple stored in LN for up to three years. The results showed that more than 90% of shoot tips retrieved from LN regenerated shoots following 45 d of re-growth. This regeneration rate was similar to the results obtained from the control (non-cryopreserved) shoot tips. Souza et al. 2016 reported 44–86% survival in cultivated and wild pineapple following cryopreservation using droplet vitrification with PVS2. Following re-growth *in vitro*, plants were successfully hardened-off with 97% survival across all treatments (i.e. shoot tips stored for 0, 24 h, one, two or three years in LN). Similarly, Souza et al. (2016) also reported high survival (100%) following hardening-off of cultivated and wild pineapple after droplet vitrification. Furthermore, all plants survived transplanting into the field environment (data not shown).

Table 1 summarizes the results obtained for the field performance of shoot tips that were cryostored and for control plants. The phenotypic characteristics evaluated included plant traits after nine months of growth in the field and fruit characteristics at harvest (14 months). Overall, the results indicated no significant differences in the field performance of plants regenerated from cryopreserved shoot tips, irrespective of the storage duration in the cryogen. Furthermore, the characteristics of plants regenerated after cryostorage were similar to those that had not been cryostored. The abovementioned observations were recorded for the plant morphological indicators assessed after nine months of field growth (i.e. plant height, characteristics of the *D* leaf, stem base diameter and plant fresh weight). This first level of analysis indicated that cryopreservation did not alter plant physical characteristics. Following an additional five months, it was confirmed that the fruit characteristics (size and mass) and quality parameters (Brix, ascorbic acid, total titratable acids, overall acidity and pH) were also similar in plants stored in LN for different durations (and in the control).

Table 1

Effect of pineapple shoot tip cryopreservation for up to three years on the phenotype of regenerated adult plants and fruits (average ± SE). Statistically significant differences among the five groups of plants were not recorded (One-Way ANOVA, $p > 0.05$).

	Control plants derived from shoot tips not exposed to LN	Plants derived from shoot tips exposed to LN (time of exposure)			
		24 h	1 year	2 years	3 years
Plant traits evaluated at 9 mo of field growth					
Plant height (cm)	97.67 ± 0.27	97.60 ± 0.33	97.65 ± 0.27	97.45 ± 0.37	97.83 ± 0.28
Number of leaves per plant	43.40 ± 0.37	44.40 ± 0.54	43.40 ± 0.37	44.30 ± 0.58	43.50 ± 0.37
D leaf length (cm)	85.12 ± 0.42	84.86 ± 0.21	85.25 ± 0.38	84.85 ± 0.26	84.97 ± 0.20
D leaf width (cm)	4.52 ± 0.06	4.46 ± 0.05	4.45 ± 0.05	4.48 ± 0.05	4.52 ± 0.05
D leaf area (cm^2)	59.77 ± 0.72	59.28 ± 0.70	60.10 ± 0.80	60.06 ± 0.69	59.70 ± 0.74
D leaf fresh weight (g)	55.81 ± 0.74	55.73 ± 0.82	56.34 ± 0.65	57.15 ± 0.45	56.91 ± 0.75
Stem base diameter (cm)	9.58 ± 0.11	9.44 ± 0.10	9.72 ± 0.10	9.67 ± 0.09	9.62 ± 0.11
Plant fresh weight (g)	4746.03 ± 7.84	4746.14 ± 11.44	4740.78 ± 9.06	4762.04 ± 13.15	4735.79 ± 10.25
Fruit traits evaluated at 14 mo of plant field growth					
Fruit length (cm)	13.19 ± 0.02	13.15 ± 0.01	13.16 ± 0.01	13.17 ± 0.02	13.18 ± 0.02
Superior diameter of fruit (cm)	9.17 ± 0.01	9.16 ± 0.00	9.16 ± 0.01	9.17 ± 0.00	9.17 ± 0.01
Fruit mass with crown (g)	2.00 ± 0.02	1.98 ± 0.01	1.99 ± 0.02	1.99 ± 0.01	2.00 ± 0.02
Fruit mass without crown (g)	1.62 ± 0.02	1.61 ± 0.03	1.61 ± 0.02	1.62 ± 0.02	1.64 ± 0.02
Total content of soluble solids in fruits (^{o}Brix)	13.88 ± 0.04	13.86 ± 0.03	13.84 ± 0.02	13.87 ± 0.02	13.89 ± 0.03

	Control plants derived from shoot tips not exposed to LN	Plants derived from shoot tips exposed to LN (time of exposure)			
		24 h	1 year	2 years	3 years
Content of ascorbic acid in fruits (mg. 100 mL juice^{-1})	76.44 ± 0.12	76.46 ± 0.09	76.46 ± 0.13	76.49 ± 0.03	76.53 ± 0.05
Total titrable acidity in fruits (%)	1.68 ± 0.02	1.66 ± 0.01	1.69 ± 0.02	1.70 ± 0.01	1.69 ± 0.02
Soluble solids / acidity (mg. 100 mL juice^{-1})	8.26 ± 0.10	8.36 ± 0.06	8.20 ± 0.12	8.18 ± 0.05	8.22 ± 0.09
pH	4.38 ± 0.03	4.40 ± 0.12	4.36 ± 0.04	4.40 ± 0.08	4.47 ± 0.11

Cryopreservation (and the cryopreparative stages) imposes a number of stresses on plants which can lead not only to damage, but also to alterations that can affect the genetic integrity and subsequent morphological characteristics of germplasm. Therefore, it is critical to evaluate the performance of plants regenerated following cryopreservation in the field as investigated in the current study. In the context of pineapple, da Silva et al. (2021) investigated the efficacy of *in vitro* slow growth storage as a conservation strategy for pineapple. Those authors reported that following 10 years of slow growth *in vitro*, there were minimal incidences of somaclonal variants (as assessed in field trials over two growing seasons). In other studies, vegetative growth and plant characteristics following cryopreservation have been assessed under glasshouse conditions, for example in shallot (Wang et al. 2021) and kiwifruit (Zhang et al. 2020). However, it is important to assess the resilience of cryostored plants when grown under field conditions as this is ultimately the system used for production. Rantala et al. (2019) investigated the recovery of cryopreserved shoot tips of blackcurrant and recorded the yield of berries harvested in two consecutive seasons. As reported in the present study, those authors found no differences in the yield of cryostored and non-stored plants. Similarly, Pawłowska et al. (2019) noted that cryopreservation of wild rose shoot tips did not affect biochemical attributes or pollen characteristics following plant growth in the field. Furthermore, in the present study, the results from field trials showed that not only were the fruit characteristics of pineapples regenerated from cryostored and non-stored shoot tips similar, but also the nutritional components of fruit were similar (Table 1). In this regard, the ascorbic acid content was similar in fruit stored for 0, 24 h, one, two and three years, i.e. 76.44 ± 0.12 to 76.53 ± 0.05 mg/100 ml juice (Table 1). This provides evidence that cryopreservation did not alter the nutritional characteristics of stored germplasm.

In theory, explants stored cryogenically can be maintained for an indefinite period of time (Engelmann 1997). However, there are concerns that levels of plant regeneration could decline over time. Hence, the current study investigated the effect of storage duration in LN on plant survival. As mentioned above, no decline in plant survival, or changes in plant/fruit characteristics, were noted when shoot tips were stored for up to three years. This is in contrast to the findings reported by Rantala et al. (2019) that reported a

significant decline in the recovery rate of cryopreserved blackcurrant shoot tips from a maximum of 86% when cryostored for up to 6 days to 58% when stored for four years in LN.

To the best of our knowledge, this is the first publication evaluating pineapple agricultural traits after cryopreservation of shoot tips for up to three years. Previously, our group has studied the effects of LN on the subsequent germination and growth of common bean, tomato, maize, tobacco, sorghum, chickpea, neonotonia and teramnus seeds. These studies showed that cryostorage induced transient morphological, physiological and biochemical changes that gradually disappeared as plants developed (Cejas et al. 2012; Zevallos et al. 2014; Pérez-Rodríguez et al. 2017; Arguedas et al. 2018; Acosta et al. 2019; Villalobos-Olivera et al. 2019a; Acosta et al. 2020; Villalobos et al. 2020). The current report adds to this body of work and provides evidence for the stability, at least in terms of field performance, of storage of pineapple germplasm in LN following three years. Future studies should investigate the genetic integrity of germplasm after cryogenic exposure to ensure that no unintended changes result, as done by Wang et al. (2021) and Zhang et al. (2020) on shallot and kiwifruit, respectively. This is essential in conservation programs.

Declarations

Author contribution

AVO, YE, JM, DE, NQ, FVDS, MEMM, EH and JCL designed the research; AVO, YE, JM and DE conducted the experiment; AVO, YE, NQ, FVDS, MEMM, EH and JCL analyzed the data and wrote the paper; and JCL had primary responsibility for the final content. All authors have read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of interest

The authors declare no conflicts of interest.

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